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DIFFERENTIAL ROLE IN LIPID PEROXIDATION BETWEEN RAT P450 1A1 AND P450 1A2

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Abstract—The role of cytochrome P450 (P450) in lipid peroxidation induced by NADPH or peroxide was investigated in a reconstituted system. When cumene hydroperoxide, *t*-butyl hydroperoxide and hydrogen peroxide were used as initiators, the rates of malondialdehyde (MDA) formation were much higher in a reconstituted system containing P450 1A1 than those observed in a reconstituted system containing P450 1A2. In contrast to peroxide-induced lipid peroxidation, P450 1A2 catalysed NADPH-induced lipid peroxidation more effectively than did P450 1A1 regardless of the presence of ADP-$Fe(NO_3)_3$. Carbon monoxide inhibited NADPH-induced formation of MDA in a reconstituted system containing P450 1A2, but not P450 1A1. In addition, superoxide dismutase (SOD) was an effective inhibitor in a NADPH-induced lipid peroxidation system catalysed by P450 1A2 but not by P450 1A1. These results suggest that a peroxide-induced reaction might proceed readily with P450 1A1, whereas P450 1A2 mainly functions in NADPH-induced lipid peroxidation via generation of an active oxygen species. It is furthermore indicated that the difference in the effect of SOD in NADPH-induced lipid peroxidation depends on the P450 used.

Oxidative degradation of polyunsaturated fatty acids occurs in two sequential steps of initiation and propagation [1]. Bast and Haenen [2] have proposed that lipid peroxidation consists of two steps, lipid peroxide-independent lipid peroxidation and the ensuing lipid peroxide-dependent lipid peroxidation, and both steps include the initiation and propagation steps. Studies on the hepatic microsomal lipid peroxidation induced by NADPH as well as inhibition and reconstitution studies have shown that both microsomal enzymes NADPH-cytochrome *c* (P450) reductase and cytochrome P450 (P450‡) are involved in NADPH-induced microsomal lipid peroxidation [1, 3–6]. In addition, P450 has been demonstrated to catalyse lipid peroxidation in the presence of organic or inorganic hydroperoxide [7, 8].

Although it is well known that P450, a family of hemoproteins, plays an important role in the metabolism of endogenous and exogenous compounds, and shows distinct but overlapping substrate specificities [9–11], little has been reported on the differences for lipid peroxidation among P450 isoenzymes and most of these studies were performed in a reconstituted system containing the P450 2B isoenzyme. Some inducers of P450 were found to modulate drug-induced lipid peroxidation in mice [12]. From these facts, we postulated that the role of P450 in lipid peroxidation might be different among the P450 isoenzymes present in liver microsomes. Therefore, we investigated in a reconstituted system whether the mechanism of P450-dependent lipid peroxidation induced by NADPH and peroxides is different in rat P450 1A1 and P450 1A2 induced by 3-methylcholanthrene and other aryl hydrocarbons [9].

MATERIALS AND METHODS

Materials and animals. Superoxide dismutase (SOD), tetramethoxypropane and cytochrome *c* were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). NADPH and 2-thiobarbituric acid (TBA) were purchased from the Oriental Yeast Co. (Tokyo, Japan) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Glucose oxidase, catalase and ADP were from Boehringer (Mannheim, Germany). Cumene hydroperoxide (CHP), *t*-butyl hydroperoxide and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) were from Nacalai Tesque Inc. (Kyoto, Japan). Emulgens 911 and 913 were kindly provided by the Kao Atlas Co. (Tokyo, Japan). All other chemicals and solvents used were of reagent or analytical grade. Male Sprague–Dawley rats (100–120 g) and Japan White rabbits (2.5–3.0 kg) were purchased from Takasugi Experimental Animals Co. (Saitama, Japan). Rats were pretreated with 3-methylcholanthrene (Wako Pure Chemical Industries, Osaka, Japan, 30 mg/kg, i.p.) for 7 days before killing. Liver microsomes were prepared by differential centrifugation as described previously [13]. The microsomal pellets were finally suspended with 100 mM potassium

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‡ Abbreviations: P450, cytochrome P450; MDA, malondialdehyde; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid; CHP, cumene hydroperoxide; IgG, immunoglobulin G fraction of serum; TMPD, *N,N,N′,N′*-tetramethylphenylenediamine.

phosphate, pH 7.4. The microsomal suspensions were stored at −80° until use.

Purification of enzymes. P450 1A1 and P450 1A2 were purified from liver microsomes of 3-methylcholanthrene-pretreated rats as described previously [14]. The specific contents of purified P450 1A1 and P450 1A2 were 15.2 and 16.3 nmol/mg of protein, respectively. Anti-P450 antibody was prepared as described previously [15]. NADPH-cytochrome *c* (P450) reductase was purified from liver microsomes of phenobarbital-pretreated rats by the method of Yasukochi and Masters [16] with minor modifications. The specific activity of purified reductase was 45.0 U/mg of protein. One unit of reductase activity was defined as the amount of enzyme catalysing the reduction of one micromole cytochrome *c* per minute [17].

Assay for lipid peroxidation. The reaction mixture consisted of 0.5 mg of microsomal protein prepared from 3-methylcholanthrene-pretreated rat livers, 80 mM Na, K-phosphate (pH 7.4) and 0.2 mM NADPH in a final volume of 0.5 mL. For immunoinhibition assays, anti-P450 1A2 immunoglobulin G fraction of serum (IgG) and control IgG were added to maintain 2 mg of IgG. The immunoinhibition study was performed four times in duplicate and mean values were expressed. A typical reaction mixture for the reconstituted system consisted of 50 pmol of P450, 0.2 U of NADPH-cytochrome *c* (P450) reductase, 0.2 mM phospholipid, 80 mM Na,K-phosphate (pH 7.4) and 0.2 mM NADPH in a final volume of 0.5 mL. In the CHP-induced lipid peroxidation system, 0.2 mM CHP was used in place of NADPH-cytochrome *c* (P450) reductase and NADPH. The reactions initiated by the addition of NADPH and CHP were carried out for 10 and 2 min, respectively, with shaking under aerobic conditions at 37°. The amount of TBA-reactive substances formed was measured by the method of Burge and Aust [18] and lipid peroxidation was expressed as the level of malondialdehyde (MDA) calculated from the calibration curve using 1,1,3,3-tetramethoxypropane. P450-dependent activity was calculated by subtraction of the values obtained without P450. Lipid hydroperoxides were analysed using an iodometric assay [18].

Assay for peroxidase in a reconstituted system. The activities of peroxidases were determined by the method of Hrycay *et al.* [19] with modifications. The reaction mixture for TMPD peroxidase consisted of 100 pmol of P450, 1 mM EDTA, 250 mM sucrose, 0.2 mM TMPD, 100 mM Na,K-phosphate (pH 7.4) and 1 mM CHP in a final volume of 1 mL. The reaction was started by the addition of CHP and absorbance at 610 nm monitored at room temperature.

Succinyl cytochrome c *reduction.* Cytochrome *c* was succinylated according to the procedure of Noguchi *et al.* [20]. After gel filtration by Sephadex G25 column, succinylated cytochrome *c* was purified using a DEAE-Sephacell column [21]. The reduction of succinylated cytochrome *c* was monitored with a Hitachi 220 spectrophotometer at 25°.

Other methods. Microsomal lipids were extracted by the method of Folch [22] from hepatic microsomes of untreated rats. Phospholipids were precipitated by acetone to remove free fatty acids and stored at −80° under anaerobic conditions until use. The content of lipid phosphorus was determined by the method of Bartlett [23]. P450 content was measured by the method of Omura and Sato [24] in the presence of 20% glycerol and 0.2% Emulgen 911. Reduction rate of P450 was measured by the method of Diel *et al.* [25] with modifications using a Shimadzu 557 double beam spectrophotometer. Protein was determined as described by Lowry *et al.* [26], using bovine serum albumin as the standard.

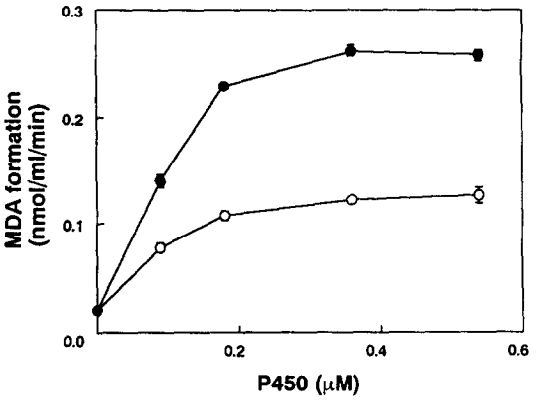

Fig. 1. NADPH-dependent lipid peroxidation catalysed by P450 1A1 or 1A2. The reaction mixture contained 0.2 U of NADPH-cytochrome *c* (P450) reductase, 0.2 mM phospholipid, 80 mM Na,K-phosphate (pH 7.4), 0.2 mM NADPH and indicated amounts of P4501A1 (○) or 1A2 (●) in a final volume of 0.5 mL. The values and SD are obtained from three or more experiments performed in duplicate.

RESULTS

NADPH- and CHP-dependent lipid peroxidation in the reconstituted system containing P450 1A1 or P450 1A2

We first investigated the effect of anti-P450 1A2 IgG on NADPH- and CHP-induced lipid peroxidation in hepatic microsomes of 3-methylcholanthrene-pretreated rats. The control activities observed in NADPH- and CHP-induced systems were 1.53 and 4.04 nmol MDA/mg/min, respectively. The activity of NADPH-induced lipid peroxidation was inhibited by anti-P450 1A2 IgG, whereas the activity of CHP-induced lipid peroxidation was unaffected by the antibody. The activities of NADPH-induced lipid peroxidation in the presence of 0.5, 1.0 and 2.0 mg of anti-P450 1A2 IgG were 0.73, 0.68 and 0.66 nmol MDA/mg/min, respectively.

The capacity of NADPH-induced lipid peroxidation was compared in a reconstituted system containing different amounts of P450 1A1 or P450 1A2. Only small amounts of TBA-reactive substances were detected without P450. Although both P450 1A1 and 1A2 were able to promote lipid peroxidation, their maximum capacities were different (Fig. 1). The rate of lipid peroxidation reached a plateau at

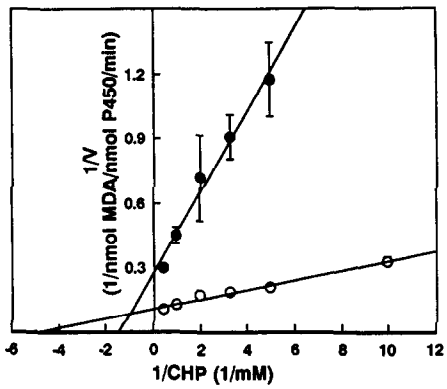

Fig. 2. Lineweaver–Burk plot for CHP-dependent lipid peroxidation catalysed by P450 1A1 or 1A2. The reaction mixture contained 50 pmol of P450 1A1 (○) or 1A2 (●), 0.2 mM phospholipid, 80 mM Na,K-phosphate (pH 7.4) and varying amounts of CHP in a final volume of 0.5 mL. The results shown are means ± SD of three or more experiments performed in duplicate.

a concentration of 0.18 μM of P450 1A2. On the other hand, P450 1A1-dependent lipid peroxidation roughly plateaued at a concentration of 0.09 μM of P450 1A1. Maximum activities of P450 1A1 and P450 1A2 under these conditions were 0.11 and 0.23 nmol MDA/mL/min, respectively. When lipid peroxide was measured as an indicator of lipid peroxidation under these conditions, activities of P450 1A1 and P450 1A2 were 1.42 and 8.32 nmol peroxide/nmol P450/min, respectively.

As it has been shown that peroxide also acts as an initiator in P450-promoted lipid peroxidation [7], we compared CHP-induced lipid peroxidation catalysed by P450 1A1 with that induced by P450 1A2. As shown in Fig. 2, P450 1A1 was more active in catalysing CHP-induced lipid peroxidation than P450 1A2 over the concentrations of CHP used. K_m and V_{max} values of P450 1A1 and P450 1A2 were 0.2 mM and 8.8 nmol MDA/nmol P450/min and 1.3 mM and 3.9 nmol MDA/nmol P450/min, respectively. In both organic hydroperoxide and inorganic hydroperoxide-induced lipid peroxidations, P450 1A1 catalysed the formation of MDA to a greater extent than P450 1A2 (Table 1).

Effects of enhancers and inhibitors on P450-catalysed lipid peroxidation in a reconstituted system

In NADPH-induced lipid peroxidation, Svingen *et al.* [1] showed that the ADP-perferryl ion acts as an initiator of the initiation step. Therefore, we investigated whether the effect of the initiator on P450-promoted lipid peroxidation was different between P450 1A1 and 1A2. As shown in Table 2, NADPH-induced MDA formation and oxygen

Table 1. Peroxide-induced lipid peroxidation catalysed by P450 1A1 and 1A2

	MDA formation (nmol MDA/nmol P450/min)	
	P450 1A1	P450 1A2
CHP (0.2 mM)	2.23	0.96
t-Butyl hydroperoxide (0.2 mM)	0.73	0.08
Hydrogen peroxide (0.2 mM)	0.82	0.30

Experimental conditions were described in Materials and Methods except that the indicated peroxide was used as inducer.

Each value represents the mean from three experiments with duplicated samples.

Table 2. Effect of ADP-Fe^{3+} on NADPH-promoted lipid peroxidation and oxygen consumption catalysed by P450 1A1 and 1A2

	MDA formation (nmol MDA/nmol P450/min)		Oxygen consumption (nmol/nmol P450/min)	
	−ADP-Fe^{3+}	+ADP-Fe^{3+}	−ADP-Fe^{3+}	+ADP-Fe^{3+}
P450 1A1	0.37	1.31	4.2	8.2
P450 1A2	1.18	2.03	9.9	10.3

The reaction mixture contained 50 pmol of P450, 0.2 U of NADPH-cytochrome *c* (P450) reductase, 0.2 mM phospholipid, 80 mM Na,K-phosphate (pH 7.4), 0.2 mM NADPH in a final volume of 0.5 mL. MDA formation and oxygen consumption were measured in the presence or absence of 2–0.1 mM ADP-$Fe(NO_3)_3$.

Each value represents the mean from two to four experiments with duplicated samples.

Other experimental conditions were described in Materials and Methods.

Table 3. Differential effect of carbon monoxide on NADPH-promoted lipid peroxidation between P450 1A1 and 1A2 in a reconstituted system

Gas phase (O_2:N_2:CO)	MDA formation (nmol/nmol P450/min) P450 1A1	P450 1A2
Experiment 1		
1:4:0	0.86 (100.0)	2.37 (100.0)
1:0:4	0.80 (93.0)	0.96 (40.5)
Experiment 2		
1:4:0	0.75 (100.0)	1.80 (100.0)
1:3:1	0.75 (100.0)	0.80 (44.4)
1:2:2	0.64 (85.3)	0.72 (40.0)
1:0:4	0.86 (114.7)	0.58 (32.2)

Incubation was performed using a silicon cap-sealed test tube, with the indicated gas phase.

Each value represents the mean of duplicated samples. Numbers in parentheses indicate per cent of control value (O_2:N_2:CO = 1:4:0).

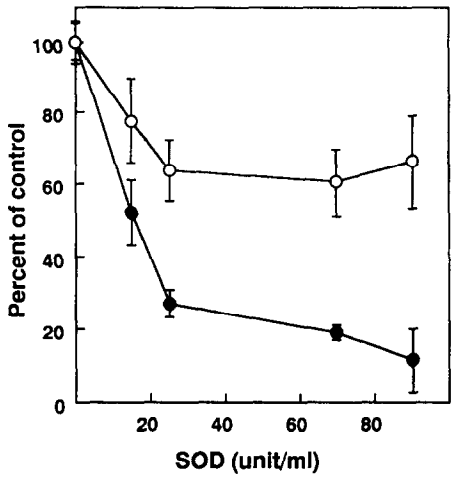

Fig. 3. Comparison of the inhibitory effect of SOD on NADPH-dependent lipid peroxidation catalysed by P450 1A1 or 1A2. The reaction mixture contained 50 pmol of P450 1A1 (○) or 1A2 (●), 0.2 U of NADPH-cytochrome *c* (P450) reductase, 0.2 mM phospholipid, 80 mM Na,K-phosphate (pH 7.4), 0.2 mM NADPH and SOD in a final volume of 0.5 mL. The amounts of SOD added are specified in the figure. The results shown are means ± SD of three or more experiments performed in duplicate. Control activities of P450 1A1 and 1A2 were 0.53 ± 0.03 and 1.13 ± 0.07 nmol MDA/nmol P450/min, respectively.

consumption in a reconstituted system containing P450 1A1 were 0.37 and 4.2 nmol MDA/nmol P450/min, respectively, in the absence of ADP-Fe^{3+}. Under the same conditions, the rates of MDA formation and oxygen consumption catalysed by P450 1A2 were 1.18 nmol and 9.9 nmol MDA/nmol P450/min, respectively. P450 1A1-dependent lipid peroxidation was more affected by the addition of ADP-Fe^{3+} when compared with P450 1A2-dependent lipid peroxidation (Table 2). In other words, although MDA formation by P450 1A1 was increased 3.5-fold by ADP-Fe^{3+}, MDA formation by P450 1A2 was increased only 1.7-fold by ADP-Fe^{3+}. When rates of oxygen consumption in P450 1A1 and 1A2 were measured, a difference in the effect of ADP-Fe^{3+} was also observed.

The finding that NADPH-induced lipid peroxidation catalysed by P450 2B1 is inhibited by carbon monoxide indicates that the activation step of oxygen is part of the mechanism of lipid peroxidation catalysed by P450 [3]. On the other hand, NADPH-induced lipid peroxidation in liver microsomes from untreated rats was shown previously to be increased by carbon monoxide [27]. Therefore, it is likely that the inhibitory effect of carbon monoxide on NADPH-induced lipid peroxidation was different among the forms of P450 used. As shown in Table 3, carbon monoxide significantly inhibited NADPH-induced lipid peroxidation in a reconstituted system containing P450 1A2 in a concentration-dependent manner, whereas in a reconstituted system containing P450 1A1, NADPH-induced MDA formation was virtually unaffected by carbon monoxide. Figure 3 shows the inhibitory effect of SOD on NADPH-induced lipid peroxidation in a reconstituted system containing P450 1A1 or P450 1A2. SOD potently inhibited MDA formation catalysed by P450 1A2 by 90%, whereas SOD inhibited MDA formation catalysed by P450 1A1 by only 35% when compared with control. Hydroxyl radical scavengers did not inhibit NADPH-induced lipid peroxidation catalysed by P450 1A1 and 1A2 (data not shown).

TMPD peroxidase activity, superoxide anion production and P450 reduction rate of P450 1A1 and 1A2

Since P450 has been proposed to act as an important factor in peroxide-dependent lipid peroxidation via its peroxidase activity [7], peroxidase activities of P450 1A1 and P450 1A2 were compared. TMPD peroxidase activities of P450 1A1 and P450 1A2 were 24.4 and 7.9 nmol/nmol P450/min, respectively (Table 4).

To clarify the reason why SOD inhibited NADPH-induced lipid peroxidation more strongly when catalysed by P450 1A2 than by P450 1A1, we compared the rate of superoxide anion radical formation in the reconstituted system containing P450 1A1 and P450 1A2, and the reduction rates of P450 1A1 and P450 1A2. As expected, P450 1A2 produced more of the superoxide anion radical than P450 1A1, and the reduction rate of P450 1A2 was 24-fold faster than that of P450 1A1.

DISCUSSION

Although several schemes for microsomal lipid peroxidation have been proposed, basically they consist of initiation and propagation steps [1, 2, 27]. From several lines of evidence, P450 is thought to have an important role in microsomal lipid peroxidation [4–7, 27], and we investigated whether the mechanism and properties of P450-dependent

Table 4. Superoxide production, NADPH peroxidase activity and reduction rate of P450 1A1 and 1A2

	TMPD peroxidase (nmol/nmol P450/min)	Succinyl cytochrome *c* reduction rate (nmol/nmol P450/min)	P450 reduction rate (nmol reduced/min)
P450 1A1	24.4	0.46	0.9
P450 1A2	7.9	2.61	22.0

TMPD peroxidase activity and succinyl cytochrome *c* reduction rate were measured as described in Materials and Methods. P450 reduction rate was measured by the method of Diel *et al.* [25] with modifications. The reaction mixture contained 0.2 mM phospholipid, 2 nmol of P450 1A1 or 1A2, 9 U of NADPH-cytochrome *c* (P450) reductase, 100 mM K-phosphate (pH 7.4), 10 mM glucose, 6 mM $MgCl_2$, 1200 U of catalase, 120 U of glucose oxidase and 1 mM NADPH in a final volume of 3 mL.

Each value represents the mean from two to four experiments with duplicated samples.

lipid peroxidation are different among P450 isoenzymes by using P450 1A1 and P450 1A2.

In preliminary experiments, we ascertained that both NADPH- and CHP-induced lipid peroxidations were enhanced by 3-methylcholanthrene pretreatment (data not shown), and NADPH-induced lipid peroxidation was inhibited by anti-P450 IgG in rat liver microsomes. In addition, it is known that 3-methylcholanthrene induces two P450s which have different properties in rat liver microsomes [9]. Therefore, these two P450 isoenzymes, P450 1A1 and P450 1A2, were used throughout this study.

P450 1A1 was a good catalyst for CHP-induced lipid peroxidation (Fig. 2, Table 1) and the value of V_{max}/K_m was 44.3 for 1A1 and 3.0 for 1A2. The value of 1A1 was 14.8-fold larger than that of 1A2. Also, P450 1A1 showed high TMPD peroxidase activity. These results suggest that a peroxide-dependent reaction occurs readily with P450 1A1.

The exact active oxygen species generated in the initiation step is still under debate (hydroxy radical, superoxide anion radical and hydrogen peroxide). P450 1A2 was a better catalyst in the NADPH-induced lipid peroxidation system than P450 1A1 (Fig. 1). The difference in the capacity for MDA formation between P450 1A1 and 1A2 in NADPH-induced lipid peroxidation was also observed when commercial phospholipids (phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine) were used in place of microsomal phospholipids (data not shown), indicating that phospholipid was not an important factor in the appearance of the difference in formation of MDA in NADPH-induced lipid peroxidation catalysed by P450 1A1 and 1A2. Carbon monoxide effectively inhibited NADPH-induced MDA formation in a reconstituted system containing P450 1A2 but did not affect it in a reconstituted system containing P450 1A1 (Table 1), and the reduction rate of P450 1A2 by NADPH via NADPH-cytochrome *c* (P450) reductase was much higher than that of P450 1A1. Weiss and Estabrook [8] have demonstrated that the ferrous form of P450 is not involved in peroxide-dependent lipid peroxidation suggesting that P450 1A2 mainly acts as an initiating factor in the lipid peroxide-independent lipid peroxidation step.

The role of an active oxygen species in lipid peroxidation is still unclear in spite of extensive studies [2, 4, 28]. Morehouse *et al.* [29] have reported that NADPH-induced lipid peroxidation in a microsomal system was independent of superoxide anion radical generation. On the other hand, Minakami *et al.* [28] and Ekstrom and Ingerman-Sundberg [4] have reported an inhibitory effect of SOD in NADPH-induced lipid peroxidation in a reconstituted system. In the present study, SOD was found to be an effective inhibitor in the NADPH-induced lipid peroxidation system catalysed by P450 1A2 but not by P450 1A1, indicating that the difference in the effect of SOD in NADPH-induced lipid peroxidation might depend on the P450 isoenzyme used. Superoxide anion production seemed to be involved in part in an NADPH-induced lipid peroxidation catalysed by P450 1A2 as in the case of P450 2E1 [6]. We demonstrated that rat P450 1A1 and P450 1A2 were involved in the various steps of lipid peroxidation, but that they exercised differential functions in the reconstituted system. However, further studies may be required to elucidate the mechanism of superoxide anion-dependent initiation of lipid peroxidation.

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