The role of lipid peroxidation in the *N*-oxidation of 4-chloroaniline

Ines GOLLY, Peter HLAVICA* and Jutta WOLF

*Institut für Pharmakologie und Toxikologie der Universität, Nussbaumstrasse 26, D-8000 München 2,
Federal Republic of Germany*

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1. Irradiation with u.v. light of aerobic aqueous media containing both rabbit liver microsomal fraction and 4-chloroaniline results in *N*-oxidation of the arylamine. The reaction is severely blocked by exhaustive extraction with organic solvents of the microsomal membranes to remove lipids. Further, scavengers of $\text{OH}^\bullet$ and $\text{O}_2^{\bullet-}$ impair the photochemical process. These findings suggest that the observed phenomenon may be closely associated with light-induced lipid peroxidation. Indeed, *N*-oxidation of 4-chloroaniline is fully preserved when either phospholipid liposomes or dispersed linoleic acid substitute for intact microsomal fraction. 2. Co-oxidation of the amine substrate occurs during iron/ascorbate-promoted lipid peroxidation also, but H_2O_2 or free $\text{OH}^\bullet$ radicals do not appear to be involved. 3. Cumene hydroperoxide-sustained rabbit liver microsomal turnover of the amine generates *N*-oxy product via O_2 -dependent and -independent pathways; propagation of lipid peroxidation is presumed to govern the former route. 4. Lipid hydroperoxides, either exogenously added to rabbit liver microsomal suspensions or enzymically formed from arachidonic acid in ram seminal-vesicle microsomal preparations, support *N*-oxidation of 4-chloroaniline. The significance, in arylamine activation, of lipid peroxidation in certain extrahepatic tissues exhibiting but low mono-oxygenase activity is discussed.

The metabolic route of *N*-oxidation of aromatic amines has been recognized to serve in the formation of more toxic and/or carcinogenic products (Kriek, 1974; Lenk & Sterzl, 1982). In mammalia, two microsomal oxygenases, namely cytochrome *P*-450 (EC 1.14.14.1) and the flavin-containing mono-oxygenase (EC 1.14.13.8), both of which are located predominantly in the liver, have been shown to catalyse oxidative attack on nitrogen-bearing groups in arylamines. Although peroxidases, too, can utilize aromatic amines as oxidizable co-substrates in the reduction of H_2O_2 , the importance, in mammalian systems, of such a function is still under debate. The subject has been reviewed in detail (Hlavica, 1982).

In addition to these pathways, arylamines might undergo *N*-oxidation during lipid peroxidation arising from either autooxidative breakdown of membrane lipids (Tappel, 1982) or stereospecific bis-dioxygenation by prostaglandin endoperoxide synthetase (EC 1.14.99.1) of arachidonic acid (Marnett & Eling, 1983). Either of these mech-

anisms may provide a complementary means for amine activation in many extrahepatic tissues exhibiting but low mono-oxygenase activity.

With the use of 4-chloroaniline as a model substrate, we now report on experiments designed to analyse the feasibility of *N*-oxidation of aromatic amines during lipid peroxidation promoted by various mechanisms in liposomal and microsomal preparations. Possible biological implications are discussed.

Materials and methods

Chemicals

Chemicals used in the present study were obtained from the following sources: ADP and catalase (EC 1.11.1.6) from Boehringer (Mannheim, Germany); superoxide dismutase (EC 1.15.1.1), indomethacin, arachidonic acid and linoleic acid from Sigma Chemie (München, Germany); 4-chloroaniline, D-mannitol and cumene hydroperoxide from Merck A.G. (Darmstadt, Germany). The hydroperoxide was purified by established procedures (Armstrong *et al.*, 1950).

* To whom correspondence should be addressed.

Preparation of microsomal fractions

Microsomal fractions from the livers of adult male and female Chinchilla rabbits, pretreated with phenobarbital (50 mg/kg body wt.) for 7 consecutive days, were prepared by the method of Jagow *et al.* (1965). Ram seminal-vesicle microsomal fractions were prepared as described by Boyd *et al.* (1983). All microsomal suspensions were washed twice with ice-cold 0.15 M-KCl. As a routine, the preparations were stored at 4°C overnight.

Extraction of microsomal phospholipids and preparation of lipid hydroperoxides

Phospholipids were isolated from rabbit liver microsomal fractions by the procedure of Folch *et al.* (1957). The final chloroform/methanol extract was concentrated to near-dryness and stored at -20°C under N₂. For preparation of liposomes, 10 mg of the material was suspended in 2 ml of 0.05 M-Tris/HCl buffer, pH 7.4, and treated for three 15 s intervals in an MSE ultrasonic disintegrator operated at full output. Linoleic acid was dispersed analogously.

Lipid hydroperoxides were prepared from isolated rabbit liver microsomal phospholipids by Methylene Blue-sensitized photo-oxidation as described by Watabe *et al.* (1982). The final condensate was taken into methanol and stored at -20°C in the dark.

Assay media

Standard assay mixtures containing 0.5 mg of rabbit liver microsomal protein/ml of suspension and 3 mM-4-chloroaniline in 0.15 M-KH₂PO₄/Na₂HPO₄ buffer, pH 7.4, were placed into quartz cuvettes (optical pathlength 1 cm) and mixed by stirring. Irradiation of the samples, positioned at a distance of 10 cm from the light-source, was carried out for 5 min at 25°C with a low-pressure mercury lamp (type NN 15/44; Haereus, Hanau, Germany) emitting at the wavelength of 254 nm; under these operating conditions, the radiant flux density was 6 mW/cm². At the end of the irradiation period, samples of the incubation media were removed and analysed for nitrosoarene. In some experiments, liposomes prepared from rabbit liver microsomal phospholipid or linoleic acid were substituted for the microsomal fraction, as specified in the appropriate legends to the Figures.

The assay media for measuring *N*-oxidation of 4-chloroaniline during iron-stimulated lipid peroxidation contained 1 mM-FeCl₃, 5 mM-ADP, 10 mM-ascorbic acid, 1 mM-4-chloroaniline and liposomal fraction equivalent to 0.9 mM rabbit liver microsomal phospholipid in 0.1 M-Tris/HCl buffer, pH 6.8. Reactions were carried out for 20 min at 37°C.

Organic-hydroperoxide-sustained microsomal *N*-oxidation of 4-chloroaniline was conducted in reaction mixtures containing rabbit liver microsomal fraction corresponding to 4 μM-cytochrome P-450, 10 mM-4-chloroaniline and 0.15 M-KH₂PO₄/Na₂HPO₄ buffer, pH 7.4. The samples were supplemented with either cumene hydroperoxide (final concentration 2 mM) or lipid hydroperoxide (final concentration 0.15–0.60 mM) dissolved in a small volume of methanol and incubated at 37°C for the times indicated.

Incubation mixtures for the study of prostaglandin endoperoxide synthetase-catalysed *N*-oxidative metabolism of 4-chloroaniline consisted of 0.1 M-KH₂PO₄/Na₂HPO₄ buffer, pH 7.8, 1 mM-4-chloroaniline, 0.1 mM-arachidonic acid and ram seminal-vesicle microsomal protein in the concentration range 0.2–1.0 mg/ml of suspension; indomethacin (final concentration 0.1 mM) was added where indicated. Reactions were carried out for 1 min at 37°C.

Analytical procedures

Cytochrome P-450 was measured as described by Omura & Sato (1964). Protein was determined by the method of Gornall *et al.* (1949) as modified by Szarkowska & Klingenberg (1963). Phospholipid was determined as indicated by Bartlett (1959). Lipid hydroperoxides and malondialdehyde were quantified as previously reported (Buege & Aust, 1978). 1-Chloro-4-nitrosobenzene was measured by the procedure of Herr & Kiese (1959).

Results and discussion

Light-induced microsomal *N*-oxidation of 4-chloroaniline

Irradiation with u.v. light of aerobic aqueous mixtures containing 4-chloroaniline and rabbit liver microsomal fraction results in oxidative transformation of the arylamine, as measured in terms of *N*-oxidation of the nitrogenous compound (Fig. 1). As can be seen, there is a strict dependence of the amount of *N*-oxy product formed on irradiation time, concentration of amine substrate and concentration of microsomal protein. Dilute microsomal suspensions form much more nitroso compound than do concentrated suspensions (Fig. 1b), and this may be a result of dispersal of the endoplasmic reticulum in the aqueous phase so that the membranes become extended and lie in close proximity to radicals generated in the aqueous medium (Wills, 1969).

The requirements for light-induced microsomal *N*-oxidation of 4-chloroaniline and the effects of various modifiers on the *N*-oxidation process are shown in Table 1. There is no appreciable turnover

Fig. 1. Dependence of light-induced *N*-oxidation of 4-chloroaniline on irradiation time, concentration of microsomal fraction and concentration of amine substrate

All reactions were carried out at 25°C in 0.15M-phosphate buffer, pH 7.4. Unless otherwise indicated, the media were supplemented with 0.5 mg of microsomal protein/ml of suspension (a and c) and 10 mM-4-chloroaniline (a and b); irradiation with u.v. light was performed for 5 min (b and c). The experimental points are means for three or four determinations.

Table 1. Light-induced rabbit liver microsomal *N*-oxidation of 4-chloroaniline

The assay media contained 3 mM-4-chloroaniline, rabbit liver microsomal protein (0.5 mg/ml of suspension), and the appropriate agent in 0.15M-phosphate buffer, pH 7.4. The samples were exposed to u.v. light for 5 min at 25°C. Results are means for three or four experiments.

System	Rate of <i>N</i> -oxidation (% of control)
Complete	100*
Dark control	<1
Minus microsomal fraction	13
Heated (5 min at 90°C)	178
Extracted with butanol/acetone†	11
Plus CoCl_2 (1 mM)	56
Plus mannitol (100 mM)	44
Plus ethanol (15%, v/v)	30
Plus superoxide dismutase (100 $\mu\text{g}/\text{ml}$)	46
Plus catalase (100 $\mu\text{g}/\text{ml}$)	132

* The control activity amounts to $7.9 (\pm 2.3 \text{ S.E.M.}) \mu\text{M}$ -1-chloro-4-nitrosobenzene formed/5 min.

† Freeze-dried rabbit liver microsomal fractions were homogenized twice with excess ice-cold butan-1-ol, rinsed with acetone and dried under vacuum.

of the arylamine in microsomal suspensions incubated in the dark; irradiation of the samples in the absence of microsomal fraction produces marginal amounts of nitroso analogue. This finding is consistent with reports by others (Pawlaczyk & Turowska, 1976; Sanniez & Pilpel, 1980) demonstrating the formation of *N*-oxidized products during photolysis of sulphanilamide in aqueous solution; it has been proposed that anilino radicals generated through photochemical activation of anilines in aqueous media might react with $\text{OH}^\bullet$ to yield the corresponding *N*-oxidized derivatives (Chignell *et al.*, 1980, 1981). The data presented in Table 1 confirm that the major part of the nitroso-arene formed arises from a reaction dependent on the presence of both light and microsomal fraction. This reaction is non-enzymic in nature, since heating of the microsomal preparations rather increases the *N*-oxygenating capacity of the system.

Attention is drawn to the role in the photolytic reaction of microsomal lipids: when experiments analogous to those described above are conducted with microsomal preparations depleted of lipid by treatment with organic solvents, the rate of *N*-oxidation is just the same as that in pure aqueous

media (Table 1). On the other hand, light-induced transformation of 4-chloroaniline is fully preserved when either liposomes made from extracted microsomal phospholipid or dispersed linoleic acid substitute for intact liver microsomal fraction, and this process is associated with lipid peroxidation, as evidenced by the production of malondialdehyde (Fig. 2). In accord with these observations, CoCl_2 , an inhibitor of lipid peroxidation (Wills, 1969), decelerates the photochemical reaction when catalysed in microsomal suspensions (Table 1). Similarly, light-dependent amine oxidation is blocked by the presence of mannitol, ethanol or superoxide dismutase (Table 1). This points at the involvement in the reaction of $\text{OH}^\bullet$ and $\text{O}_2^{\bullet-}$ species, known promoters of lipid peroxidation (Lai & Piette, 1978; Thomas *et al.*, 1982) most probably generated by photolysis of the air-saturated

Fig. 2. Light-induced *N*-oxidation of 4-chloroaniline and lipid peroxidation in phospholipid liposomes and micelles of linoleic acid

Reaction mixtures containing 3 mM 4-chloroaniline and various amounts of phospholipid liposomes in 0.15 M-phosphate buffer, pH 7.4, were exposed to u.v. light for 5 min at 25°C. The experimental points, which are means for three or four determinations, indicate the amounts of nitrosoarene (●) and malondialdehyde (○) formed during irradiation. In some experiments, linoleic acid (■) substituted for phospholipid.

aqueous incubation media (Holroyd & Bielski, 1978). The possibility that $\text{OH}^\bullet$ originates from an iron-catalysed Haber-Weiss reaction (McCord & Day, 1978) can be ruled out, since amine oxidation is insensitive to catalase (Table 1). Since high concentrations of the scavengers tested effect only partial inhibition of the *N*-oxygenating activity, additional mechanisms might serve in initiating lipid peroxidation. Thus ozone formed by irradiation of O_2 with u.v. light reacts with unsaturated fatty acids to produce radicals (Pryor, 1981).

Taken together, 4-chloroaniline undergoes co-oxidation during light-induced peroxidation of microsomal lipids (Wilbur *et al.*, 1949), but the detailed reaction sequence remains unclear. Lipid hydroperoxides (LOOH) themselves can be ruled out as possible mediators of *N*-oxidation of the arylamine, since incubation of 4-chloroaniline with LOOH does not yield appreciable amounts of nitrosoarene (Fig. 4). Hence, by virtue of its hydrogen-donor property, the amine substrate is presumed to interact with radical intermediates ($\text{L}^\bullet$, $\text{LO}^\bullet$, $\text{LOO}^\bullet$), and this explains the inhibitory action of aniline on lipid peroxidation (Miles *et al.*, 1980). Anilino radicals generated in this way may be trapped by O_2 (Sanniez & Pilpel, 1980) or combine with $\text{OH}^\bullet$ (Chignell *et al.*, 1981) to form *N*-oxy product.

Ascorbate- and hydroperoxide-dependent *N*-oxidation of 4-chloroaniline in liposomes and microsomal fractions

Further studies on the role of lipid peroxidation in the bioactivation of 4-chloroaniline were carried out with reaction media consisting of liposomes, ADP-chelated Fe^{3+} and ascorbic acid. Similar model systems have been found to be effective in promoting lipid peroxidation (Ottolenghi, 1959; Devasagayam *et al.*, 1983), and their biological significance is inferred from the observation that small amounts of non-protein-bound iron, complexed with phosphate or phosphate esters, are indeed available to intracellular membranes (Halliwell & Gutteridge, 1984); moreover, the concentration of ascorbate appears to control the extent of lipid peroxidation *in vivo* (Leung *et al.*, 1981).

The data summarized in Table 2 show that co-oxidation of 4-chloroaniline is strongly dependent on the presence of both microsomal phospholipid and ascorbic acid. High concentrations of catalase and mannitol do not inhibit this process, disproving the involvement of H_2O_2 or free $\text{OH}^\bullet$. This finding is compatible with results reported by Svingen *et al.* (1979); we propose that, in the presence of reducing equivalents, ADP- Fe^{3+} initiates lipid peroxidation via an ADP-iron-oxygen complex.

Table 2. *Effect of modifiers on the ADP-Fe³⁺-dependent N-oxidation of 4-chloroaniline in phospholipid liposomes*

The incubation mixtures contained 1 mM-FeCl₃, 5 mM-ADP, 10 mM-ascorbic acid, 1 mM-4-chloroaniline and liposomal fraction equivalent to 0.9 mM-phospholipid in 0.1 M-Tris/HCl buffer, pH 6.8. Reactions were carried out for 20 min at 37°C. The data are the means for four to six determinations.

System	Rate of <i>N</i> -oxidation (% of control)
Complete	100*
Minus phospholipid	15
Minus ascorbic acid	17
Plus mannitol (100 mM)	78
Plus catalase (100 µg/ml)	144

* The control activity amounts to 0.7 (± 0.4 S.E.M.) µM-1-chloro-4-nitrosobenzene formed/20 min.

Simple complexes of iron as well as certain types of haem compounds, such as cytochrome *P*-450, propagate lipid peroxidation by catalysing decomposition of lipid hydroperoxides (LOOH) produced during the initiation reaction to yield reactive intermediates (LO[•], LOO[•]) that stimulate chain reactions by abstracting hydrogen atoms (Svingen *et al.*, 1979; Halliwell & Gutteridge, 1984). As it would be difficult to assess lipid hydroperoxide breakdown in a matrix where additional LOOH can be continuously formed, cumene hydroperoxide and a rabbit liver microsomal system were used to probe the co-oxidation of 4-chloroaniline during the propagation step. The choice of the organic hydroperoxide was based on its ability to undergo cytochrome *P*-450-catalysed homolytic scission generating cumyloxy (CuO[•]) radicals (Griffin, 1980), which may stimulate lipid peroxidation (Sies & Sommer, 1975).

When reaction mixtures containing liver microsomal fraction, 4-chloroaniline and cumene hydroperoxide are incubated under anaerobic conditions, there is a time-dependent transformation of the arylamine to the nitroso derivative (Fig. 3); boiling of the microsomal preparations abolishes the catalytic activity (results not shown). The observed turnover is ascribed to the peroxidase-type function of microsomal cytochrome *P*-450 mediating amine oxidation via an iron-bound active oxygen species arising from peroxide-bond cleavage (Hlavica *et al.*, 1983). When the reaction is allowed to proceed under an atmosphere of O₂, the rate of *N*-oxidation is significantly enhanced (Fig. 3). This favours a cascade in which an amino free radical formed through attack by a complex range of reactive propagation products, such as CuO[•], L[•], LO[•] and LOO[•], incorporates molecular oxygen and the resultant intermediate is subsequently transformed into the end product. An alternative

Fig. 3. *Role of molecular oxygen during cumene hydroperoxide-sustained N-oxidation of 4-chloroaniline in rabbit liver microsomal fractions*

Rabbit liver microsomal fractions were diluted with 0.15 M-phosphate buffer, pH 7.4, to give a final concentration equivalent to 4 µM-cytochrome *P*-450. The suspensions were supplemented with 10 mM-4-chloroaniline and gassed for 5 min with either N₂ (○) or O₂ (●). Reactions were started by the addition of cumene hydroperoxide (final concentration 2 mM) and run at 37°C for the times indicated. The values are the averages ± S.E.M. of three experiments.

mechanism permitting insertion into the substrate molecule of atmospheric oxygen is offered by the reaction of L[•] with O₂ to yield endogenous lipid hydroperoxides serving as oxygen donors in the haemoprotein-dependent peroxidative *N*-oxidation of 4-chloroaniline. Indeed, exogenous lipid hydroperoxides prepared from native microsomal phospholipids sustain amine oxidation in intact microsomal fractions (Fig. 4). However, it has to be kept in mind that cytochrome *P*-450 is not unique in supporting hydroperoxide-mediated oxidations. Arachidonic acid is known to be converted enzymically into a series of hydroperoxy acids by the microsomal prostaglandin synthetase system, and this process is associated with co-oxidation of many foreign compounds (Marnett & Eling, 1983). In agreement with this, 4-chloroaniline undergoes arachidonate-dependent *N*-oxidation in ram seminal-vesicle microsomal preparations devoid of detectable amounts of cytochrome *P*-450 (Fig. 5), and the reaction is strongly blocked by the presence of indomethacin, a typical inhibitor of prostaglandin synthetase.

Fig. 4. Lipid hydroperoxide-supported *N*-oxidation of 4-chloroaniline in rabbit liver microsomal fractions

Rabbit liver microsomal preparations were adjusted with 0.15M-phosphate buffer, pH 7.4, to a final concentration equivalent to 4 µM-cytochrome *P*-450. After incorporation into the media of 10 mM-4-chloroaniline and various amounts of lipid hydroperoxide, the samples were incubated for 15 min at 37°C (●). Controls lacking microsomal fraction were run simultaneously (○). The data are means for three determinations.

The data presented throughout this work pose the question as to the significance of lipid peroxidation in the bioactivation of aromatic amines. Thus certain types of lipophilic arylamines known to produce skin tumours (Lenk & Sterzl, 1982) might enter the lipid phase of skin to undergo transformation into *N*-oxidized proximate carcinogens during lipid peroxidation triggered by exposure to u.v. light (Wilbur *et al.*, 1949) or sunlight. A good case can be also made that prostaglandin synthetase-catalysed lipid peroxidation plays a pivotal role in urinary-tract carcinogenicity of aromatic amines: the prostaglandin synthetase system is abundant in kidney medulla (Zenser *et al.*, 1979) and mediates *N*-oxidation of a series of arylamines, for which the urinary bladder is a target organ (Kadlubar *et al.*, 1982; Boyd *et al.*, 1983). Further studies are necessary to elucidate the biological importance of such mechanisms.

Fig. 5. Arachidonate-dependent *N*-oxidation of 4-chloroaniline in ram seminal-vesicle microsomal preparations

Ram seminal-vesicle microsomal suspensions in 0.1 M-phosphate buffer, pH 7.8, were fortified with 0.1 mM-arachidonic acid and 1 mM-4-chloroaniline and incubated for 1 min at 37°C in the absence (●) or in the presence (○) of 0.1 mM-indomethacin. The points are the averages for six experiments.

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