Cytochrome P450 oxidase activity and its role in NADPH dependent lipid peroxidation

Aalt Bast, Jan W. Brenninkmeijer, E. Maria Savenije-Chapel and Jan Noordhoek

Department of Pharmacology and Pharmacotherapy, Faculty of Pharmacy, State University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht, The Netherlands

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A comparison is made between microsomal NADPH-dependent H_2O_2 production and malondialdehyde (MDA) formation in rat liver microsomes, obtained from phenobarbital pretreated rats. An increase in H_2O_2 formation was observed during NADPH-dependent disposition (10 min) of 100 μM diazepam (33%) and 2 mM hexobarbital (69%). In contrast orphenadrine (100 μM) and its mono-*N*-demethylated metabolite tofenacine (100 μM) decreased the H_2O_2 formation (35% and 55%, respectively). However, all these substrates were found to inhibit NADPH-dependent lipid peroxidation (60 min), estimated by measuring MDA formation, to various extents. These data strongly suggest that the oxidase activity of cytochrome P450 (H_2O_2 production) is not involved in a rate-limiting step in NADPH-dependent lipid peroxidation.

Cytochrome P450 oxidase *NADPH* *Lipid peroxidation*

1. INTRODUCTION

Three types of biochemical reactions have been established in which the cytochrome P450 system is involved [1]. The stoichiometry of these functions of cytochrome P450 is shown in fig.1. The monooxygenase function of cytochrome P450 has been studied extensively. The exact function and mechanism of the oxidase and peroxidase activity of cytochrome P450 is unknown. With regard to the oxidase activity however, it has been suggested that the H_2O_2 formed during drug metabolism, originates from the decay of oxycytochrome P450, via superoxide anion radicals [2]. Since the participation of several reduced forms of oxygen in NADPH-dependent lipid peroxidation has been proposed [3,4], the oxidase activity of cytochrome P450 might be involved in microsomal lipid peroxidation. In fact, cytochrome P450 has indeed been suggested to catalyze the propagation of lipid peroxidation [5]. However, the catalytic role of cytochrome P450 in peroxidation of lipid in liver microsomes as well as the participation of superoxide anion radicals in microsomal lipid peroxidation has also been challenged [6–8].

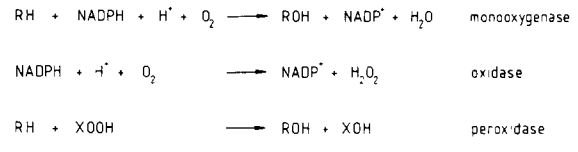

Fig.1. Proposed stoichiometry of the monooxygenase, oxidase and peroxidase functions of cytochrome P450: RH, ROH and XOOH are the substrate, the product and a peroxy compound, respectively.

Here, we compared the effect of several cytochrome P450 substrates on the oxidase activity of cytochrome P450 with their effect on NADPH-dependent lipid peroxidation. In this way the involvement of the oxidase activity in a rate-limiting step of NADPH-dependent microsomal lipid peroxidation was excluded. Furthermore, the results emphasize the regulatory function of substrates in the oxidase activity of cytochrome P450.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Orphenadrine and tofenacine were generous gifts from Gist-Brocades (Delft) and diazepam a gift from Hoffmann-La Roche (Mijdrecht). Hexobarbital (Evipan® –Natrium) was from Bayer (Leverkusen); thiobarbituric acid (monohydrate) from E. Merck (Darmstadt); $NADP^+$, glucose 6-phosphate (disodium salt), glucose 6-phosphate dehydrogenase and catalase (from beef liver) were obtained from Boehringer (Mannheim). All other chemicals used were of analytical grade purity.

2.2 *Preparation of microsomes and pretreatment of animals*

Hepatic microsomes were prepared from 250–300 g male Wistar rats as in [9]. Phenobarbital treatment consisted of 3 daily i.p. injections (80 mg/kg). On day 4 the rats were killed by decapitation.

2.3. *Enzyme assays*

The incubation conditions were essentially identical for all measurements. Microsomes (from 1/12 g liver/ml incubate) were incubated at 37°C with shaking air being freely admitted. The incubate (in 50 mM phosphate buffer (pH 7.4) with 0.1 mM EDTA unless stated otherwise) contained 0.5 mM $NADP^+$, 4.2 mM $MgCl_2$, 4.2 mM glucose 6-phosphate, 0.3 IU/ml incubate of glucose 6-phosphate dehydrogenase and substrate. The substrate concentrations and the incubation times were as indicated in the figures.

The H_2O_2 was determined as formaldehyde which is formed in the presence of methanol (50 mM) by the peroxidative reaction of catalase (3000 mU) according to [10]. Formaldehyde was determined according to [11].

Lipid peroxidation was determined by measuring malondialdehyde by means of the thiobarbituric acid assay, according to [12]. The concentration of malondialdehyde was calculated using an extinction coefficient of $1.56 \times 10^5\ M^{-1}.cm^{-1}$.

Microsomal protein was assayed as in [13], using crystalline bovine serum albumin as standard.

3. RESULTS

A linear rate of H_2O_2 formation is observed in a liver microsomal suspension in the presence of an NADPH generating system in the absence of substrate (fig.3). The H_2O_2 formation thus obtained represents endogenous H_2O_2. Depending on the substrate used, the H_2O_2 production can either be decreased or activated (fig.2A). Diazepam (100 μM) enhanced H_2O_2 formation. Hexobarbital (2 mM) which is known to stimulate H_2O_2 production [14] was used as a reference compound. In contrast 100 μM of orphenadrine or its *N*-demethylated metabolite, tofenacine, attenuated the endogenous H_2O_2 formation by ~35% and 55%, respectively (fig.2A). Such a drastic inhibition of H_2O_2 generation had not been reported; therefore, we evaluated this further by in-

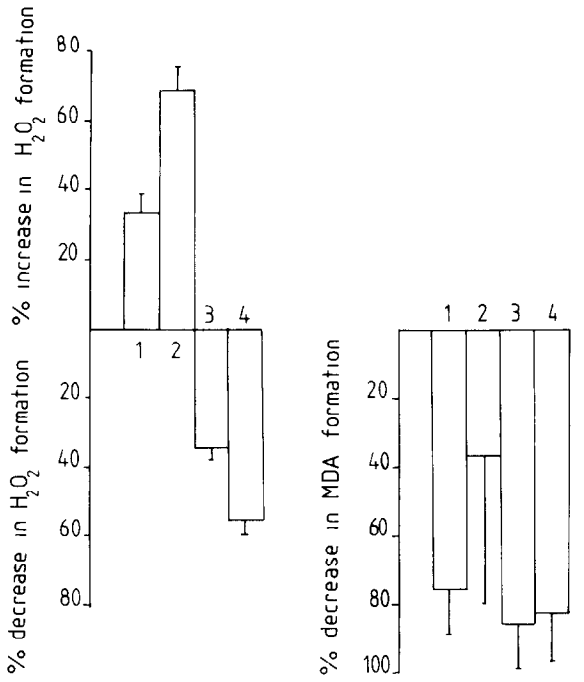

Fig.2. (A) The percentage change in H_2O_2 production using an incubation time of 10 min: (1) diazepam (100 μM); (2) hexobarbital (2 mM); (3) orphenadrine (100 μM); (4) tofenacine (100 μM). Hepatic microsomes of rats which were pretreated with phenobarbital were used. Data represent the mean (± SD) of at least 2 duplicate experiments.

Fig.2. (B) The percentage decrease in malondialdehyde using an incubation time of 60 min. The incubation was performed in an EDTA-free phosphate buffer medium; further as in fig.2A.

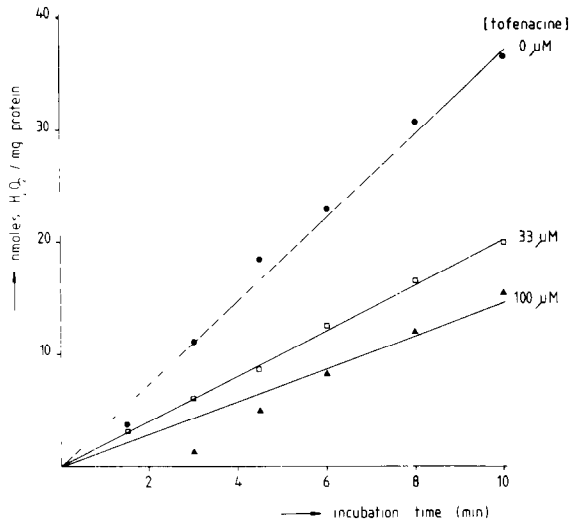

Fig.3. Time-dependent H_2O_2 formation in liver microsomes obtained from rats which were pretreated with phenobarbital. The concentration-dependent inhibitory effect of tofenacine is indicated. Data represent the mean of 3 expt.

vestigating the dose-dependent inhibition of H_2O_2 formation by tofenacine as a function of time (fig. 3). Endogenous H_2O_2 formation could apparently not be inhibited completely, since supplementation of the incubate with 200 μM of tofenacine resulted in the same inhibitory effect as compared with 100 μM of tofenacine (not shown).

Furthermore, a comparison was made between the effect of these compounds on H_2O_2 formation and on NADPH-dependent lipid peroxidation. The latter was measured by estimation of malondialdehyde (MDA). All substrates used were found to inhibit MDA formation. Again tofenacine was chosen to investigate this inhibition further (fig.4). The data show that the microsomal MDA formation, promoted by NADPH, could be blocked extensively by tofenacine in a concentration-dependent manner. Tofenacine at 200 μM inhibited NADPH-dependent MDA formation completely (not shown).

4. DISCUSSION

Three main items with regard to the oxidase activity of cytochrome P450 (fig.1) have thus far not

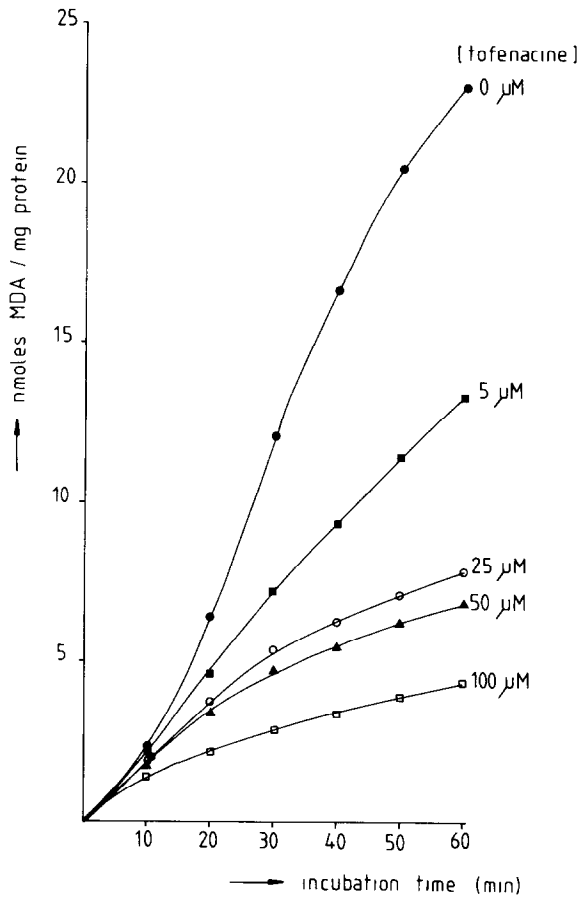

Fig.4. Time-dependent malondialdehyde formation in liver microsomes incubated in an EDTA-free phosphate buffer. Liver microsomes were obtained from rats which were pretreated with phenobarbital. The concentration-dependent inhibitory effect of tofenacine is indicated. Data represent the mean of 3 duplicate experiments.

been solved unambiguously. These items are: what is the mechanism of endogenous NADPH-dependent H_2O_2 formation (H_2O_2 formation without substrate), what regulates the 'uncoupling' effect of substrates on the cytochrome P450 reaction cycle (H_2O_2 formation with substrate) and finally what is the biological effect of microsomal H_2O_2 (or superoxide anion radical) formation.

It has been suggested that the H_2O_2 originates from the decay of oxycytochrome P450 in combination with the subsequent disproportionation of the superoxide anion radicals released [2]. The contribution of NADPH cytochrome P450 reductase

to the microsomal endogenous H_2O_2 formation has not been firmly established. However, the one-electron reduction of oxygen, forming superoxide anion radicals, may be directly catalyzed by the reductase [15,16]. Superoxide anions thus formed may dismutate to H_2O_2.

This finding, that both orphenadrine and tofenacine substantially decreased endogenous H_2O_2 formation, obviously provides an interesting tool to further investigate the intrinsic regulation of the oxidase activity. The observation that tofenacine cannot inhibit H_2O_2 formation completely, indeed suggests that several mechanisms are involved in endogenous H_2O_2 production. The inhibition is not due to an effect on the catalase/H_2O_2/methanol reaction (unpublished). Moreover, NADPH-dependent cytochrome *c* reduction remains uninfluenced by orphenadrine or tofenacine (not shown). The fact that orphenadrine and tofenacine, in the presence of NADPH form a metabolic intermediate which irreversibly binds to ferrouscytochrome P450 [17] is not responsible for the inhibition of the oxidase activity:

(1) Because orphenadrine and tofenacine produce this complexation of cytochrome P450 to a different extent [17], whereas the inhibition of H_2O_2 formation is approximately of the same magnitude (fig.2A).

(2) In [18] using benzphetamine as substrate a lack of relationship between NADPH catalyzed ferrouscytochrome P450 complexation and benzphetamine-induced increase of H_2O_2 production was shown.

Tofenacine elicits a ligand binding with hepatic microsomal ferricytochrome P450 obtained from phenobarbital pre-treated rats [19]. This interaction might prevent oxygen binding and thereby impede cytochrome P450 catalyzed oxygen activation. Further detailed mechanistic aspects of the extrinsic regulation of the oxidase activity are hardly known [20].

Here, emphasis was placed on the biological effect of the oxidase activity of cytochrome P450. It has frequently been suggested that 'activated' oxygen species are involved in lipid peroxidation [3,4]. However, comparison of H_2O_2 formation with microsomal malondialdehyde formation demonstrates that both phenomena are not interconnected (fig.2–4). This strongly suggests that the oxidase activity or the interaction of superoxide anion with H_2O_2 (via the iron catalyzed Haber–Weiss reaction) is not responsible for a rate-limiting step in NADPH-dependent lipid peroxidation.

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