N-Oxidation of 4-chloroaniline by prostaglandin synthase

Redox cycling of radical intermediate(s)

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1. 4-Chloroaniline undergoes *N*-oxidation in ram seminal-vesicle microsomal preparations supplemented with arachidonic acid to yield *N*-(4-chlorophenyl)-hydroxylamine and 1-chloro-4-nitrosobenzene. H_2O_2 also supports metabolism of the amine substrate to the same organic-solvent-extractable products, suggesting that the hydroperoxidase activity of prostaglandin synthase is responsible for the co-oxidation. 2. Analysis of the reaction mixtures by e.s.r. spectrometry reveals the formation of a radical intermediate bearing the characteristics of a strongly immobilized nitroxide. 3. Arylamine-stimulated $\text{O}_2^{\cdot-}$ release can be observed when the arachidonic acid-containing incubation media are supplemented with NADPH. Redox cycling of the nitroxide/hydroxylamine couple is presumed to represent the major source of $\text{O}_2^{\cdot-}$, but additional mechanisms, such as redox changes of nitro anion radicals resulting from potential further metabolism of 1-chloro-4-nitrosobenzene, cannot be excluded. 4. The concerted action of carrier-bound nitroxides and $\text{O}_2^{\cdot-}$ in initiating damage of cellular macromolecules is discussed.

The metabolic route of *N*-oxidation of primary aromatic amines has been recognized to serve in the formation of more toxic and/or carcinogenic products (Weisburger & Weisburger, 1973). The most extensively studied enzyme catalysing bio-activation of this type of nitrogenous compounds is the cytochrome *P*-450-dependent microsomal mono-oxygenase system located predominantly in the liver (Hlavica, 1982).

Recently, interest has focused on microsomal prostaglandin synthase (EC 1.14.99.1), which may provide a complementary system for amine activation in many extrahepatic tissues containing only small amounts of mono-oxygenase(s). In fact, a series of aromatic amines have been detected to act as reducing cofactors for the hydroperoxidase activity of prostaglandin synthase, undergoing co-oxidation in this process to yield the corresponding nitroso and nitro derivatives (Kadlubar *et al.*, 1982; Boyd *et al.*, 1983). Moreover, one-electron oxidation of arylamines by this enzyme may give rise to formation of reactive electrophiles, such as amino free cation radicals (Joseph *et al.*, 1983),

and this is regarded as a crucial step leading to damage to cellular macromolecules.

With the use of 4-chloroaniline as a model substrate, we report on the occurrence, during prostaglandin synthase-mediated *N*-oxidation of the amine, of a free-radical species, which was identified by e.s.r. spectrometry as a strongly immobilized nitroxide. Further, NADPH-dependent $\text{O}_2^{\cdot-}$ production during co-oxidation of the arylamine is also reported.

Experimental

Chemicals

Chemicals used in the present study were obtained from the following sources: NADP^+ , NADPH, horse heart cytochrome *c*, glucose 6-phosphate and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from Boehringer (Mannheim, Germany); superoxide dismutase (EC 1.15.1.1) from bovine blood [specific activity, as defined by McCord & Fridovich (1969), 3000 units/mg of protein], arachidonic acid, indomethacin, succinic anhydride and 2,4,6-trinitrobenzenesulphonic acid from Sigma Chemie (Taufkirchen, Germany); 4-chloroaniline, 1-chloro-4-nitrosobenzene and

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1-chloro-4-nitrobenzene from Fluka A.G. (Neu-Ulm, Germany); 2,2,6,6-tetramethylpiperidine-*N*-oxyl and H_2O_2 (30%, v/v) from EGA-Chemie (Steinheim, Germany).

Preparation of microsomal fraction

Ram seminal vesicles were obtained from a slaughterhouse and stored at -20°C until use. Microsomal fractions were prepared after thawing of the tissue as described by Boyd *et al.* (1983). As a routine, the microsomal pellets were suspended in 0.1 M- $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.8, and stored at 4°C overnight. These preparations were devoid of detectable amounts of cytochrome *P*-450, but contained $0.65(\pm 0.03 \text{ S.E.M.}; n = 12)$ nmol of cytochrome *b*₅/mg of protein and $37(\pm 8 \text{ S.E.M.}; n = 8)$ units of NADPH-cytochrome *c* reductase (EC 1.6.2.4) per mg of protein, as measured by established procedures (Omura & Sato, 1964; Masters *et al.*, 1968); one unit of reductase activity is defined as 1 nmol of ferricytochrome *c* reduced/min.

Succinylation (3-carboxypropionylation) of cytochrome c

Partial succinylation of cytochrome *c* was performed as indicated by Finkelstein *et al.* (1981). The degree of modification of lysine residues in the cytochrome molecule, as estimated by titration with 2,4,6-trinitrobenzenesulphonic acid (Fields, 1972), ranged from 47 to 49%. The modified haemoprotein was reduced by detergent-solubilized NADPH-cytochrome *c* reductase at a rate 7% of that observed with native preparations; the reactivity towards $\text{O}_2^{\cdot-}$, as tested in a xanthine oxidase/xanthine system (Finkelstein *et al.*, 1981), was 47% that of untreated cytochrome *c*.

Microsomal incubations

The reaction mixtures for measuring microsomal *N*-oxidation of 4-chloroaniline contained 0.1 M- $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.8, 0.1 mM-arachidonic acid, 1 mM-4-chloroaniline and ram seminal-vesicle microsomal protein as indicated. The samples were incubated for 1 min at 37°C in the absence or in the presence of 0.1 mM-indomethacin. In some experiments, either 0.1 mM- H_2O_2 or NADPH-generating system composed as described below substituted for arachidonic acid.

The assay media for determining microsomal reduction of 1-chloro-4-nitrosobenzene consisted of 0.1 M- $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.8, 1.2 mM-NADP⁺, 10 mM-glucose 6-phosphate, 6 mM-MgCl₂, 5 μg of glucose-6-phosphate dehydrogenase/ml of mixture, 0.2 mM-nitroso compound and the amounts of ram seminal-vesicle microsomal fraction indicated. Reactions were carried out for 20 min at 37°C . Controls serving to assess

the extent of non-enzymic reduction by NADPH of the nitrosoarene were run simultaneously by using heat-inactivated microsomal preparations.

Spectral measurements

Substrate-induced NADPH-sustained formation of $\text{O}_2^{\cdot-}$ in microsomal preparations was analysed as follows: assay mixtures containing 0.5 mg of ram seminal-vesicle microsomal protein/ml of suspension, 0.1 mM-arachidonic acid, 0.1 mM-NADPH and 20 μM -succinylated ferricytochrome *c* in 50 mM- $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.7, were equally divided between two cuvettes. 4-Chloroaniline (final concn. 1 mM) was added to the contents of the sample cell, and the time course of change in A_{550} was recorded with a Cary 219 spectrophotometer at 25°C ; optical path length was 1 cm. A set of analogous experiments was carried out after incorporation into the contents of both cuvettes of superoxide dismutase (100 μg /ml of mixture). Alternatively, arachidonic acid was omitted from the incubation media, and reactions were started by the addition, in the absence or in the presence of $\text{O}_2^{\cdot-}$ scavenger, of either 1-chloro-4-nitrosobenzene (final concn. 10 μM) or 1-chloro-4-nitrobenzene (final concn. 1 mM) to the experimental cell.

Nitroxide free radicals were generated in a system containing 3.3 mg of ram seminal-vesicle microsomal protein/ml of suspension, 1.3 mM-arachidonic acid and 3.3 mM-4-chloroaniline in 0.1 M- $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.8. After incubation for 5 min at 25°C , the microsomal fraction was collected by centrifugation for 20 min at 100 000 *g*. The microsomal pellet was transferred to a quartz capillary tube, and e.s.r. spectra were recorded at room temperature with a Bruker ER 200 SRD spectrometer equipped with a TM cavity; 2,2,6,6-tetramethylpiperidine-*N*-oxyl was used as an external standard.

Other analytical procedures

Protein was determined by the method of Gornall *et al.* (1949) as modified by Szarkowska & Klingenberg (1963). 4-Chloroaniline was measured as indicated by Brodie & Axelrod (1948). *N*-Oxidized products were quantified as nitrosoarene detectable after treatment of the reaction mixtures with $\text{K}_3\text{Fe}(\text{CN})_6$ (Herr & Kiese, 1959).

Results and discussion

Arachidonic acid-dependent microsomal N-oxidation of 4-chloroaniline: detection of a nitroxide intermediate

Addition of 4-chloroaniline to aerobic ram seminal-vesicle microsomal suspensions supplemented with arachidonic acid results in *N*-

oxidation of the arylamine (Fig. 1); transformation of the amine substrate does not occur when heat-inactivated microsomal fraction is used (results not shown). *N*-Oxidation is severely blocked by the presence of indomethacin (Fig. 1), suggesting the metabolic process to be catalysed by the prostaglandin synthase system. In agreement with this, H_2O_2 applied instead of arachidonic acid also sustains amine oxidation (Fig. 1). Microsomal cytochrome *P*-450 can be ruled out to account for the observed peroxidase activity, since the microsomal preparations used were devoid of measurable amounts of the pigment. Accordingly, ram seminal-vesicle microsomal fractions fail to effect NADPH/ O_2 -dependent *N*-oxidation of 4-chloroaniline (Fig. 1), a reaction typical of cytochrome *P*-450 (Hlavica *et al.*, 1983).

Analysis of the incubation mixtures with and without prior $K_3Fe(CN)_6$ treatment reveals that 19% of the material trapped by the colorimetric reagent originates from *N*-(4-chlorophenyl)hydroxyl-

amine formed during arachidonic acid-supported co-oxidation of 4-chloroaniline, the rest being attributable to the corresponding nitroso derivative. The low steady-state concentration of the hydroxylamine suggests rapid conversion of the compound into the nitroso analogue via both enzyme-controlled and non-enzymic oxidations. This process gives rise to the formation of a radical intermediate characterized by the e.s.r. spectrum depicted in Fig. 2(c). The signal ($g = 2.0050$), exhibiting a marked high-field transition, is typical of a strongly immobilized nitroxide free radical (Floyd *et al.*, 1978; Ritter *et al.*, 1983). Support in favour of this comes from comparison of the metabolically generated spectrum with that of the unbound (Fig. 2a) and partially membrane-associated (Fig. 2b) stable 2,2,6,6-tetramethylpiperidine-*N*-oxyl radical used as an external standard. The enzymic nature of nitroxide formation is indicated by the inability of boiled microsomal fraction to sustain radical production (Fig. 2d).

Part of the nitroxide radicals formed might result from pseudo-Diels-Alder addition of 1-chloro-4-nitrosobenzene to unsaturated double bonds in fatty acids, according to the model proposed by Sullivan (1966). Indeed, Griffin (1980) observed a weak e.s.r. spectral change on incorporation of nitrosobenzene into rat liver microsomal fractions.

Substrate-induced microsomal formation of superoxide anion in the presence of arachidonic acid and NADPH

Microsomal adducts of aromatic nitroxides, together with the corresponding *N*-hydroxy derivatives, have been proposed to form redox couples serving in the NADPH/ O_2 -dependent production of superoxide anion (Stier *et al.*, 1980). The simultaneous occurrence, during prostaglandin synthase-catalysed *N*-oxidation of 4-chloroaniline, of hydroxylamine and nitroxide intermediates, as well as the occurrence, in ram seminal-vesicle microsomal fractions, of significant amounts of NADPH-cytochrome *c* reductase prompted us to investigate the amine-induced release of $O_2^{\cdot-}$ in aerobic microsomal preparations supplemented with arachidonic acid and NADPH.

Partially succinylated ferricytochrome *c* was used as a scavenger for $O_2^{\cdot-}$; the modified haemoprotein is known to be no longer recognized as a substrate by cytochrome *c* oxidase or cytochrome *b*₅-dependent reductase(s) (Kuthan *et al.*, 1982). Involvement of $O_2^{\cdot-}$ was ascertained by the application of superoxide dismutase (McCord *et al.*, 1977; Kuthan *et al.*, 1982). The high final concentration (100 μ g/ml) of the enzyme used affords maximal suppression of ferricytochrome *c* reduction, as evidenced by the ability of the enzymic scavenger to decrease the initial rate of electron transfer in

Fig. 1. *N*-Oxidation of 4-chloroaniline by ram seminal-vesicle microsomal fractions

Assay mixtures containing 0.1 mM-arachidonic acid, 1 mM-4-chloroaniline and various amounts of microsomal protein in 0.1 M-phosphate buffer, pH 7.8, were incubated for 1 min at 37°C in the absence (○) or in the presence (●) of 0.1 mM-indomethacin. In some experiments, either 0.1 mM- H_2O_2 (□) or NADPH/ O_2 (△) was applied in place of arachidonic acid. The data are means of three to six determinations.

Fig. 2. Typical 9.35 GHz *e.s.r.* spectrum arising from oxidation of 4-chloroaniline by prostaglandin synthase (a) Spectrum resulting from the addition of excess 2,2,6,6-tetramethylpiperidine-*N*-oxyl (final concn. 0.4 mM) to a suspension containing 3.3 mg of ram seminal-vesicle microsomal protein/ml of 0.1 M-phosphate buffer, pH 7.8. (b) Identical with (a), except that excess unbound spin label was removed by the addition to the assay medium of NiSO_4 (final concn. 200 mM). (c) Spectrum generated on the inclusion of 4-chloroaniline in a ram seminal-vesicle microsomal suspension supplemented with arachidonic acid; for details see the Experimental section. (d) Identical with (c), except that boiled microsomal fraction was used. The instrumental conditions were as follows: magnetic field, 0.33 T; scan range, 10 mT; modulation amplitude, 0.25 mT; nominal microwave power, 20 mW; receiver gain, 3.2×10^3 for (a), 16×10^4 for (b) and 16×10^5 for (c) and (d); scan time, 100 s; time constant, 0.1 s.

the systems described in Figs. 3(a) and 5(a) to the extent of unspecific, superoxide dismutase-insensitive, reduction of the electron acceptor (Fig. 3b); boiling of the dismutase abolishes its inhibitory action (results not shown).

It has been claimed that peroxidation of ferrocytochrome *c* by bovine blood superoxide dis-

mutase may give rise to faulty conclusions when the enzyme is added as a probe for $\text{O}_2^{\cdot-}$ to H_2O_2 producing systems, such as aerobic microsomal suspensions containing NADPH (Hodgson & Fridovich, 1975b). However, several lines of evidence show that this mechanism does not perturb our measurements. (a) Significant accumulation, in the presence of NADPH/ O_2 , of H_2O_2 in the assay mixtures is unlikely to occur, since incorporation into the media of the reduced nicotinamide nucleotide does not trigger *N*-oxidation of 4-chloroaniline, a reaction readily supported by H_2O_2 in ram seminal-vesicle microsomal fractions (Fig. 1). (b) Since HO_2^- rather than H_2O_2 is likely to combine with Cu,Zn superoxide dismutases to generate the reactive complex (Blech & Borders, 1983), significant peroxidative activity is only observed in the alkaline range of pH ($\text{pK}_{\text{a}}(\text{H}_2\text{O}_2) = 11.6$); at the H^+ concentration used in the present optical measurements (pH 7.7), the rate of peroxidative attack on sensitive groups is negligible (Hodgson & Fridovich, 1975a; Blech & Borders, 1983). (c) Superoxide dismutase-induced changes in A_{550} are balanced, owing to equal distribution between the sample and reference cell of the basic incubation mixtures. If part of the dismutase effects observed results from peroxidation of ferrocytochrome *c*, the amine substrate (Fig. 3a) as well as its metabolite (Fig. 5a) are expected to enhance H_2O_2 formation or facilitate oxidation of the succinylated haemoprotein. Stimulation by these compounds of auto-decomposition of oxygenated ferrocytochrome *P*-450 (Ullrich & Kuthan, 1980) can be dismissed as accounting for extra H_2O_2 production; as pointed out above, the microsomal membranes used throughout the present study were deficient in cytochrome *P*-450. Similarly, we were unable to detect interference by 4-chloroaniline with microsomal NADPH-cytochrome *c* reductase to augment the rate of auto-oxidation of the enzyme (Grover & Piette, 1981). Further, amines rather inhibit superoxide dismutase-mediated peroxidation of ferrocytochrome *c* by being peroxidized in competition with the latter (Hodgson & Fridovich, 1975b). In accord with these observations, superoxide dismutase fails to affect cytochrome *c* reduction when added to incubation media containing NADPH and 4-chloroaniline (Fig. 3b).

In the light of these results, the influence of superoxide dismutase on the arylamine-promoted reduction of modified ferricytochrome *c* (Fig. 3a) can be interpreted to reflect predominantly the involvement of $\text{O}_2^{\cdot-}$. The observed release of the oxygen free radical is believed to be closely related to oxidative transformation of the amine substrate by the prostaglandin synthase system. This view is supported by the inability of 4-chloroaniline to

Fig. 3. Amine-induced reduction of succinylated ferricytochrome *c* by ram seminal-vesicle microsomal preparations in the absence (○, □ and △) and in the presence (●, ■ and ▲) of superoxide dismutase

Ram seminal-vesicle microsomal suspensions supplemented with arachidonic acid, NADPH and succinylated ferricytochrome *c* as specified in the Experimental section were equally divided between two cuvettes. 4-Chloroaniline (final concn. 1 mM) was added to the contents of the sample cell and the change in A_{550} monitored at 25°C (a). In some experiments, either arachidonic acid (b) or NADPH (c) was omitted from the assay media. Where indicated, superoxide dismutase was added to give a final concentration of 100 µg/ml of mixture. The experimental points are means of three determinations.

elicit the formation of superoxide dismutase-sensitive material when arachidonic acid is omitted from the assay mixtures (Fig. 3b). Further, $O_2^{\cdot -}$ production is fully dependent on the presence of reducing equivalents (Fig. 3c). These findings hint at the functioning of a redox cycle.

As shown in Fig. 3, part of the succinylated ferrocycytochrome *c* formed arises from mechanism(s) insensitive to the presence of superoxide dismutase. These probably include direct electron transfer to cytochrome *c* from aminophenols (Nagasawa & Gutmann, 1959) generated along with the *N*-oxidized products through enzymic and non-enzymic ring hydroxylation of 4-chloroaniline or isomerization of the corresponding aryl-hydroxylamine (Booth & Boyland, 1964). On the other hand, some reduction of cytochrome *c* by nitroxide radicals cannot be excluded (Ritter *et al.*, 1983). It is noteworthy that, whatever the nature of the process(es) involved, reduction of ferricytochrome *c* proceeds at higher rate in the absence than in the presence of NADPH (cf. Figs. 3b and 3c). This phenomenon might be related to further NADPH-dependent metabolism of the compound(s) responsible for cytochrome *c* reduction; in the absence of NADPH, accumulation of the material might act to accelerate electron transfer to the haemoprotein.

Ultimate proof of the ability of *N*-oxidized derivatives of 4-chloroaniline to sustain NADPH-

Fig. 4. Enzymic reduction of 1-chloro-4-nitrosobenzene in ram seminal-vesicle microsomal fractions.

Reaction mixtures containing NADPH-generating system, 0.2 mM-1-chloro-4-nitrosobenzene and various amounts of ram seminal-vesicle microsomal protein in 0.1 M-phosphate buffer, pH 7.8, were incubated for 20 min at 37°C. The data are corrected for non-enzymic reduction by NADPH of the nitrosoarene. The experimental points are means of five determinations.

driven $O_2^{\cdot-}$ formation in ram seminal-vesicle microsomal fractions comes from experiments with 1-chloro-4-nitrosobenzene. Although oxygen is known to be inhibitory to NADPH-supported microsomal reduction of nitroso- and nitro-arenes (Hlavica, 1982), catalytic amounts of the nitroso compound undergo enzyme-controlled aerobic reduction to the parent amine at a measurable rate (Fig. 4). This metabolic event is associated with the release of $O_2^{\cdot-}$, most probably through autoxidation of the hydroxylamine intermediate, as evidenced by the marked inhibition by superoxide dismutase the reduction of succinylated ferricytochrome *c* (Fig. 5a).

It has to be kept in mind that metabolically formed nitrosoarene might be further oxidized in the arachidonic acid-containing reaction mixtures to yield the nitro analogue (Boyd *et al.*, 1983). Nitroaromatics, in their turn, can undergo NADPH-cytochrome *c* reductase-supported conversion into the corresponding nitro anion radical species (Holtzman *et al.*, 1981); these are attacked by oxygen, regenerating the parent drug and forming $O_2^{\cdot-}$ (Perez-Reyes *et al.*, 1980). Indeed, ad-

dition of 1-chloro-4-nitrobenzene to aerobic microsomal suspensions supplemented with NADPH results in some $O_2^{\cdot-}$ release (Fig. 5b). However, it should be noted that nitro anion radicals exhibit considerably higher reactivity than $O_2^{\cdot-}$ towards ferricytochrome *c* (Simic *et al.*, 1975), and this might simulate lower efficiency of superoxide dismutase.

In summary, our results establish evidence for the NADPH-dependent formation, during prostaglandin synthase-mediated co-oxidation of 4-chloroaniline, of superoxide anion. Although redox cycling of the nitroxide/hydroxylamine couple arising from *N*-oxidation of the arylamine is believed to represent the major source of $O_2^{\cdot-}$ (Scheme 1), additional mechanisms have to be envisaged. These include redox changes of nitro anion radicals potentially formed in the assay media or oxidation of metabolically generated aminophenols to the corresponding semiquinone-imine radicals. The latter might oxidize NADPH to the pyridinium free radical, which reacts with oxygen to produce $O_2^{\cdot-}$ (Mason & Chignell, 1982).

Although 4-chloroaniline belongs to the group of

Fig. 5. Nitrosoarene- and nitroarene-dependent reduction of succinylated ferricytochrome *c* by ram seminal-vesicle microsomal preparations in the absence (○ and □) and in the presence (● and ■) of superoxide dismutase

Ram seminal-vesicle microsomal suspensions supplemented with NADPH and succinylated ferricytochrome *c* as specified in the Experimental section were equally divided between two cuvettes. 1-Chloro-4-nitrosobenzene (a; final concn. 10 μ M) or 1-chloro-4-nitrobenzene (b; final concn. 1 mM) was added to the contents of the sample cell and the change in A_{550} recorded at 25°C. Where indicated, superoxide dismutase was included in the media to give a final concentration of 100 μ g/ml of mixture. The experimental points are means of three determinations.

Scheme 1. Production of $\text{O}_2^{\cdot-}$ through redox cycling of the nitroxide/hydroxylamine couple

non-carcinogenic aromatic amines, it can be regarded as a model with which to assess general principles governing the bioactivation of amine compounds. With respect to this, the occurrence, in our metabolic system, of a carrier-bound primary aromatic nitroxide is of special interest, since this type of adduct, owing to its longevity, has been proposed to combine with DNA in living cells to cause distortion of the macromolecule (Stier *et al.*, 1982). Additional damage of critical cellular constituents may be afforded by the simultaneous action of $\text{OH}^{\cdot}$ generated from $\text{O}_2^{\cdot-}$ via the iron-catalysed Haber–Weiss mechanism (McCord & Day, 1978). Further work is needed to elucidate the biological importance of such processes in arylamine-induced cytotoxicity.

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