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LIPID PEROXIDATION AS A POSSIBLE CAUSE OF OCHRATOXIN A TOXICITY

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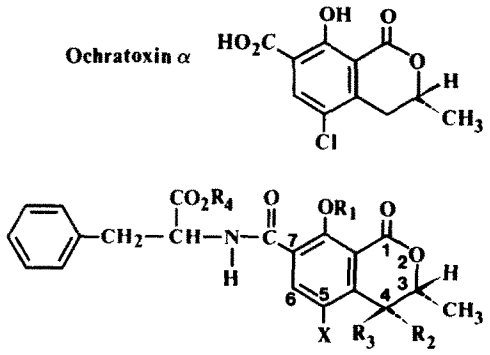
Abstract—Addition of the mycotoxin ochratoxin A (OA), a nephrotoxic carcinogen, to rat liver microsomes greatly enhanced the rate of NADPH or ascorbate-dependent lipid peroxidation as measured by malondialdehyde formation. NADPH-dependent lipid peroxidation in kidney microsomes was similarly enhanced by OA. The process required the presence of trace amounts of iron but cytochrome P-450 and free active oxygen species appeared not to be involved.

The efficiency of several ochratoxins (ochratoxins A, B, C, α and *O*-methyl-ochratoxin C) to enhance lipid peroxidation was related to the presence and reactivity of the phenolic hydroxyl group. Furthermore, the ability of these ochratoxins to enhance lipid peroxidation in microsomes correlated precisely with their known toxicities in chicks. Administration of ochratoxin A to rats also resulted in enhanced lipid peroxidation *in vivo* as evidenced by a seven-fold increase in the rate of ethane exhalation.

These results suggest that lipid peroxidation may play a role in the observed toxicity of ochratoxin A in animals; a mechanism is proposed.



Ochratoxin A: X = Cl; $R_1 = R_2 = R_3 = R_4 = H$
Ochratoxin B: X = H ; $R_1 = R_2 = R_3 = R_4 = H$
Ochratoxin C: X = Cl; $R_1 = R_2 = R_3 = H$; $R_4 = CH_3$
O-Methyl-ochratoxin C: X = Cl; $R_2 = R_3 = H$; $R_1 = R_4 = CH_3$
(4R)-4-hydroxyochratoxin A: X = Cl; $R_1 = R_3 = R_4 = H$; $R_2 = OH$
(4S)-4-hydroxyochratoxin A: X = Cl; $R_1 = R_2 = R_4 = H$; $R_3 = OH$

Fig. 1. Chemical structures of the various ochratoxins.

Ochratoxin A (OA)†, a dihydroisocoumarin-containing mycotoxin (Fig. 1), is produced by members of the *Aspergillus ochraceus* group and some species of the *Penicillium* group. The occurrence of OA in food and feed is widespread in various climates around the world and it has been detected in cereals, beans and peanuts at levels ranging from 9–27,500 μg/kg [1].

Ingestion of OA has been shown to induce nephropathy in several species [2] while dietary feeding induced renal adenomas and hepatocellular carcinomas in mice [3]. OA is suspected of being the main etiologic agent responsible for endemic Balkan nephropathy and associated urinary tract tumours in humans [4]. OA did not produce genetic or related effects in a variety of *in vitro* short term tests [5–7] but it has been shown to induce single strand breaks in DNA of liver, kidney and spleen of mice [8].

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† Abbreviations used: BHT, butylated hydroxytoluene; b.w., body weight; MDA, malondialdehyde; OA, ochratoxin A; r.s., regenerating system; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid; TCA, trichloroacetic acid.

Moreover, there are numerous reports indicating that administration of OA to rodents alters a variety of biochemical parameters in the liver, kidney and urinary tract [9–11].

Despite its carcinogenic and toxic properties, the mechanism of its action is not known. Moreover, to our knowledge, there is no report in the literature indicating that OA binds covalently to DNA. OA has been shown to be metabolized by rat liver microsomes to (4*S*)-4-hydroxy-OA and (4*R*)-4-hydroxy-OA [12] while with rabbit liver microsomes, an additional metabolite, 10-hydroxy-OA is formed [13]. We have previously shown that 4-hydroxylation of OA shows polymorphism in rat strains phenotyped as extensive and poor metabolizers of debrisoquine [14].

In this communication we show that OA enhances lipid peroxidation when added to rat liver microsomes *in vitro* or when administered to rats *in vivo*. Furthermore, the ability of OA and its analogues to enhance lipid peroxidation correlated well with their known toxicity.

MATERIALS AND METHODS

Chemicals. Ascorbic acid, BHT, metyrapone, α-naphthoflavone, DL-isocitric acid, isocitric dehydrogenase (type IV from porcine heart), $NADP^+$, NADPH, OA, SOD (lyophilized powder from bovine erythrocytes) and TBA were purchased from Sigma Chemical Co. (St. Louis, MO). Desferal was obtained from Ciba-Geigy (Rueil-Malmaison, France), OB was a gift from Dr. A. Pohland, FDA, Washington, U.S.A. All other chemicals were of the highest grade commercially available.

Animals. Male Wistar rats (150–250 g) were obtained from a local supplier (IFFA-CREDO, Lyon). Animals were maintained at 21° on a 12 hr light and dark cycle and had free access to standard laboratory chow and water. Liver and kidney microsomes were prepared from overnight fed animals by differential centrifugation of liver and kidney homogenates [15]. Protein was estimated by the Lowry method using bovine serum albumin as standard [16].

Preparation of OA analogues and metabolites (for structures see Fig. 1). Oα was prepared by refluxing OA with 6 N HCl [17]. OM-OC was prepared by treating OA in chloroform with an excess of ethereal diazomethane as described by Van der Merwe *et al.* [17]. OC was prepared by esterification of the carboxyl group of OA in the presence of 14% boron trifluoride in methanol according to a method described by Nesheim [18]. (4*S*)-4OH-OA and (4*R*)-4OH-OA were prepared by metabolizing OA with rat liver microsomes in the presence of NADPH and separating the metabolites by TLC [12]. The two epimers were present in a ratio of 1:2 respectively but were pooled together for the purpose of the present experiment. All compounds appeared as single spots on TLC and had a UV absorbance spectrum in accordance with published values.

In vitro *lipid peroxidation*. Unless otherwise indicated, all incubations were carried out in duplicate at 37° with shaking and contained (per ml): potassium phosphate pH 7.4 (100 μmol), microsomal protein (2 mg) and OA or the indicated analogue (125 nmol). Lipid peroxidation was initiated by the addition of either NADPH (1 μmol) or a NADPH regenerating system (consisting of 0.4 μmol $NADP^+$, 5 μmol $MgCl_2$, 5 μmol DL-isocitrate and 0.65 units of isocitric dehydrogenase) or ascorbate (1 μmol)–$FeSO_4$ (5 nmol). Lipid peroxidation was terminated at various time points 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% TCA. After addition of 500 μl of 50 mM TBA, the tubes were heated in a boiling water bath for 15 min, cooled, centrifuged and the absorbance of the MDA–TBA adduct was read at 535 nm [19]. Various agents, when included in the incubation mix, were added before initiation of lipid peroxidation.

In vivo *lipid peroxidation*. Male Wistar rats (150–250 g) were intubated with OA (6 mg/kg b.w.; p.o.) in sodium bicarbonate solution (10 mM; ~1 ml). Control rats received an equivalent amount of sodium bicarbonate alone. Lipid peroxidation was estimated by measuring the levels of ethane exhaled by the rats. For this purpose, control and treated rats (two per group) were immediately placed for 2 hr in separate all-glass exposure chambers (2 l. capacity) which contained sodalime for CO_2 absorption and a desiccant [20]. At timed intervals after the start of the experiment, the air within each chamber was stirred with the aid of a 50 ml syringe prior to withdrawal of 1 ml of air for analysis. Ethane concentrations were determined by gas chromatography using a Perkin–Elmer Sigma 2000 gas chromatograph equipped with a Porapak QS column (2 m; $\frac{1}{8}$″) and a flame ionization detector. Results are expressed as nM ethane in the desiccator/kg body weight of rats. The relatively short retention time of ethane (1.70 min) at an oven temperature of 80° permitted frequent samplings (~every 10 min) during the course of the experiment. Calibration was performed using known amounts of ethane.

RESULTS

Effect of OA on in vitro *lipid peroxidation*

Addition of OA to liver microsomes stimulated both the enzymatic and the chemically-induced lipid peroxidation. Figure 2 shows the time course of microsomal lipid peroxidation induced by a NADPH r.s., NADPH or ascorbate/Fe^{2+} in the presence or absence of 125 μm OA. In the absence of OA, no lipid peroxidation was observed for up to 60 min in the presence of a NADPH r.s. (Fig. 2A). When initiated by NADPH (Fig. 2B) or ascorbate/Fe^{2+} (Fig. 2C), the onset of lipid peroxidation was rather slow. Thus, at the end of 20 min, lipid peroxidation initiated by NADPH or ascorbate/Fe^{2+} resulted in the formation of only 1.1 nmol and 0.65 nmol of MDA per mg protein while at the end of 60 min, the corresponding MDA values were 10.17 nmol and 5.83 nmol respectively. In contrast, addition of 125 μM OA led to a rapid stimulation of MDA formation. Thus, at the end of 20 min, approximately 1.77 nmol, 18.96 nmol and 12.65 nmol of MDA were formed when lipid peroxidation was initiated by a NADPH r.s., NADPH or ascorbate/Fe^{2+} respect-

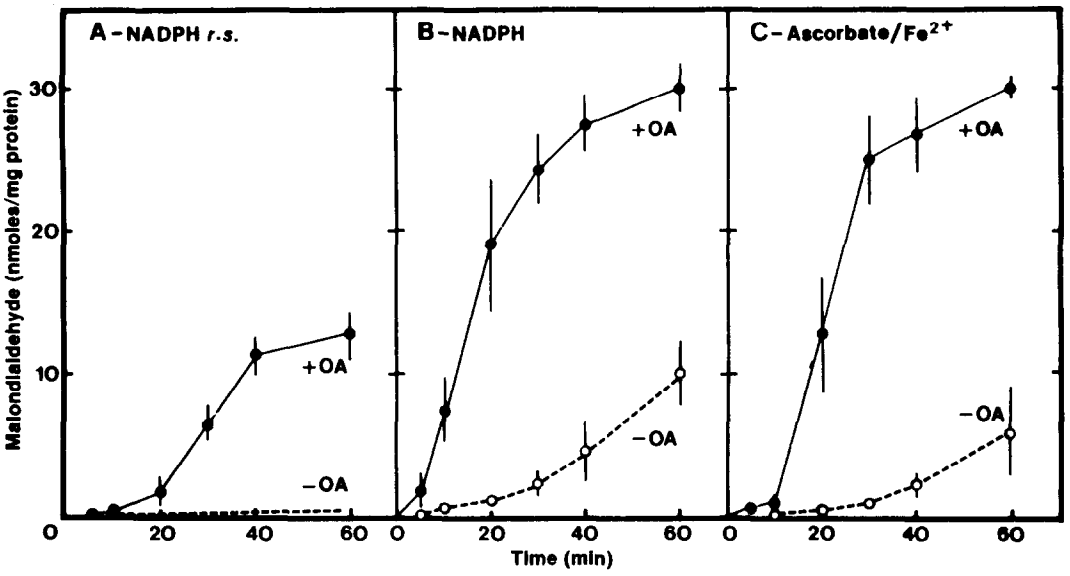

Fig. 2. Enhancement of liver microsomal lipid peroxidation in the presence of OA. Incubations were carried out in 0.1 M phosphate buffer (pH 7.4) and contained (per ml): 2 mg microsomal protein and 125 nmol of OA. Lipid peroxidation was initiated by the addition of a NADPH regenerating system (A), NADPH (1 mM final conc.; B) or Ascorbate/Fe^{2+} (1 mM/5 μM final conc.; C). Details are as described in Materials and Methods. Each point represents the mean ± SD of duplicate incubations from at least five different experiments.

ively (Fig. 2). Maximum MDA values of around 30 nmol/mg protein were observed at the end of 60 min but only in the presence of NADPH or ascorbate/Fe^{2+} (Fig. 2B and 2C).

The effect of varying concentrations of OA on stimulation of lipid peroxidation is shown in Fig. 3. Maximum stimulation of lipid peroxidation initiated by NADPH or ascorbate/Fe^{2+} required the presence of 60 μM or 125 μM OA respectively. These values pertain only to the 20 min time point shown in Fig. 3 and are likely to be lower if lipid peroxidation is measured for longer time periods.

Table 1 shows the effect of a variety of agents—active oxygen scavengers, antioxidants, cytochrome P-450 inhibitors and a Fe^{3+} chelator—on NADPH and ascorbate/Fe^{2+}-dependent MDA formation in the presence of 125 μM OA. In general, there was no effect of SOD, catalase, mannitol or dimethyl-

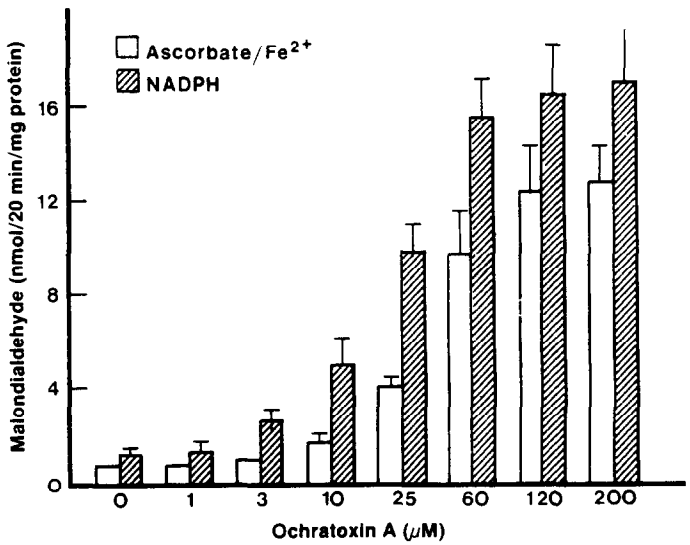

Fig. 3. Effect of the OA concentration on lipid peroxidation. Incubations were carried out for 20 min in 0.1 M phosphate buffer (pH 7.4) and contained 2 mg/ml microsomal protein and varying amounts of OA (0–200 μM). Each bar represents the mean ± SD of duplicate incubations from two different experiments.

Table 1. Effect of various agents on ochratoxin A stimulated malondialdehyde formation

Addition to system	Malondialdehyde formed* NADPH	Ascorbate
No addition	(100)	(100)
SOD (20 μg)	97	96
Catalase (20 μg)	103	98
Mannitol (100 mM)	93	104
Dimethylsulfoxide (100 mM)	89	96
BHT (25 μM)	0	0
Desferal (50 μM)	1	1
Metyrapone (500 μM)	110	102
α-Naphthoflavone (100 μM)	9	46

* Incubations were carried out in duplicate for 20 min at 37° in 1 ml of 0.1 M phosphate buffer (pH 7.4) and contained 2 mg microsomal protein and 125 μM OA. The various agents were added as aqueous solutions or in 5 μl of methanol. Lipid peroxidation was initiated by the addition of 1 mM NADPH or 1 mM ascorbate + 5 μM $FeSO_4$. All concentrations indicated are final concentrations in the reaction medium. Details are as described in Materials and Methods.

Values are expressed relative to "No addition" taken as 100, which corresponded to 16.9 and 12.2 nmols MDA/20 min for NADPH and ascorbate respectively. The entire experiment was repeated with similar results.

sulfoxide indicating lack of involvement of free superoxide anions, hydrogen peroxide or hydroxyl radicals. As expected, the antioxidant BHT and the iron chelator desferal [21] virtually abolished lipid peroxidation. The cytochrome P-450 inhibitor metyrapone was without any effect but α-naphthoflavone (100 μM) inhibited the NADPH-dependent lipid peroxidation by 90%. However, the inhibitory effect of α-naphthoflavone is probably due to its antioxidant properties since it also inhibited the ascorbate/Fe^{2+} dependent lipid peroxidation by 54%.

Lipid peroxidation by various OA analogues and metabolites

Table 2 compares the ability of OA, OB (no Cl at position 5), OC (OA methyl ester), OM-OC (O-methyl-OA-methylester), Oα (hydrolyzed OA; 5-chloro-3,4-dihydro-8-hydroxy-3-methyl-isocoumarin-7-carboxylic acid) and 4-OH-OA in stimulating lipid peroxidation in the presence of NADPH or ascorbate/Fe^{2+}. Oα and OM-OC each possessed very feeble activity and produced a marginal stimulation (~25%) at the end of 60 min in the case of both NADPH and ascorbate/Fe^{2+}. OB was partially effective; its response was much slower than that of OA but it produced the same amount of MDA at the end of 60 min. Only OC was able to stimulate the NADPH-dependent lipid peroxidation at a rate comparable to that of OA while its ability to stimulate the ascorbate/Fe^{2+} dependent lipid peroxidation exceeded that of OA. A mixture of the two OA metabolites (4*S*)-4-OH-OA and (4*R*)-4-OH-OA (in a ratio of 1:2) tested at a concentration of 2.5 μM, was completely devoid of any activity. The concentration of 2.5 μM was chosen since this is the sum of the two epimers formed at the end of 60 min under our incubation conditions (data not included).

Alteration of the pH exerted a considerable influence on the ability of OA and its analogues to stimulate lipid peroxidation (Fig. 4). In the NADPH-dependent lipid peroxidation, OA exhibited a typically bell-shaped response with maximum activity at pH 7.0. OC also behaved similarly showing maximum activity at pH 7.0, although it had high activity

Table 2. Ability of various ochratoxin A analogues and metabolites to stimulate lipid peroxidation

Compound (125 μM)	Malondialdehyde (nmol/mg protein) NADPH 10 min	NADPH 20 min	NADPH 60 min	Ascorbate 10 min	Ascorbate 20 min	Ascorbate 60 min
None	0.4	1.2	11.5	0.1	0.8	8.2
OA	8.9	21.5	31.8	2.1	16.0	30.2
OB	1.6	10.3	29.7	0.3	3.2	30.0
OC	8.1	19.0	31.5	5.3	21.6	31.3
OM-OC	0.5	1.8	15.7	0.3	2.1	10.1
Oα	0.4	2.6	15.4	0.3	1.1	10.3
4-OH-OA (2.5 μM)	0.4	1.3	10.9	0.2	1.0	8.9

Incubations were carried out in duplicate as described in Materials and Methods. All concentrations represent final concentrations in the reaction medium. Values shown are an average of duplicates which differed from each other by less than 10%. The experiment was repeated in its entirety with similar results.

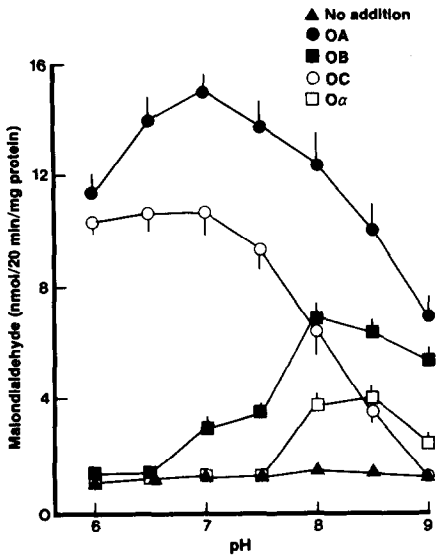

Fig. 4. Effect of pH on enhancement of lipid peroxidation by the various ochratoxins. Incubations were carried out for 20 min in 0.1 M phosphate buffer of different pHs (6.0–9.0) in the presence of 2 mg/ml microsomal protein. Each of the 4 ochratoxins was tested at a concentration of 125 μM in each buffer. Results are means ± SD of duplicate incubations from two separate experiments.

at pH 6.0 and 6.5 as well. In contrast, OB showed no activity at pH 6.0 and 6.5 and only a modest increase at pH 7.0 and 7.5 before peaking at pH 8.0 and then declining. Oα demonstrated maximum activity at pH 8.5 and good activity at pH 8.0 but was devoid of activity at any other pH (Fig. 4).

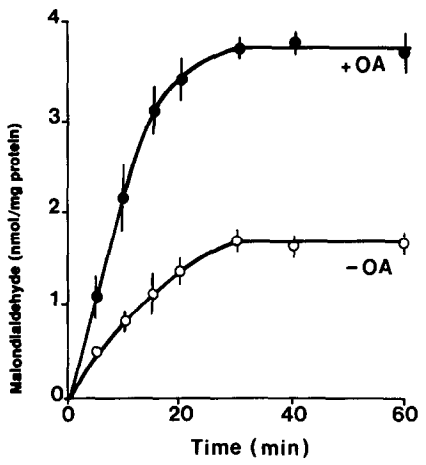

Fig. 5. Enhancement of kidney microsomal lipid peroxidation in the presence of OA. Incubations were carried out in 0.1 M phosphate buffer (pH 7.4) and contained (per ml): 2 mg kidney microsomal protein and 125 nmol of OA. Lipid peroxidation was initiated by the addition of a NADPH regenerating system. Details are as described in the Materials and Methods. Each point represents the mean ± SD of duplicate incubations from two separate experiments.

Addition of OA to kidney microsomes also enhanced the rate of NADPH dependent MDA formation (Fig. 5). However, this increase was modest (~3-fold) in comparison to the enhancement observed with liver microsomes.

Effect of OA on in vivo *lipid peroxidation*

Figure 6 compares the ability of control and OA treated rats to exhale ethane. Control rats, having

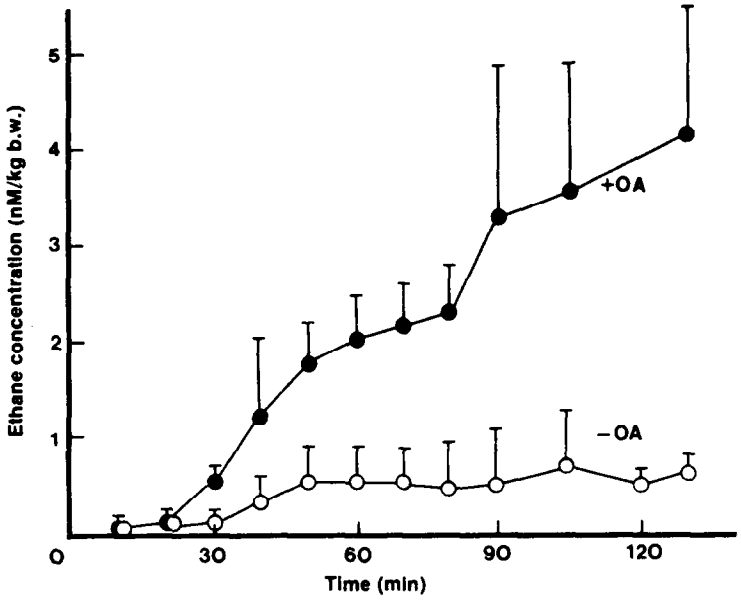

Fig. 6. Effect of OA administration on exhalation of endogenous ethane. Male Wistar rats were injected with OA (6 mg/kg b.w.) in 10 mM sodium bicarbonate or with sodium bicarbonate alone just before being placed (2 rats/group) in a closed exposure chamber. Exhalation of ethane into the atmosphere of the chamber was measured at 10 min intervals by gas chromatography as described in Materials and Methods. Data are expressed as nM ethanc/kg b.w. and each point represents the mean ± SD of six rats.

received only the vehicle 10 mM sodium bicarbonate, exhaled low amounts of ethane at a constant rate throughout the duration of the experiment (0.6 nM/kg b.w. at the end of 130 min). In contrast, rats treated with OA (6 mg/kg b.w.) commenced exhaling larger amounts of ethane after about 20 min and this level steadily increased by 130 min to an average of 4.15 nM/kg b.w. or about seven times that of control rats. Measurement of ethane exhalation was discontinued at the end of this time period because of possible discomfort to the animals in the enclosed glass chamber. The same animals, when reintroduced into their respective chambers after staying for 2 hr in a normal cage, still gave an ethane exhalation ratio of about 7:1 (OA treated:control) at the end of 6 hr. However, this ratio fell to about 2.5:1 at the end of 26 hr of OA treatment (data not shown).

DISCUSSION

The results of the present study clearly show that OA possesses the ability to enhance lipid peroxidation when added to liver or kidney microsomes *in vitro* or when administered to rats *in vivo*. *In vitro* lipid peroxidation was monitored by the TBA method which measures mostly MDA formation. Although a good correlation between MDA formation and other indices of lipid peroxidation like chemiluminescence, formation of fluorescent products and evolution of ethane has been observed [22], uncritical use of MDA measurement is sometimes believed to lead to artifactual results [23]. For this purpose, we have also, on occasion, measured fluorescent products [24] and have observed a similar pattern of induction (data not included). In any case, the large stimulation in ethane exhalation observed in OA-treated rats (Fig. 6) lends strong support to the observation that OA stimulates lipid peroxidation. It is now universally accepted that measurement of ethane exhalation represents a sensitive, convenient and non-invasive monitor for *in vivo* lipid peroxidation [22, 23, 25]. However, the method does not identify the organs responsible for ethane production.

We have measured the stimulatory effect of OA on the NADPH-dependent microsomal lipid peroxidation in phosphate buffer and in the absence of added iron. These conditions are standard for a study of the mixed function oxidation of a variety of xenobiotics [26, 27] including OA [12, 13]. However, they differ significantly from the usual conditions used to study lipid peroxidation, i.e. Tris–chloride buffer and the presence of 1–2 mM ADP and 40–100 μM Fe^{2+} [28, 29]. Isolated microsomes are known to contain small amounts of iron [30] and it is clear that this iron mediates the OA stimulated lipid peroxidation since addition of desferal, a known chelator of iron [21], completely abolished MDA formation (Table 1). In the absence of OA, there was a considerable lag before the NADPH-dependent peroxidation proceeded (Fig. 2). This is probably due to unavoidable auto-oxidation which is accelerated by NADPH-dependent enzymes, with release of traces of iron from the heme moieties of cytochrome P-450 or contaminating hemoglobin [23]. Even though the ascorbate-dependent lipid peroxidation was carried out in the presence of 5 μM Fe^{2+}, the rate of peroxidation was slow in the absence of added OA (Fig. 2). This may be due to the formation of a weak association between iron and phosphate of the buffer which may impair the reactivity of iron towards microsomal lipids [30]. The low rate of lipid peroxidation observed in the presence of the NADPH r.s. as opposed to NADPH was at first puzzling. A detailed investigation of the effect of the individual components of the NADPH r.s. on MDA formation (data not shown) revealed that the enzyme isocitric dehydrogenase (Sigma, type IV from bovine heart) was the cause of the inhibition. This is most likely due to the presence of EDTA in the enzyme solution provided by the manufacturer. In any case, this does not negate the results obtained.

It is now generally accepted that the NADPH-dependent microsomal lipid peroxidation involves the flavoprotein NADPH–cytochrome P-450 reductase which serves to reduce Fe^{3+} to Fe^{2+}, either free or chelated [23, 28]. Ascorbate linked lipid peroxidation is largely non-enzymic since ascorbate can chemically reduce Fe^{3+} to Fe^{2+}. The lack of inhibition of lipid peroxidation observed in the presence of metyrapone, a commonly used cytochrome P-450 inhibitor (Table 1), is in agreement with the fact that cytochrome P-450 is not involved. Inhibition of MDA formation by α-naphthoflavone, another cytochrome P-450 (P-448) inhibitor, is probably due to its antioxidant properties since it also inhibited the ascorbate dependent reaction in boiled microsomes by about 50% (data not shown). Miles *et al.* [31] have shown that a number of cytochrome P-450 substrates including aniline, SKF-525A, aminopyrine, benzo(*a*)pyrene and ethylmorphine all inhibit lipid peroxidation due to their antioxidant properties. Similarly, the lack of effect of superoxide dismutase, catalase, mannitol or dimethyl sulfoxide rules out dependence on freely diffusible active oxygen species like superoxide anions, hydrogen peroxide or hydroxyl radicals. These findings are in complete agreement with previously published reports [23, 28, 32].

A comparison of the abilities of OA and its analogues in stimulating lipid peroxidation (Table 2) yields some interesting features. The importance of the phenolic hydroxyl group at C-8 is readily apparent from the fact that OA, OB and OC, but not OM-OC which has the C-8 phenolic group blocked, are able to enhance lipid peroxidation with varying degrees of efficiency. Similarly, the presence of a chlorine atom at C-5 appears to be important since the lack of it in OB results in a substantial reduction of the ability to enhance MDA formation as compared to OA or OC (Table 2). In like manner, the importance of phenylalanine (or perhaps the absence of a free carboxyl group at C-7) is evident from the observation that OA, OB and OC, but not Oα, possess the ability to stimulate lipid peroxidation.

Chu *et al.* [33] studied the structural requirements for ochratoxin intoxication in one-day-old chicks. They found that the LD_{50}s of OA, OC and OB were ~150 μg, 216 μg and 1900 μg, respectively. In their assay, OM-OC and Oα were found to be non-toxic. This is precisely the order of potency we observed

of the same ochratoxins with respect to enhancement of lipid peroxidation (Table 2). Chu *et al.* [33] attributed the differences in the toxicity of the various ochratoxins to the reactivities of the phenolic group at C-8. They measured the apparent dissociation constants of the phenolic hydroxyl of OA, OB, OC and Oα and found them to be 7.05, 7.95, 7.14 and 11.0 respectively [33, 34]. We therefore examined the effect of pH on the ability of the various ochratoxins to enhance lipid peroxidation. From Fig. 4 it is clear that NADPH-dependent MDA formation is maximal at pH 7.0 in case of OA and OC and at pH 8.0 for OB. The activity with Oα is low but peaks at around pH 8.5. It would thus seem that ionization of the phenolic hydroxyl group is important for enhancement of lipid peroxidation and a ratio of 1:1 for the ionized: protonated phenolic hydroxyl group appears to be optimum. Wills [35] has shown that the NADPH dependent microsomal lipid peroxidation exhibits a broad pH optimum between 6.0 and 7.4 but that the activity declines at higher pHs. It is thus likely that the lower activity observed with Oα at pH 9.0 is probably due to a strong inhibition of lipid peroxidation at this pH. When the experiment was repeated with ascorbate/Fe^{2+}, no definitive results were obtained (data not included). This may be due to the fact that ascorbate-dependent lipid peroxidation has a sharp pH optimum of 6.0 and that the activity declines rapidly at more alkaline pHs [35].

A great number of organic chemicals including therapeutic drugs have been shown to stimulate lipid peroxidation *in vitro* and *in vivo* [23 for review]. Prominent among these are the haloalkanes including carbon tetrachloride. In general, redox cycling compounds (e.g. paraquat, adriamycin, bleomycin, nitrofurantoin) are metabolized by enzymes to radical intermediates which easily re-oxidize in the presence of molecular oxygen yielding active oxygen species which are believed to initiate lipid peroxidation [23]. However, the role of lipid peroxidation in the toxicity of redox cycling compounds is still controversial. In preliminary experiments, we have observed no enhancement of superoxide anion formation in the presence of OA (data not shown). In the case of carbon tetrachloride or cocaine, the agent initiating lipid peroxidation appears to be a reactive metabolite of the substrate formed in a cytochrome P-450 dependent reaction [36–38]. In the case of OA, cytochrome P-450 appears not to be involved since enhancement of lipid peroxidation was observed in the ascorbate dependent system as well (Fig. 2). Also, a mixture of (4*S*)- and (4*R*)-4-hydroxy-OA, the only metabolites of OA known to be formed by rat liver microsomes [12], completely failed to enhance lipid peroxidation, at least in the concentrations they are formed in during microsomal incubations. It is thus tempting to speculate that OA enhances microsomal lipid peroxidation by facilitating the NADPH or ascorbate-dependent reduction of Fe^{3+} (ferric) ions to Fe^{2+} (ferrous) ions and/or by facilitating the initiation step of lipid peroxidation (Fig. 7). A chelated form of Fe^{3+}–O_2^+ has been proposed [28, 39] as the initiating species of lipid peroxidation. In preliminary experiments, we have obtained spectral evidence for the formation of a complex between OA and Fe^{3+} much akin to that observed between doxorubicin and Fe^{3+}. However, it must be emphasized that the mechanism of OA induced lipid peroxidation *in vivo* may be different and could involve the mediation of cytochrome P-450.

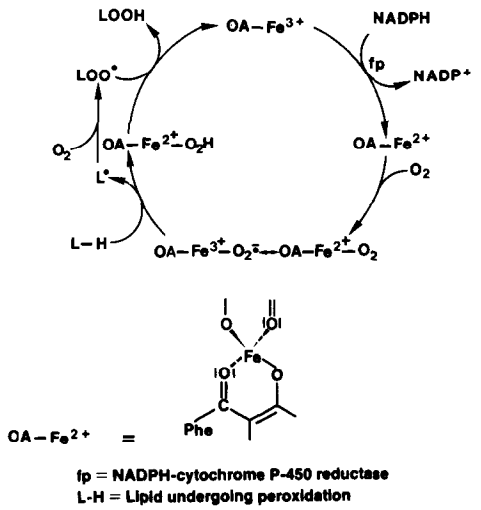

Fig. 7. Scheme showing possible sites of enhancement of lipid peroxidation by OA. OA–Fe^{3+} represents the iron–ochratoxin A complex which is reduced enzymatically by the flavoprotein NADPH–cytochrome P-450 reductase. All subsequent steps including the binding of oxygen, the formation of lipid radicals and lipid hydroperoxides are non-enzymatic.

The peroxidation of polyunsaturated fatty acids present in the membrane lipids has been proposed as a mechanism by which a number of foreign compounds produce structural tissue injury [40–42]. For certain chemicals, such as haloalkanes, it is clear that lipid peroxidation may have a critical role in bringing about tissue damage. However, for others like bromobenzene and paracetamol, the importance of lipid peroxidation is a matter of controversy [43–45] and it has been suggested that lipid peroxidation in such cases is a consequence of cell death rather than its cause.

At present, we are actively investigating the mechanism of OA enhanced lipid peroxidation both *in vitro* and *in vivo*. Despite the good correlation observed between toxicity in chicks and the potency of ochratoxins to induce lipid peroxidation, it remains to be demonstrated whether or not the toxic effects observed in rodents following OA administration are due to the onset of lipid peroxidation.

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