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# RAT LIVER MICROSOMAL NADPH-SUPPORTED OXIDASE ACTIVITY AND LIPID PEROXIDATION DEPENDENT ON ETHANOL-INDUCIBLE CYTOCHROME P-450 (P-450IIE1)

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**Abstract**—The liver microsomal ethanol-inducible cytochrome P-450 (P-450IIE1) form is known to exhibit a high rate of oxidase activity in the absence of substrate and it was therefore of interest to evaluate whether this form of P-450 could contribute to microsomal and liposomal NADPH-dependent oxidase activity and lipid peroxidation. The rate of microsomal NADPH-consumption, $O_2^-$-formation, $H_2O_2$-production and generation of thiobarbituric acid (TBA) reactive substances correlated to the amount of P-450IIE1 in 28 microsomal samples from variously treated rats. Anti-P-450IIE1 IgG inhibited, compared to control IgG, microsomal $H_2O_2$-formation by 45% in microsomes from acetone-treated rats and by 22% in control microsomes. NADPH-dependent generation of TBA-reactive products was completely inhibited by these antibodies, whereas preimmune IgG was essentially without effect. Liposomes containing reductase and P-450IIE1 were peroxidized in a superoxide dismutase (SOD) sensitive reaction at a 5–10-fold higher rate than membranes containing 3 other forms of cytochrome P-450. Lipid peroxidation in reconstituted vesicles dependent on the presence of P-450IIB1 was by contrast not inhibited by SOD. Microsomal peroxidase activities, using 15-(*S*)-hydroperoxy-5-*cis*-8,11,13-*trans*-eicosatetraenoic acid as a substrate were high in microsomes from phenobarbital- or ethanol-treated rats but low in membranes from isoniazid-treated rats, having the highest relative level of P-450IIE1. It is suggested that the oxidase activity of P-450IIE1 contributes to microsomal NADPH-dependent lipid peroxidation. The combined action of the oxidase activity by P-450IIE1 and the peroxidase activities by P-450IIB1 and other forms of P-450 may be important for the high rate of lipid peroxidation observed in e.g. microsomes from ethanol- or acetone-treated rats. The possible importance of cytochrome P-450IIE1-dependent lipid peroxidation *in vivo* after ethanol abuse is discussed.

The cytochrome P-450 system possesses an oxidase activity in liver microsomes, first observed by Gillette *et al.* [1], whereby $H_2O_2$ is produced. Both NADPH-cytochrome P-450 reductase and cytochrome P-450 have been postulated to be responsible for the $H_2O_2$ formation. Evidence in favour of the fact that cytochrome P-450 is the major contributor of microsomal $H_2O_2$ generation are that cytochrome P-450 substrates can stimulate [2] and that CO [3], tofenacin [4] and metyrapone [5] can inhibit the microsomal $H_2O_2$ production. The capability of type II ligands, such as metyrapone, to inhibit microsomal $H_2O_2$ formation is probably connected with their ability to prevent oxygen binding and reduction. A correlation between the ligand-induced spectral shift of cytochrome P-450 and inhibition of $H_2O_2$ production has been described [4, 6]. Furthermore, $H_2O_2$ is produced in reconstituted cytochrome P-450 containing systems [7] and much evidence has been presented that it originates from the cytochrome P-450 component [8–14]. A plausible mechanism for the $H_2O_2$ production appears to be autooxidation of the oxy-cytochrome P-450 complex, yielding $O_2^-$, with the subsequent dismutation to $H_2O_2$ [3, 8, 9].

Ethanol-inducible cytochrome P-450, which is constitutively in its high spin form, is known to exhibit an especially high rate of oxidase activity [10, 11, 13]. Ethanol-feeding of rats causes a 3-fold increase in the rate of microsomal NADPH consumption [11], which corresponds to the increase of the rat liver ethanol-inducible form of cytochrome P-450 (P-450IIE1)† seen in microsomes from ethanol-treated rats [15]. As a consequence of the oxidase activity dependent on ethanol-inducible cytochrome P-450, mainly $H_2O_2$ is formed, although the stoichiometry between the amount of NADPH consumed and the $H_2O_2$ formed is not completely balanced [10]. This discrepancy has been explained by the occurrence of a 4-electron transfer to molecular oxygen, causing presumably formation of water [13].

The participation of cytochrome P-450 in microsomal lipid peroxidation has been under debate (cf. [12, 16, 17]). Five different roles for the hemoprotein in this respect can be considered: (1) as a catalyst of the formation of metabolic intermediates of xenobiotics (e.g. $CCl_4$) being capable of initiating lipid peroxidation; (2) as a reductant of compounds undergoing redox-cycling reactions, which thereby generate oxygen radicals acting as initiators of lipid peroxidation; (3) as a peroxidase utilizing lipid hydroperoxides as substrates, thereby being responsible for the formation of peroxyl, alkoxy and

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† Abbreviations used: P-450IIE1, ethanol-inducible cytochrome P-450 from rat liver microsomes (P-450j); P-450IIB1, phenobarbital-inducible cytochrome P-450 from rat liver microsomes (P-450b); P-450IIC11, cytochrome P-450h; $Me_2SO$, dimethylsulphoxide; 15-HPETE, 15-(*S*)-hydroperoxy-5-*cis*-8,11,13-*trans*-eicosatetraenoic acid; SOD, superoxide dismutase; TBA, thiobarbituric acid.

hydroxyl radicals, which can initiate or propagate lipid peroxidation; (4) as a source for oxygen radicals, mainly $O_2^-$, being capable of initiating lipid peroxidation in the presence of non-heme iron; and (5) as a reductant of lipid peroxides, which as a consequence are transformed to inactive products, with respect to lipid peroxidation.

We have previously described a mechanism of lipid peroxidation in reconstituted membrane vesicles dependent on the oxidase activity of rabbit liver cytochrome P-450IIB1 [12, 18]. It was therefore of interest to evaluate to what extent microsomal lipid peroxidation could be dependent on cytochrome P-450-induced oxidase activity in intact microsomes and, in particular, whether cytochrome P-450IIE1 could contribute to this activity. Here, we give immunological evidence for the participation of P-450IIE1 in microsomal formation of $H_2O_2$ and in microsomal NADPH-dependent lipid peroxidation. We also present evidence suggesting that the oxidase activity of this P-450 isozyme effectively contributes to lipid peroxidation in reconstituted membrane vesicles.

## MATERIALS AND METHODS

### *Materials*

NADPH, superoxide dismutase (from bovine erythrocytes, specific activity 3 100 units/mg), mannitol and cytochrome *c* were obtained from Sigma Chemical Co. (St Louis, MO). EDTA was from Fluka (Buchs, Switzerland) and imidazole, 2-propanol, aniline and dimethylsulphoxide ($Me_2SO$) were purchased from Merck (Darmstadt, F.R.G.). Liquid alcohol diet and liquid control diet [19] were obtained from Bioserv (Frenchtown, NJ). Rat liver cytochrome P-450IIB1, IIC11 and IIE1 [11, 20] and NADPH-cytochrome P-450 reductase [21] were purified as previously described. Microsomal phospholipids were extracted according to Bligh and Dyer [22] from liver microsomes of phenobarbital-treated rabbits and stored under nitrogen, at −20°. 15-(*S*)-Hydroperoxy-5-*cis*-8,11,13-*trans*-eicosatetraenoic acid (15-HPETE) was kindly donated by Dr Maria Kumlin, Karolinska Institute.

### *Methods*

*Animals.* Male Sprague–Dawley rats (170 g) were used. Some rats were fed the alcohol liquid diet or control liquid diet as described by DeCarli and Lieber [19] for 20 days. The control group was pair-fed. Other rats were starved and treated with acetone (5 ml/kg, given as 33% (v/v) acetone in 0.9% (w/v) NaCl) intragastrically for two days [20]. Other groups of rats were treated with three daily intraperitoneal injections of imidazole (250 mg/kg), phenobarbital (80 mg/kg), isoniazid (250 mg/kg) or 2-propanol (2 ml/kg, given as 20% (v/v) in water). Rats treated with dimethylsulphoxide (2 ml/kg) were injected i.p. twice daily for three days. All rats except the pair-fed and starved animals received food and water *ad lib.*

*Preparation of liver microsomes and reconstituted membrane vesicles.* Livers were homogenized in 10 mM sodium/potassium phosphate buffer, pH 7.4, containing 1.14% KCl. The microsomes were prepared by ultracentrifugation and washed once before suspension in 50 mM potassium phosphate buffer, pH 7.4 [20]. Unilamellar phospholipid vesicles were prepared by the cholate gel filtration technique [21] in Chelex-100-treated 15 mM potassium phosphate buffer, pH 7.4 containing 50 mM KCl. The vesicles consisted of microsomal phospholipids, NADPH-cytochrome P-450 reductase and cytochrome P-450 in a molar ratio of 1200:0.4:1.

*Assays.* Rat liver cytochrome P-450IIE1 was quantified by Western-blot as previously described [15]. The activity of NADPH-cytochrome P-450 reductase was determined at 30° in 0.33 M potassium phosphate buffer, pH 7.6, by monitoring the reduction of cytochrome *c* at 550 nm. NADPH-oxidation was measured spectrophotometrically, at 37° by following the decrease in absorbance at 340 nm. Production of $O_2^-$ was detected using succinylated cytochrome *c* as described by Ingelman-Sundberg and Johansson [10]. The system was calibrated for the quenching observed by the microsomes themselves. $H_2O_2$ was determined by the ferrous thiocyanate method [2] and the incubations contained microsomes corresponding to 1 mg of microsomal protein and azide (1 mM) in 50 mM potassium phosphate buffer, pH 7.4. The reactions were started by the addition of 0.3 mg NADPH and terminated after 4 min by 1 ml 10% (w/v) TCA. Thiobarbituric acid (TBA) reactive products were measured according to Bernheim *et al.* [23]. Liver microsomes corresponding to 0.5 mg of protein in 50 mM potassium phosphate buffer (Chelex-100-treated), pH 7.4, were incubated in the presence of 0.23 mM NADPH for 10 min at 30° before analysis of TBA-reactive substances. Similar incubations with reconstituted membrane vesicles contained 0.1 nmol cytochrome P-450 and were carried out for 5 min at 30°. Lipid hydroperoxides was analysed using the iodometric assay as described by Buege and Aust [24]. For this assay, microsomes corresponding to 3 mg of microsomal protein were incubated in the presence of 1 mg NADPH for 15 min at 30°. The reaction was terminated by the addition of 5 ml chloroform: methanol (2:1, v/v). The peroxidase activity of cytochromes P-450 was measured with 15-HPETE as a substrate. The incubations contained microsomes corresponding to 0.25 mg of protein, 100 μM of substrate in 0.25 ml 50 mM potassium phosphate buffer, pH 7.4, and were performed for 30 sec at 25°. The reactions were stopped by the addition of 2 volumes of methanol and the samples were analysed using straight phase HPLC. A μPorasil column was used with hexane:2-propanol:acetic acid (99:1:0.1, by vol.) as the mobile phase. The flow rate was 1 ml/min and the absorbance of the eluate at 235 nm was detected.

Appropriate blanks to the different analysis were performed by the addition of NADPH after termination of the reactions. Statistical analysis was performed using Students *t*-test and P-values less than 0.05 were considered significant.

## RESULTS

### *Microsomal NADPH-dependent oxidase activity*

The amount of cytochrome P-450IIE1, the rate of

Table 1. Cytochrome P-450 contents, NADPH cytochrome P-450 reductase activities, NADPH-dependent microsomal oxidase activities and rate of NADPH-dependent lipid peroxidation in microsomes from variously treated rats*

| Treatment | Total P-450 (nmol/mg) | P-450IIE1 | P-450-reductase (Units/mg) | NADPH-oxidation | $O_2^-$-formation (nmol product/mg, min) | $H_2O_2$ formation | TBA-react. subst. |
|---|---|---|---|---|---|---|---|
| — | 0.65 ± 0.11 | 0.05 ± 0.01 | 62 ± 4.9 | 11.8 ± 2.8 | 2.2 ± 1.0 | 2.3 ± 0.8 | 0.51 ± 0.15 |
| Acetone | 1.50 ± 0.11 | 0.45 ± 0.03‖ | 123 ± 4.4 | 25.3 ± 3.5‖ | 5.2 ± 1.9§ | 7.4 ± 0.6‖ | 1.16 ± 0.24‖ |
| 2-Propanol | 0.53 ± 0.07 | 0.11 ± 0.03‡ | 99 ± 6.8 | 16.8 ± 3.1 | 2.5 ± 0.2 | 4.3 ± 0.4‖ | 0.42 ± 0.03 |
| Imidazole | 0.96 ± 0.15 | 0.12 ± 0.02§ | 87 ± 11.4 | 18.1 ± 0.8§ | 2.8 ± 0.7 | 4.7 ± 0.7‖ | 0.42 ± 0.05 |
| $Me_2SO$ | 0.89 ± 0.07 | 0.21 ± 0.06§ | 84 ± 16.0 | 27.5 ± 2.3‖ | 2.9 ± 0.4 | 4.6 ± 0.8‖ | 0.46 ± 0.04 |
| Ethanol | 1.65 ± 0.16 | 0.51 ± 0.08‖ | 108 ± 5.9 | 31.6 ± 3.5‖ | 5.2 ± 0.6‖ | 6.6 ± 0.7‖ | 0.57 ± 0.10‖ |
| Control† | 0.64 ± 0.07 | 0.045 ± 0.01 | 93 ± 9.1 | 10.8 ± 1.9 | 2.0 ± 0.9 | 2.6 ± 0.6 | 0.11 ± 0.01 |

* The results represent mean ± SD of experiments performed with microsomes from four different animals in each group.
† The animals were pair-fed the liquid control diet.
‡ $P < 0.05$.
§ $P < 0.01$.
‖ $P < 0.001$ compared to control.

microsomal NADPH-cytochrome *c* reductase activity and the rate of microsomal NADPH consumption, $H_2O_2$-formation and $O_2^-$-production was monitored in membrane preparations from acetone-, ethanol-, imidazole-, isopropanol-, $Me_2SO$-treated and control animals (Table 1 and Fig. 1). Ethanol- or acetone-treatment caused an almost 10-fold increase in the level of P-450IIE1 and a 2-fold enhancement in the NADPH-cytochrome P-450 reductase activity. The amount of P-450IIE1 was increased 2–4-fold after treatment with propanol, imidazole or $Me_2SO$, but these agents did not affect the level of NADPH-cytochrome P-450 reductase by more than 50%. Microsomes from acetone-treated rats produced 2.5-fold higher amounts of $O_2^-$, compared to control, and a similar difference was observed between microsomes from ethanol-treated animals and pair-fed controls. No significant increase in $O_2^-$ formation was evident in microsomes from 2-propanol-, imidazole- or $Me_2SO$-treated animals, compared to control. Linear regression analysis of

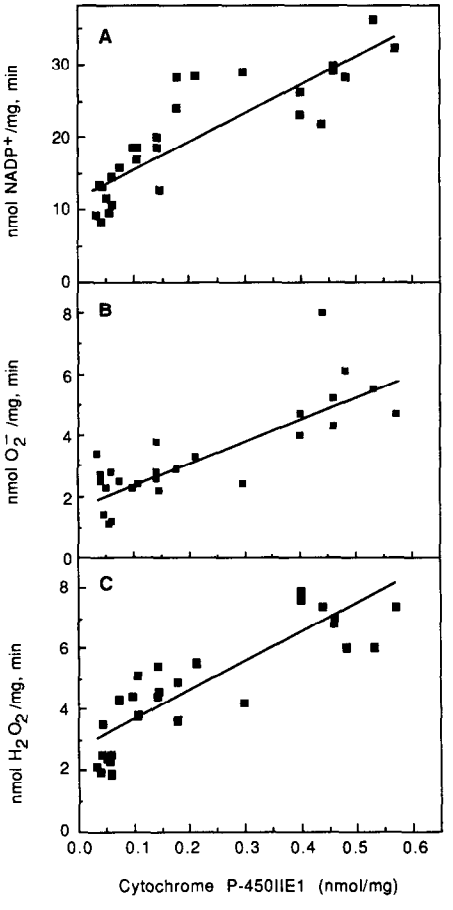


Fig. 1. NADPH oxidase activity (A), $O_2^-$ production (B) and $H_2O_2$ formation (C) in liver microsomes prepared from rats treated with various chemicals as a function of the amount of cytochrome P-450IIE1 in the membranes. $O_2^-$-production was measured with succinylated cytochrome *c* and $H_2O_2$ was determined by the ferrous thiocyanate method. For details see Methods. Each data point represents one animal.

the relation between the amount of cytochrome P-450IIE1 in the membranes and the rate of superoxide radical production, revealed a correlation coefficient of 0.82 (N = 28, Fig. 1).

The rate of formation of $H_2O_2$ was higher in liver microsomes from treated rats, compared to controls (Table 1). Microsomes from acetone-treated animals produced over three-fold more $H_2O_2$ than the appropriate controls. The corresponding difference between microsomes from ethanol-treated and pair-fed rats, was 2.5-fold and in membrane preparations from 2-propanol-, imidazole- and $Me_2SO$-treated rats, the rate of production of $H_2O_2$ was twice the controls. Linear regression analysis revealed a correlation ($r = 0.86$, N = 28) between the amount P-450IIE1 in the different membrane preparations and the rate of microsomal $H_2O_2$-production (Fig. 1).

Similar differences between the various types of microsomes were also observed when the rate of microsomal NADPH consumption was monitored (Table 1). Regression analysis of the relationship between the amount of P-450IIE1 and the rate of microsomal consumption of NADPH gave a correlation coefficient of 0.86 (N = 28, Fig. 1).

Addition of anti P-450IIE1-IgG cf. [20] to incubation mixtures containing microsomes from acetone-treated rat inhibited $H_2O_2$-formation by 64%, whereas preimmune IgG decreased the amount of $H_2O_2$ formed by 20% (Fig. 2). Thus the specific inhibition of anti P-450IIE1-IgG was about 44%. The corresponding inhibition by anti P-450IIE1-IgG in control microsomes was 22% (not shown in figure).

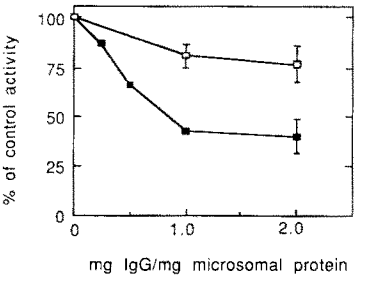


Fig. 2. Effect of anti P-450IIE1 IgG (■—■) or control IgG (□—□) on the rate of microsomal $H_2O_2$ production. Microsomes were prepared from acetone-treated rat liver. $H_2O_2$ was detected with the ferrous thiocyanate method, for details see Methods. The rate of $H_2O_2$ production in the absence of IgG was 7.2 nmol/mg/min. The results shown are means ± SD of three different experiments or mean of two different experiments performed in duplicate.

*Microsomal NADPH-dependent lipid peroxidation*

The rate of NADPH-dependent formation of TBA-reactive substances was 2-fold higher in microsomes from acetone-treated rats, compared to control, whereas an almost 5-fold higher rate of lipid peroxidation was caused by treatment of rats with the liquid ethanol diet, compared to pair-fed controls (Table 1). In addition, pretreatment of the rats with acetone also caused a 2-fold enhanced rate of lipid hydroperoxide formation, compared to control and equalled 0.28 nmol per mg microsomal protein per min (not shown). Pretreatment of the rats with 2-propanol, imidazole or $Me_2SO$ caused an approximately 2-fold increase in the amount of P-450IIE1, but did not significantly influence the rate of formation of TBA-reactive products (Table 1). Linear regression analysis of the relationship between the amount of P-450IIE1 in the microsomes and the rate of formation of TBA-reactive products, revealed a correlation coefficient of 0.68 (N = 28), whereas a correlation coefficient of 0.54 was reached between the NADPH-cytochrome P-450 reductase activity and the rate of formation of TBA-reactive products.

*Effect of EDTA, scavengers of oxygen radicals and P-450 ligands on microsomal NADPH-dependent lipid peroxidation*

Addition of the hydroxyl radical scavangers mannitol or $Me_2SO$ to incubation systems containing rat liver microsomes from either control or acetone-treated animals, did not significantly influence the

Table 2. Effect of radical scavengers, P-450 ligands and EDTA on NADPH-dependent lipid peroxidation in liver microsomes from acetone-treated and control rats

| Addition | TBA-reactive products (nmol/mg/min) Acetone | % Inhibition | Control | % Inhibition |
|---|---|---|---|---|
| None | 0.84 | — | 0.37 | — |
| Mannitol, 500 mM | 1.42 | — | | |
| $Me_2SO$, 300 mM | 0.64 | 24 | 0.37 | 4 |
| Ethanol, 100 mM | 0.79 | 6 | 0.36 | 4 |
| Imidazole, 5 mM | 0.88 | — | 0.55 | — |
| SKF-525A, 200 μM† | 0.24 | 72 | 0.08 | 79 |
| EDTA, 10 μM | <0.01 | 100 | 0.01 | 98 |
| Fe:EDTA, 10 μM | 2.74 | — | 0.87 | — |

* Incubations were performed with microsomes corresponding to 0.5 mg protein in Chelex-100-treated 50 mM potassium phosphate buffer, pH 7.4 as outlined under Materials and Methods. The values represent the mean of 2–4 different experiments performed in duplicate.

† SKF-525 A (200 μM) completely inhibited NADPH-dependent lipid peroxidation in reconstituted membranes containing only NADPH-cytochrome P-450 and phospholipids (molar ratio 1:3000) in the presence of 100 μM Fe(III)-EDTA (2:1) and 0.1 mM NADPH (not shown) indicating an antioxidant property of this chemical.

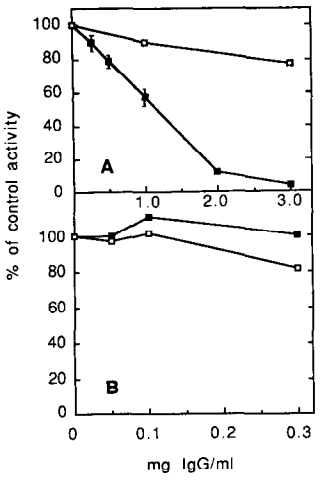


Fig. 3. Effect of anti P-450IIE1 IgG (■—■) or control IgG (□—□) on the rate of NADPH-dependent lipid peroxidation in microsomes prepared from acetone-treated rat liver (A) and in reductase-containing membranes supported with 100 μM Fe-EDTA (B). Incubations contained microsomal protein corresponding to 0.5 mg in 50 mM potassium phosphate buffer, pH 7.4 and 0.25 mM NADPH (A), or 50 U reductase-vesicles and 0.1 mM NADPH (B). The rate of formation of thiobarbituric acid reactive products in the absence of IgG was 11.1 nmol/mg/min and 4.2 nmol/nmol red/min. The results in A are from five different experiments and in B from two different experiments. The SD is indicated where three or more experiments were performed.

rate of formation of TBA-reactive products (Table 2). EDTA (10 μM) completely inhibited the lipid peroxidation, whereas Fe(III):EDTA (2:1) at 10 μM concentration stimulated the reaction threefold in microsomes from acetone-treated rats and more than two-fold in control microsomes (Table 2).

*Effect of anti P-450IIE1 IgG on the rate of microsomal lipid peroxidation*

Polyclonal antibodies against P-450IIE1 inhibited, in a dose-dependent manner, the microsomal NADPH-dependent lipid peroxidation (Fig. 3A). At 4 mg of anti-P-450IIE1 IgG per mg of microsomal protein, the reaction occurring in liver microsomes prepared from acetone-treated rats was almost completely inhibited, whereas at the same concentration, preimmune IgG was almost without effect. In control experiments, anti-P-450IIE1 IgG was added to reconstituted membrane vesicles containing NADPH-cytochrome P-450 reductase and phospholipids, and the formation of TBA-reactive products in the presence of Fe:EDTA and NADPH was measured. No inhibition was seen up to 6 mg IgG/nmol reductase by either anti- or control IgG (Fig. 3B). At higher concentrations of antibodies, both control IgG and anti P-450IIE1-IgG inhibited the reductase-dependent lipid peroxidation in a concomitant manner (not shown in figure).

*Lipid peroxidation in reconstituted systems*

Different purified isozymes of rat and rabbit P-450 were incorporated into membrane vesicles containing NADPH-cytochrome P-450 reductase and microsomal phospholipids and the formation of TBA reactive products was measured. Vesicles containing P-450IIE1 or IIB1 produced more than 5–10-fold more TBA-reactive substances than membranes containing the other isozymes (Table 3). Addition of 10 μM EDTA completely inhibited both the P-450IIE1- and P-450IIB1-dependent lipid peroxidation in the reconstituted membranes. Superoxide dismutase (SOD) specifically inhibited the rate of lipid peroxidation dependent on P-450IIE1, but did not affect the P-450IIB1-dependent lipid peroxidation (Table 3).

Table 3. NADPH-dependent lipid peroxidation in reconstituted membrane vesicles containing NADPH-cytochrome P-450 reductase and various forms of microsomal cytochromes P-450*

| Type of P-450 | Rate of lipid peroxidation (nmol/nmol P-450/min) |
|---|---|
| P-450IIE (rat) | 2.7 |
| +SOD 10 μg | 0.19 |
| +EDTA 10 μM | <0.01 |
| P-450IIB1 (rabbit) | 0.73‡ |
| P-450IA (rabbit) | 0.56‡ |
| P-450IIC11 (rat) | 0.19 |
| P-450IIB1 (rat) | 3.3 |
| +SOD 10 μg | 3.2 |
| +EDTA 10 μM | <0.01 |
| P-450-reductase† | 0.04‡ |

* The molar ratio between cytochrome P-450 and reductase was 4:1. Lipid peroxidation was measured as formation of thiobarbituric acid reactive products. Values are means from 2–18 different experiments made in duplicate.

† The rate of lipid peroxidation is here expressed as nmol of TBA-reactive substances/0.25 nmol reductase per min.

‡ The values were taken from Ekström and Ingelman-Sundberg [18].

*Microsomal peroxidase activities*

The relative contribution of phenobarbital-inducible and ethanol-inducible cytochromes P-450 to microsomal lipid peroxidation by their peroxidase activities, was evaluated using 15-HPETE as a substrate (Table 4). Liver microsomes from phenobarbital-treated rats exhibited the

Table 4. Peroxidase activities in liver microsomes isolated from variously treated rats using 15-HPETE as substrate. The conditions were as described under Methods. The results represent mean values of 2–4 different experiments performed in duplicate

| Type of microsomes | nmol 15-HPETE converted per mg/min | per nmol P-450/min |
|---|---|---|
| Control | 25 | 39 |
| Phenobarbital | 77 | 44 |
| Isoniazid | 8 | 15 |
| Ethanol | 52 | 35 |
| Control, pair-fed | 11 | 18 |

highest rate of peroxidase activity and microsomes from isoniazid-treated rats, the lowest rate. Relatively large differences were apparent when the activities were expressed per mg of microsomal protein, whereas less differences were seen in relation to the amount of cytochrome P-450 in the membranes. Microsomes from isoniazid-treated rats, containing the highest relative amount of P-450IIE1 (0.6 nmol of apoprotein/nmol of P-450) had the lowest rate of peroxidase activity when related to the P-450 content. The results indicate that the microsomal peroxidase activity mainly is related to the total amount of cytochrome P-450 in the membranes, but that ethanol-inducible P-450 exhibits a lesser capacity for the activity compared to other forms of microsomal cytochrome P-450.

## DISCUSSION

The results presented indicate a role for cytochrome P-450IIE1 in microsomal NADPH-dependent oxidase activity. With the aid of polyclonal antibodies against cytochrome P-450IIE1, immunological evidence for the participation of this cytochrome in microsomal NADPH-dependent formation of $H_2O_2$ was obtained. Anti-P-450IIE1 IgG inhibited $H_2O_2$-formation in microsomes from acetone-treated rats to the same extent as previously noted with this IgG-fraction and microsomal NADPH-dependent ethanol oxidation [20]. Correlations were reached between the rate of microsomal oxidase activity, as measured by NADPH-oxidation, $H_2O_2$-formation or $O_2^-$ production and the content of P-450IIE1 in the microsomal membranes from several differently treated rats.

Immunological evidence was also reached for the participation of P-450IIE1 in microsomal lipid peroxidation, although the mechanism of inhibition requires further investigation. Differences were registered between ethanol-treated and pair-fed rats and acetone-treated and control rats, respectively, with regard to the rate of NADPH-dependent lipid peroxidation (Table 1). The discrepancies in rate of lipid peroxidation between the two control groups, might be caused by changes in the amount and type of lipids present in the respective membranes, due to the different diets used in the two cases (cf. [25]). The microsomal NADPH-dependent lipid peroxidation was not inhibited by scavengers of hydroxyl radicals such as mannitol, $Me_2SO$ and ethanol, but was completely depressed in the presence of small amounts of EDTA. In reconstituted membranes, cytochrome P-450IIE1 caused a much higher rate of $O_2^-$-dependent and EDTA-sensitive lipid peroxidation than four other forms of cytochrome P-450 and, in the microsomes, a correlation was reached between the rate of $O_2^-$ and P-450IIE1 content. Thus, taken together the data support the concept that the oxidase activity of P-450IIE1 may be of importance for NADPH-dependent lipid peroxidation in microsomes from acetone- or ethanol-treated rats. These results are compatible with the mechanism of rabbit liver cytochrome P-450IIB1-dependent lipid peroxidation previously described [12].

A central point of interest is to what extent various forms of cytochrome P-450 contribute to microsomal lipid peroxidation by their peroxidase activities. Microsomes from isoniazid-treated rats, which had a high content of P-450IIE1, exhibited the lowest rate of peroxidase activity with 15-HPETE as substrate, whereas microsomes from phenobarbital-treated rats, having very small amounts of P-450IIE1 [20], peroxidized 15-HPETE at the highest rate. These results, reached in microsomes, are also compatible with the data from the reconstituted membranous systems (Table 3). Lipid peroxidation dependent on P-450IIE1 was completely sensitive to SOD, whereas P-450IIB1-dependent lipid peroxidation was not. This indicates that P-450IIB1 contributes to liposomal lipid peroxidation mainly by its peroxidase activity, whereas P-450IIE1-dependent formation of $O_2^-$ is of critical importance for this type of stimulation of lipid peroxidation. Acetone and ethanol-treatment of rats is known to cause induction of both P-450IIE1 and P-450IIB1 in the liver microsomes [20]. Because of the high rate of lipid peroxidation, compared to appropriate controls, in microsomes from ethanol- and acetone-treated rats, one might suggest that the combined action of the oxidase activity of P-450IIE1 and the peroxidase activity of P-450IIB1 and other forms of P-450 is of crucial importance for generation of the TBA-reactive substances in these microsomes.

Since P-450IIE1 is an ethanol-inducible enzyme one might consider the possibility that induction of this isozyme might be related to the occurrence of ethanol-induced liver damage. Excessive and chronic use of alcohol by humans results in liver damage characterized by fatty infiltration, which can lead to fibrotic degeneration and necrosis [26, 27]. The ethanol-dependent hepatotoxic effects are predominantly seen in the centrilobular region of the liver [28] and evidence exists to suggest that the ethanol-dependent liver damage is associated with an enhanced amount of lipid peroxides [29, 30]. Reinke *et al.* [31] have recently identified, using the spin trap $\alpha$-2,4,6-trimethoxyphenyl-*N*-*t*-butylnitrone, carbon centered radicals in the hepatic endoplasmic reticulum formed *in vivo* in ethanol-treated but not in control rats. Furthermore, the radical formation was synergistically influenced by a high fat diet. Starvation, and probably a low carbohydrate diet, is known to have a pronounced synergistic effect on the extent of induction of P-450IIE1 in rat liver [20] and is also known to synergistically potentiate ethanol-induced liver damage [28]. The level of immunodetectable cytochrome P-450IIE1 in liver microsomes from ethanol-treated rats is about 0.5 nmol/mg of microsomal protein ([15], Table 1). We recently found that this isozyme is almost exclusively localized in the centrilobular region of the liver [32], whereas cytochrome P-450 as a whole is more evenly distributed [33]. Thus, the heterolobular distribution of P-450IIE1 make concentrations of this isozyme of 50 $\mu$M possible in those regions preferentially affected by ethanol-treatment. This means that, on the basis of the results here presented, the rate of $O_2^-$ formation and P-450-dependent lipid peroxidation might possibly be of a significance in the centrilobular region after chronic ethanol treatment.

Further studies are needed in order to directly link the action of cytochrome P-450IIE1 in the centrilobular liver regions with tissue damage under *in vivo* conditions.

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