Pergamon

Biochemical Pharmacology, Vol. 47, No. 2, pp. 247–251, 1994.

0006-2952/94 $6.00 + 0.00

TRIMETHADIONE METABOLISM, A USEFUL INDICATOR FOR ASSESSING HEPATIC DRUG-OXIDIZING CAPACITY

MAYUMI NAKAMURA, EINOSUKE TANAKA,* SHOGO MISAWA,† TSUTOMU SHIMADA,† SUSUMU IMAOKA‡ and YOSHIHIKO FUNAE‡

Institute of Community Medicine, University of Tsukuba, Tsukuba-shi, Japan, †Osaka Prefectural Institute of Public Health, Osaka, Japan and ‡Laboratory of Chemistry, Osaka City University Medical School, Osaka, Japan

(*Received* 24 *June* 1993; *accepted* 30 *September* 1993)

Abstract—The metabolism of trimethadione (TMO), a useful indicator of hepatic drug-oxidizing capacity in rats and humans, was studied using 14 different forms of rat cytochrome P450 (CYP1A1, 1A2, 2A1, 2A2, 2B1, 2B2, 2C6, 2C7, 2C11, 2C12, 2C13, 2E1, 3A2 and 4A2) and three forms of human cytochrome P450 (CYP1A2, 2C and 3A4). TMO N-demethylation was increased by treating rats with phenobarbital. CYP2C11 and 2B1 had high TMO N-demethylase activity, but 1A1 and 1A2 had low activity. Antibodies raised to CYP2C11 and 2B1/2 inhibited TMO N-demethylation in hepatic microsomes of untreated and phenobarbital-treated rats, respectively. In a reconstituted system, human CYP3A4 and 2C produced efficiently dimethadione (DMO), but CYP1A2 did not catalyse TMO N-demethylation. Antibodies raised to CYP3A2 and 2C11 inhibited TMO N-demethylation in human hepatic microsomes. These results indicated that the N-demethylation of TMO is catalysed mainly by CYP2C11 and 2B1 in rat hepatic microsomes, and that human CYP3A4 and an unspecified isoform of the 2C subfamilies contribute to TMO N-demethylation in human liver.

Keywords: cytochrome P450, trimethadione, liver, rat, human, *in vitro*

Liver transplantation and therapy using drugs are now used widely in the treatment of liver failure in acute and chronic liver disease. These developments have created a need for assessing liver function. Antipyrine has been used to estimate drug-metabolizing activity in animals [1, 2] and humans [3–9], but the correlations between the half-life of antipyrine and those of other drugs have been poor, probably because of the complexity of antipyrine metabolism [10].

Trimethadione (TMO§) (Fig. 1), an anticonvulsant agent, may be a more suitable candidate for estimating drug-metabolizing activity. It is rapidly absorbed from the gastrointestinal tract, distributed into the total body fluids [11], and is extensively N-demethylated to dimethadione (DMO) by P450-dependent monooxygenases in liver microsomes [12]. Neither TMO nor DMO is bound to plasma proteins or any other macromolecules in biological materials [13], and the disappearance of TMO from plasma follows first-order kinetics according to a simple one-compartment model system. Therefore, we propose a TMO tolerance test, like antipyrine, as a useful indicator for assessment of hepatic drug-oxidizing capacity, which involves N-demethylation, in rats [14] and humans [15].

Recently we reported that TMO metabolism was enhanced by phenobarbital, dexamethasone and Aroclor 1254 treatment *in vitro* and *in vivo* [16].

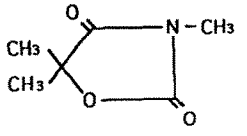

Fig. 1. Structure of trimethadione.

However, there are no data on TMO metabolism by different forms of purified P450 in reconstituted systems from rats and human microsomes. We have purified several liver microsomal P450 isozymes from humans and from untreated rats or those treated with phenobarbital [17–19]. The present studies were designed to identify the specific forms of P450 involved in the metabolism of TMO in a reconstituted system using purified P450 isozymes from rats and humans.

MATERIALS AND METHODS

Chemicals. TMO was supplied by the Dainippon Pharmaceutical Co. Ltd (Osaka, Japan). DMO was obtained from Tokyo Kasei (Tokyo, Japan). Dilauroylphosphatidylcholine and NADPH were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Other reagents and organic solvents were obtained from Wako Pure Chemical Industries (Tokyo, Japan).

Preparation of microsomes and purification of P450s. Male and female Sprague–Dawley rats weighing 200–250 g were obtained from Japan Clea (Tokyo, Japan). Phenobarbital (80 mg/kg, dissolved

* Corresponding author: Dr Einosuke Tanaka, Institute of Community Medicine, University of Tsukuba, Tsukuba-shi, Ibaraki-ken 305, Japan.

§ Abbreviations: P450, cytochrome P450; TMO, trimethadione; DMO, dimethadione.

saline) was given i.p. daily for 3 or 4 days. Hepatic microsomes were prepared as reported elsewhere [17]. CYP2C11, 2A2, 2C12, 3A2, 2B1, 1A1, 1A2 and 2A1 were purified from hepatic microsomes of rats and 4A2 was purified from renal microsomes as described previously [17–19]. Purification of NADPH-cytochrome P450 reductase and cytochrome b_5 have been described elsewhere [20]. Human hepatic microsomes were prepared in a similar manner to those of the rat [17]. Human CYPs, CYP3A4, CYP2C sub-families (not identified as CYP2C8 or 2C9) and CYP1A2 were purified by the method of Shimada *et al.* [21].

Assay of TMO metabolites. In the microsomal system, TMO *N*-demethylase activity was determined by incubating hepatic microsomes from controls or rats treated with phenobarbital, containing 200 or 500 μg of microsomal protein in a final volume of 1.0 mL (0.1 M potassium phosphate buffer, pH 7.4) at 37° for 30 min. In the reconstituted systems, 30 pmol of purified P450, was incubated with 5 μg of dilauroylphosphatidylcholine, TMO (0.5 or 5 mM), with or without cytochrome b_5 in a final volume of 1.0 mL of Tris–HCl buffer, pH 7.4 at 37° for 30 min. In the inhibition study, immunoglobulin G (IgG) antibodies were pre-incubated with the microsomal fraction for 10 min at 37°. The remaining components were added and the reaction initiated. Antibodies against purified CYP2C11, 2B1/2, 3A2 and 1A1 were raised in a female Japanese white rabbit (Saga, Japan) and the IgGs were prepared as described previously [22]. These reactions were started by adding NADPH (0.2 μmol), and stopped by 0.25 mL of 15% zinc sulphate and saturated barium hydroxide. The formaldehyde formed in the demethylation in rat liver was determined according to the method of Nash [23]. On the other hand, TMO metabolism was determined from the amount of DMO produced (nmol of produced DMO/min/nmol P450) by gas chromatography [24]. Substrate concentrations (0.5 or 5 mM) were chosen as described previously [25]. Two to five preparations of microsomes or purified P450s were measured.

RESULTS

Metabolic activity of TMO N-demethylation in rat hepatic microsomes

The TMO N-demethylation activity in hepatic microsomes of untreated male rats and in those from rats treated with phenobarbital were 0.46 and 2.12 nmol/min/mg protein, respectively. Hepatic microsomes of rats treated with phenobarbital were about 4.6-fold more active than those of untreated rats. An increased TMO metabolism was seen in response to phenobarbital treatment. Lineweaver–Burk plots at substrate concentrations of 0.5–5 mM TMO were constructed. With respect to TMO N-demethylation, untreated microsomes had a single high K_m value (3.27 mM), whereas microsomes from phenobarbital-treated rats had two K_m values (0.67 and 1.59 mM). This indicates that TMO *N*-demethylase activity was effected by at least two P450 isozymes, and that TMO metabolism was catalysed by different forms of P450 in untreated and phenobarbital-treated liver microsomes. As untreated and phenobarbital-treated liver microsomes have multiple forms of P450, we studied the metabolism of TMO by several different purified and reconstituted P450 isozymes.

Table 1. TMO N-demethylation activity of purified P450s from rats

P450 isozymes	0.5 mM TMO		5 mM TMO	
	$-b_5$	$+b_5$	$-b_5$	$+b_5$
1A1	8.0	7.4	5.0	5.2
1A2	9.4	9.0	9.4	6.4
2A1	7.8	8.4	10.8	13.8
2A2	14.2	14.8	13.2	12.2
2B1	18.6	17.2	21.6	21.2
2B2	7.8	8.4	6.4	6.8
2C6	8.2	8.0	8.0	8.4
2C7	9.2	9.0	9.6	10.4
2C11	14.6	17.0	18.2	19.2
2C12	14.2	15.4	13.2	14.6
2C13	8.0	8.0	9.4	8.4
2E1	12.0	9.6	10.8	13.8
3A2	5.0	6.2	5.2	5.0
4A2	5.2	5.0	3.8	3.4

Values (N = 2–5) are nmol product/min/nmol P450. A reconstituted system containing P450 (30 pmol), rat NADPH-P450 reductase (0.3 U), dilaurolylphosphatidylcholine (10 μg), TMO (0.5 or 5 mM), NADPH (0.2 μmol) with or without b_5 (30 pmol) was assayed.

TMO N-demethylation catalysed by purified P450s from rats

The rates of DMO formation in the reconstituted system with purified P450s at two substrate concentrations (low: 0.5 and high: 5 mM) are shown in Table 1. TMO N-demethylation was catalysed by CYP2B1 which was the major form in liver microsomes of rats treated with phenobarbital. The activities of CYP2C11 (the male-specific form) and 2C12 (the female-specific form) were of similar magnitude but CYP1A1 and 1A2, the 3-methylcholanthrene-inducible forms, catalysed this reaction moderately. The activity of CYP2A2 was high but this isozyme was present only in small amounts in untreated male hepatic microsomes [26]. When cytochrome b_5 was added to the reconstituted system, the TMO N-demethylation by P450 isozymes were not markedly affected. These results indicate that TMO N-demethylation (the formation of DMO) was catalysed by all P450 isozymes, but particularly by CYP2C11 and 2B1.

Inhibition of rat hepatic microsomes by antibodies

The results of inhibition studies with antibodies raised to CYP2C11, 2B1/2 and 1A1 on the formation of DMO in microsomes from untreated and phenobarbital-treated male rats are shown in Fig. 2. Antibody raised to CYP2C11 inhibited the formation of DMO in microsomes from untreated male rats by 70%, but antibodies raised to CYP2B1/2 and 1A1 caused a mere 20% inhibition (Fig. 2a). The CYP2B1/2 antibody inhibited the activity of

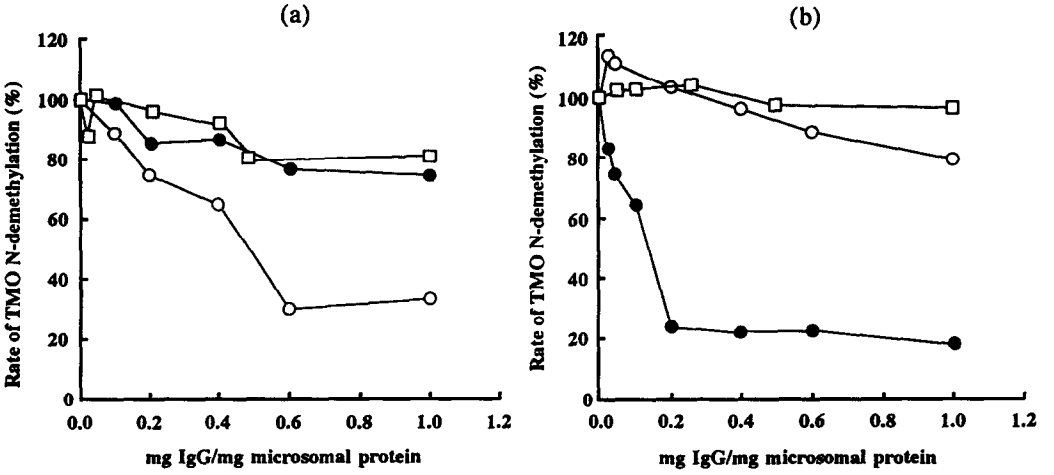

Fig. 2. The effects of P450 antibodies on TMO N-demethylation activity of rat hepatic microsomes. The designated amount of P450 IgG antibodies were incubated with the microsomal fraction for 10 min at 37°. NADPH was added and the reaction was initiated. The effect of P450 antibodies on the TMO N-demethylation activity of rat hepatic (a) untreated and (b) phenobarbital-treated microsomes are shown. Values are expressed by relative activity when the activity with control IgG is set at 100%. Antibodies: (○) anti CYP2C11; (●) anti CYP2B1/2 and (□) anti CYP1A1.

liver microsomes from male rats treated with phenobarbital almost completely, but the CYP2C11 and 1A1 antibodies did not (Fig. 2b). Inhibition of the formation of DMO by antibodies against CYP2C11 and 2B1/2 indicated that the reaction was catalysed by CYP2C11 in liver microsomes from untreated rats, and by CYP2B1 in liver microsomes from phenobarbital-treated rats. The N-demethylation of TMO was greater with microsomes isolated from phenobarbital-treated rats. We previously reported the CYP2C11 is the major form of P450 in the hepatic microsomes of untreated rats, and that CYP2B1 is a minor component [26]. However, the expression of CYP2B1 in liver microsomes from phenobarbital-treated rats was high, and that of CYP2C11 was decreased to about 20% of the total P450 content [27]. These results are consistent with the high rates of TMO N-demethylation catalysed by CYP2C11 and 2B1 in untreated and phenobarbital-treated rat hepatic microsomes, respectively.

TMO metabolism by human P450s

With human hepatic microsomes, Lineweaver–Burk plots gave linear plots and two K_m values (0.81 and 1.73 mM), under the same conditions as those used for rats. Human and rat hepatic microsomes had similar K_m values, which indicates that TMO was metabolized by similar P450 isozymes.

We studied the TMO metabolism by three P450s purified from human hepatic microsomes, including two major enzymes, CYP3A4 and 2C (immunochemically related to rat CYP3A2 and 2C12, respectively), and CYP1A2 (immunochemically related to rat CYP1A2) [17, 28]. CYP3A4 and 2C catalysed the N-demethylation of TMO efficiently, but CYP1A2 did not (Table 2). CYP3A4 had high TMO N-demethylation activity in the modified reconstituted system, which included cytochrome b_5, sodium cholate and a phospholipid mixture of dilauroylphosphatidylcholine, dioleoylphosphatidylcholine and phosphatidylserine (1:1:1), conditions that are necessary for CYP3A4 activity in a reconstituted system [29]. The addition of cytochrome b_5 to the reconstituted system enhanced the catalytic activity of CYP3A4. The catalytic activities of CYP3A4 and 2C in TMO demethylation were higher than those of human CYP1A2 (Table 2). To confirm which P450 isozymes catalysed TMO demethylation, inhibition studies were carried out. The rat CYP3A2 antibody reacts with human CYP3A4 but not with human CYP2C or 1A2 [29]. The inhibition studies with antibody against rat CYP3A2, 2C11 and 1A1 on TMO *N*-demethylase activity in human hepatic microsomes are shown in Fig. 3. Antibodies raised to CYP3A2 and 2C11 inhibited TMO *N*-demethylase activity by 50%, but the CYP1A1 antibody did not cause inhibition. These results indicate that CYP3A4

Table 2. TMO N-demethylation activities of purified human P450s

P450 isozymes	TMO N-demethylation activity
CYP3A4 ($P\text{-}450_{NF}$)	4.4
CYP2C ($P\text{-}450_{MP}$)	2.4
CYP1A2 ($P\text{-}450_{PA}$)	0.6

The catalytic activities of three purified human P450s (CYP3A4, CYP2C and CYP1A2) measured reconstructively. The substrate concentration was 5 mM TMO. The reaction mixture was analysed as described in Materials and Methods. The values (N = 2–5) are expressed as nmol product/min/nmol P450.

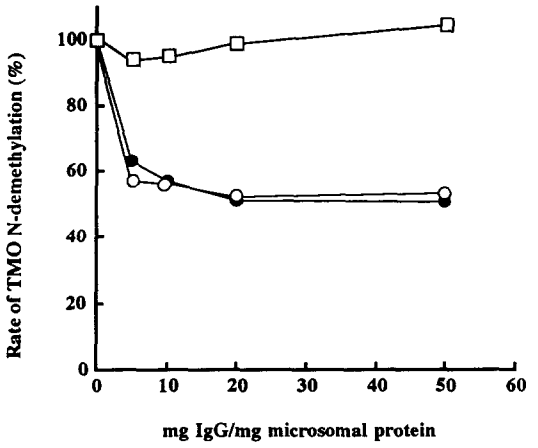

Fig. 3. The effects of P450 antibodies on TMO N-demethylation activity of human hepatic microsomes. The indicated amounts of P450 IgG antibodies were incubated with human microsomes under the same conditions as described in the legend to Fig. 1. The effects of P450 antibodies on the TMO N-demethylation activity of human hepatic microsomes are shown. Values are expressed by relative activity when the activity with control IgG is set at 100%. Antibodies: (○) anti CYP2C11; (●) anti CYP3A2 and (□) anti CYP1A1.

and 2C are important enzymes that metabolize TMO in human hepatic microsomes.

DISCUSSION

The principal aim of this study was to identify the specific forms of P450 involved in the metabolism of TMO in a reconstituted system using purified P450 forms from rats and humans. The reconstitution study showed that DMO, the only metabolite of TMO, was formed mainly by CYP2C11 and 2B1 in untreated and phenobarbital-treated rat hepatic microsomes, respectively (Table 1). The CYP2C11 antibody inhibited the formation of DMO in the microsomes from untreated male rats by 80%, whereas the CYP2B1/2 and 1A1 antibodies did so by only 20% (Fig. 2a). The CYP2B1/2 antibody almost completely inhibited the activity of microsomes from male rats treated with phenobarbital, but the CYP2C11 and 1A1 antibodies had little effect (Fig. 2b). These results are consistent with the report of Tanaka *et al.* [16], who demonstrated that the formation of DMO was induced by phenobarbital treatment in rats. These results indicate the CYP2C11 and 2B1 are involved in the N-demethylation of TMO. The formation of DMO and TMO is useful in quantifying the hepatic drug-oxidizing function of CYP2C11, which is the major form of P450 in untreated male rats. CYP2C12, the major form in the adult female rat liver, also had high TMO N-demethylation activity in the reconstituted system (Table 1). The amount of CYP2B1 in hepatic microsomes of untreated male rats was low but its expression was induced more than 50-fold by phenobarbital [27]. It is also induced by other drugs [30].

TMO metabolism by purified human hepatic P450s has not previously been reported. We found that human CYP3A4 and 2C contribute to TMO N-demethylation (Table 2). When a high concentration of TMO (5 mM) was used as substrate for human P450s, the TMO N-demethylation activities of the isozymes hardly changed compared with the low concentration (0.5 mM) (Table 1). In the reconstituted system, the catalytic activities of CYP3A4 and 2C were high, but that of CYP1A2 was low. Therefore, CYP3A4 and 2C appear to be important catalysts of TMO metabolism in human hepatic microsomes. In the human liver, CYP3A4 plays apparently a major role in the oxidation of many drugs [31] and can be induced by xenobiotic chemicals [32]. CYP2C is expressed constitutively in the human liver to a high degree [33]. In the erythromycin breath test, nine patients with severe liver disease had decreased levels of CYP1A2, 2C8, 2C9 and 3A [34]. We conclude therefore, that TMO N-demethylation is catalysed by P450 isozymes; in particular CYP2C11 and 2B1 of the rat and CYP3A4 and an unspecified isoform (presumably of the 2C sub-family) in humans contribute to this metabolism.

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