Biochemical Pharmacology, Vol. 45, No. 8, pp. 1563–1569, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

RAPID COMMUNICATIONS

OXIDATION OF ACETAMINOPHEN TO *N*-ACETYL-*p*-AMINOBENZOQUINONE IMINE BY HUMAN CYP3A4

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(Accepted 11 March 1993)

Abstract — We have investigated: (a) the formation of *N*-acetyl-*p*-aminobenzoquinone imine (NAPQI) from acetaminophen (APAP) by reconstituted human liver CYP3A4, (b) the kinetics of NAPQI formation in microsomes prepared from four human livers varying in CYP1A2, 2E1 and 3A4 content determined by Western blot analysis, (c) the contribution of CYP3A4 to the total formation of NAPQI from 0.1 mM APAP in human liver microsomes using troleandomycin as a specific inhibitor, and (d) the relationship between the contribution of CYP3A4 to NAPQI formation and relative CYP3A4 content. The K_m of CYP3A4 for APAP was found to be approximately 0.15 mM, similar to concentrations observed in humans after therapeutic doses of the drug. The kinetics of formation of NAPQI in human liver microsomes were complex; the lower K_m was similar to that found for reconstituted CYP3A4. The contribution of CYP3A4 to total NAPQI formation varied from 1 to 20% among livers, and correlated with the relative CYP3A4 content, $r^2 = 0.88$, $P < 0.05$. Our findings indicate that CYP3A4, the major P450 isoform in human liver and enterocytes, contributes appreciably to the formation of the cytotoxic metabolite NAPQI at therapeutically relevant concentrations of APAP and suggest that APAP may be a previously unrecognized inhibitor of this enzyme.

The formation of *N*-acetyl-*p*-aminobenzoquinone imine (NAPQI)† from acetaminophen (APAP) has been studied previously by Raucy *et al.* [1] in reconstituted CYP2E1 and in human microsomes probed with monospecific antibodies to CYP1A2 and 2E1. They demonstrated that these two forms of cytochrome P450 account for virtually all the NAPQI formed from 10 mM APAP in human liver microsomes. The contribution of various animal forms of cytochrome P450 to the formation of NAPQI has also been studied. Rabbit CYP1A2 and 2E1 and several other rabbit forms of cytochrome P450 all catalyze the reaction [2]. Nine rat forms of cytochrome P450 catalyze the reaction, including those thus far shown to oxidize APAP to NAPQI in humans [3]. The most efficient rat P450 in the oxidation of 1 mM APAP to NAPQI appears to be CYP3A1 [4].

The multiplicity of rat and rabbit cytochromes P450 capable of forming NAPQI from APAP, and the very efficient activity of CYP3A1 in the rat, suggested that the human ortholog, CYP3A4, might contribute to the formation of NAPQI in humans, particularly at concentrations of APAP that would be achieved following therapeutic doses, which are much lower than those examined previously. We report the results of studies of NAPQI formation from APAP by reconstituted human liver CYP3A4, and studies in human liver microsomes using isoform-specific inhibitors of cytochrome P450. The results show that CYP3A4 formed NAPQI from APAP with a K_m (*ca.* 0.15 mM) that did not greatly exceed concentrations produced by a 1 g dose of the drug (*ca.* 0.1 mM). The contribution among livers of CYP3A4 to the total formation of NAPQI from 0.1 mM APAP varied substantially, as might be

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† Abbreviations: APAP, acetaminophen; DDC, dithiodiethylcarbamate; DTT, dithiothreitol; GSH, glutathione; IOD, integrated optical density; NAPQI, *N*-acetyl-*p*-aminobenzoquinone imine; and TAO, troleandomycin.

expected for a constitutive and inducible enzyme. Furthermore, the proximity of the K_m to concentrations obtainable after therapeutic doses of the drug suggests that APAP may be a previously unrecognized clinically significant inhibitor of other metabolic reactions catalyzed by CYP3A4.

MATERIALS AND METHODS

Chemicals. APAP, glutathione (GSH), dithiothreitol (DTT), NADPH (tetrasodium salt, type IV), dithiodiethylcarbamate (DDC), troleandomycin (TAO), EDTA, phenylmethylsulfonyl fluoride, flavin mononucleotide and 2',5'-ADP-agarose were purchased from the Sigma Chemical Co. (St. Louis, MO). Glycerol was from J.T. Baker, Inc. (Phillipsburgh, NJ). Emulgen 911 was a gift from Kao-Atlas (Tokyo, Japan). DEAE-Sepharose was purchased from Pharmacia LKB (Uppsala, Sweden).

Preparation of human liver microsomes. Intact (whole) human livers were obtained from organ donors and stored as previously described [5]. Microsomes were prepared from homogenates of liver by differential centrifugation and stored in 100 mM potassium phosphate buffer, pH 7.4, in 1- to 2-mL portions at -70°. Microsomal protein concentration was determined by the method of Lowry *et al.* [6]. Total microsomal cytochrome P450 content was determined from the reduced minus oxidized carbon monoxide difference spectra [7].

Purification of rabbit cytochrome *b*5. Rabbit liver microsomal protein was isolated by differential centrifugation from livers of phenobarbital-induced rabbits. All purification steps were conducted at 4°. Approximately 1000 mg of total microsomal protein was solubilized in phosphate buffer containing 0.2% Emulgen 911 and subjected to lauryl-Sepharose chromatography [8]. The late eluting protein fraction containing *b*5 was dialyzed exhaustively against 5 mM potassium phosphate buffer, pH 7.5, containing 25% glycerol, 0.5% Emulgen 911, 0.1 mM DTT and 0.1 mM EDTA. All subsequent elution buffers contained the same components. A 2.5 x 25 cm column of DEAE-Sepharose was prepared and equilibrated with the above dialysis buffer. The dialyzed *b*5 fraction was loaded onto the DEAE column, and washed with 400 mL of 50 mM potassium phosphate buffer. Cytochrome *b*5 eluted from the column approximately midway through a linear 50 to 500 mM potassium phosphate gradient (1000 mL total volume). Elution fractions containing *b*5 were pooled and dialyzed exhaustively against 20 mM Tris-acetate buffer, pH 7.5, with 20% glycerol. A 2.15 x 15 cm Toso Haas DEAE-5PW anion exchange HPLC column (Phenomenex, Rancho Palos Verde, CA) was equilibrated with the Tris dialysis buffer mentioned above which also contained 0.4% Emulgen 911. The *b*5 pool was loaded onto the HPLC column and the column washed with 80 mL of equilibration buffer. The heme protein eluted from the column approximately midway through a linear 0 to 500 mM sodium acetate gradient in Tris-acetate equilibration buffer (500 mL total volume). Those fractions judged pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were pooled, subjected to cholate-Emulgen 911 exchange on a small hydroxyapatite column, dialyzed against 20 mM potassium phosphate, pH 7.4, 25% glycerol, and stored at -70°.

Purification of cytochrome P450 reductase. All purification steps were conducted at 4°. Approximately 1500 mg of phenobarbital-induced rabbit liver microsomal protein was solubilized in 50 mM potassium phosphate buffer, pH 7.5, containing 20% glycerol, 0.5% Emulgen 911, 1 mM DTT, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 5 μM flavin mononucleotide. These same components and pH were present in all subsequent elution buffers. A 2.6 x 30 cm column of DEAE-Sepharose was packed and equilibrated with 50 mM potassium phosphate buffer, total soluble protein was loaded onto the column and the column was washed with 150 mL of 50 mM potassium phosphate buffer. A protein fraction enriched in cytochrome P450 reductase eluted from the column with 0.5 M KCl in 50 mM potassium phosphate buffer. Final purification was accomplished with the use of 2',5'-ADP-agarose according to the procedure described by Shephard *et al.* [9].

Western blot analysis of microsomal P450. Human liver microsomal CYP2E1 and 3A4* contents were quantitated by Western blot analysis [5,8] using specific polyclonal rabbit antibodies prepared against human CYP2E1 [5] and CYP3A4 [10] for primary immunodetection. Human liver microsomal CYP1A2 content also was quantitated by Western blot analysis using a rabbit polyclonal antibody raised against purified rat CYP1A1 [11] for primary

* The analysis for CYP3A4 also includes the highly homologous and electrophoretically indistinguishable isoform, CYP3A3.

immunodetection of a major protein band that co-migrated with authentic cDNA-expressed CYP1A2 (Gentest, Woburn, MA). These data are reported as relative band IOD/nmol total P450.

Human liver microsomal incubations. Microsomal incubations contained APAP (0.05 to 20 mM when kinetic constants were determined and 0.1 mM otherwise), inhibitor (if included), 2 mg/mL microsomal protein, 2 mM NADPH and 5 mM GSH in 100 mM potassium phosphate buffer, pH 7.4 (final volume 0.5 mL). Final inhibitor concentrations were 100 μM TAO or 150 μM DDC. TAO was dissolved in methanol; the final methanol concentration in incubations containing TAO was 0.05% (v/v), which did not inhibit NAPQI formation in the absence of TAO. After a 5-min preincubation of microsomes and inhibitor, 1 mM NADPH was added and the reaction continued for another 20 min to allow inhibitory complex to form. NAPQI formation was initiated by the addition of APAP, GSH and 1 mM NADPH. The reaction was stopped 10 min later by the addition of 200 μL of 2 M perchloric acid. The mixture was centrifuged for 2 min at 13,000 *g* and 400 μL of the supernatant was transferred to a tube containing 400 μL of 1 M potassium phosphate and allowed to sit at 4° for 1 hr. Samples were then centrifuged for 2 min at 13,000 *g*, and 2 μL of the supernatant was analyzed for APAP-3-GSH (the NAPQI-GSH adduct) by HPLC.

Reconstituted CYP3A4 incubations. CYP3A4 was purified from human liver microsomes as described previously [10]. The enzyme was reconstituted as described by Halvorson *et al.* [12] for CYP3A1. Final incubation conditions were: 0.15 nmol CYP3A4; 0.45 nmol rabbit cytochrome P450 reductase; 0.15 nmol rabbit cytochrome b_5; 100 μg liver microsomal lipid, prepared as described by Folch *et al.* [13]; 25 μg Emulgen 911; 5 mM GSH; 2 mM NADPH and sufficient 100 mM potassium phosphate, pH 7.4, to yield a volume of 0.5 mL. Preincubation, initiation, incubation, termination and analysis of product were as described for microsomal incubations.

HPLC analysis of APAP-3-GSH. Liquid chromatography was performed on a Hewlett-Packard 1050 system with an Alltech Econosphere C_{18} column (5 μm, 4.6 mm x 10 cm) and an ESA electrochemical detector. The mobile phase was 9% methanol in 50 mM ammonium phosphate, pH 6.0, delivered at 1.0 mL/min. The guard cell on the detector was set at 0.7 V to oxidize the mobile phase. The first cell was set at 0.25 V and the second analytical cell was set at 0.42 V to maximize the response to APAP-3-GSH and minimize the response to APAP. The response time was 4 sec and the gain was 1200 for the second detector cell.

Data analysis. The untransformed velocity versus concentration data from reconstituted CYP3A4 were fit to a single enzyme Michaelis-Menten function, and those from DDC-inhibited human liver microsomes were fit to a two-enzyme function to obtain estimates of V_{max} and K_m using BMDP (Los Angeles, CA) statistical software. Appropriate models were selected by examination of residuals for randomness and the F-ratio test. Eadie-Hofstee plots were constructed to visually demonstrate the contribution of more than one enzyme to NAPQI formation in the microsomal system.

RESULTS

Reconstituted human CYP3A4 formed NAPQI from APAP with a V_{max} of 1.50 ± 0.06 nmol/nmol P450/min and a K_m of 142 ± 33 μM (Fig. 1). The kinetics of NAPQI formation were next examined in human liver microsomes to determine if evidence of this CYP3A4-catalyzed process could be found. This was conducted in human livers 105, 107, 108 and 109, selected to yield a spectrum of isozyme complement based on the Western blot analysis (Table 1).

Figure 2 shows the formation of NAPQI as a function of APAP concentration in a representative human liver microsomal preparation. The Eadie-Hofstee plot clearly indicates that more than one enzyme contributes to the process. In the presence of 150 μM DDC, a selective mechanism-based inhibitor of CYP2E1 [14], a biphasic Eadie-Hofstee plot was still seen.

Table 2 lists the values of the kinetic constants in the absence and presence of DDC. Under both conditions, the data were best fit by a two-enzyme model. If a third component was included, the regression analysis did not converge. Because DDC is a mechanism-based inhibitor of CYP2E1 the inhibited component was identified through effects on V_{max}. DDC caused a decrease in V_{max1} in only two of the four livers. The effect of DDC on V_{max1} in all livers other than 105 was modest. DDC substantially decreased V_{max2} in each of the four livers, with a mean decrease of 67%. DDC, a selective inhibitor of CYP2E1, thus inhibited the high K_m process.

In the absence and presence of DDC, the mean values of K_{m1} were 0.153 and 0.253 mM, respectively, agreeing well with the value determined in reconstituted human liver CYP3A4. The mean value of K_{m2}, regardless of the presence of DDC, was more than 25 times the K_m of CYP3A4.

Table 3 shows the apparent contribution of CYP3A4 to the total formation of NAPQI in each liver, and the relationship between the contribution of CYP3A4 and its relative abundance. The inhibition of NAPQI formation by TAO varied from 1% in the liver with the least CYP3A4 to 20% in the liver with the most CYP3A4. The correlation between percent inhibition and relative abundance of CYP3A4 was strong, $r^2 = 0.88$, and significant, $P < 0.05$.

Table 1. Cytochrome P450 isoform content in human livers

Liver	1A2	2E1	3A4
	(relative immunoblot density, IOD / nmol total P450)		
101	18.8	73.2	88.1
103	38.1	48.8	53.9
104	2.7	285	52.1
105	30.4	60.4	81.3
106	39.4	158	152
107	34.5	368	46.4
108	7.9	137	52.7
109	7.7	184	143
110	31.7	95.6	34.8
111	18.9	69.6	33.3
113	14.9	55.6	49.4
114	30.7	126	52.1
115	9.5	102	41.4
116	31.1	100	61.6
118	4.2	342	32.9
119	16.4	107	115

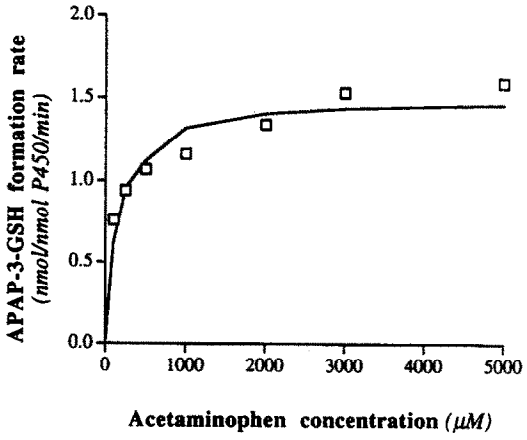

Fig. 1. NAPQI formation by human liver CYP3A4. The mean of duplicate incubations is plotted.

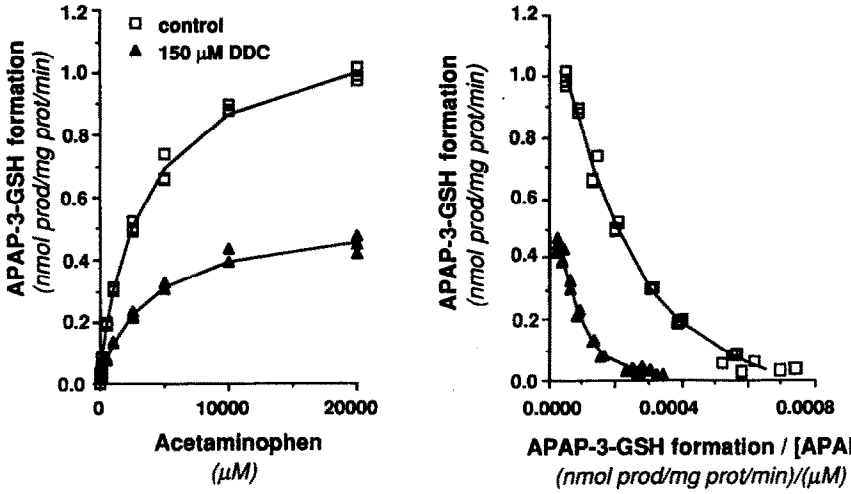

Fig. 2. Formation of NAPQI from APAP in microsomes prepared from human liver 105 in the absence and presence of DDC. An Eadie-Hofstee plot is shown on the right. Individual results from 3 replicate incubations at each APAP concentration are plotted.

Table 2. Kinetic constants for the formation of NAPQI in human liver microsomes in the absence and presence of 150 μM DDC

	$V_{max}1$ (nmol/mg prot/min)		$V_{max}2$ (nmol/mg prot/min)		K_m1 (mM)		K_m2 (mM)	
Liver	-DDC	+DDC	-DDC	+DDC	-DDC	+DDC	-DDC	+DDC
105	0.139	0.034	1.05	0.511	0.284	0.112	4.40	4.15
107	0.027	0.028	1.37	0.275	0.021	0.500	3.59	5.67
108	0.025	0.037	0.885	0.254	0.029	0.219	3.62	2.98
109	0.080	0.056	0.639	0.258	0.280	0.181	3.38	4.99
Mean	0.0678	0.388	0.986	0.325	0.153	0.253	3.75	4.45
SD	0.0539	0.0121	0.0311	0.0385	0.148	0.171	0.448	1.16

Table 3. Relative content of CYP3A4 and inhibition of NAPQI formation from 0.1 mM APAP in human liver microsomes

		Velocity †		
Liver	CYP3A4* (% max liver)	-TAO (pmol/mg prot/min)	+TAO (pmol/mg prot/min)	Inhibition† (%)
105	57	61 ± 8	53 ± 3	12 ± 8
107	32	48 ± 6	47 ± 4	1 ± 8
108	37	52 ± 2	46 ± 2	8 ± 4
109	100	61 ± 5	49 ± 1	20 ± 2

* From data in Table 1. Liver 109 had maximum CYP3A4 content.

† Values are means ± SD of three determinations.

DISCUSSION

Raucy *et al.* [1] have demonstrated previously that human CYP1A2 and 2E1 catalyze the formation of NAPQI from APAP. They used 10 mM APAP in their microsomal incubations and concluded that the two isoforms examined accounted for virtually all the NAPQI formed. We have shown that reconstituted CYP3A4 also catalyzed the formation of NAPQI with a K_m of approximately 0.15 mM. The contribution of CYP3A4 to total human liver microsomal NAPQI formation at 0.1 mM APAP was determined using TAO as a specific inhibitor of the enzyme [15,16]. CYP3A4-catalyzed NAPQI formation (identified as that portion inhibited by TAO)* correlated with relative CYP3A4 content, and varied from 1 to 20% among the livers examined. The medical record prior to procurement of the livers used in this study did not indicate that the donors were exposed to known inducers of CYP1A2, 2E1, or 3A4. The variability of isoform content and relative contribution of CYP3A4 to total NAPQI formation therefore seems to be due to constitutive variation of these forms, and may be even greater following treatment with inducers. Our findings also suggest that CYP1A2 has a relatively high K_m for APAP, in that a high K_m process remained in

* Chemical inhibitors of CYP3A4, such as TAO and gestodene, sometimes underestimate the degree of inhibition observed with anti-CYP3A4 antibodies [16,17].

addition to one with a K_m approximately equal to that of reconstituted CYP3A4 in the presence of DDC, a specific mechanism-based inhibitor of CYP2E1 [14]. CYP2E1 apparently has a relatively high K_m as well, in that DDC substantially inhibited only the high K_m process. Our findings are in agreement with those of Raucy *et al.* [1], as it would be expected that the contribution of CYP3A4 to total NAPQI formation would be negligible at 10 mM APAP.

Peak APAP plasma concentration after a 1.0 g dose is approximately 0.1 mM [18]. Toxicity is usually seen when plasma APAP exceeds 2 mM in the first few hours after ingestion [19]. The contribution of CYP3A4 to the formation of NAPQI following therapeutic doses of the drug is likely to be appreciable, and probably declines in overdose. However, this situation may change in the case of P450 induction. Bray *et al.* [20] have reported recently that prior history of long-term anticonvulsant therapy increases the severity of APAP hepatotoxicity in overdose cases. Induction of enzymes that bioactivate APAP was proposed as a likely mechanistic explanation of the interaction. Indeed, the majority of patients in the study (15 of 18) received phenobarbital, phenytoin or carbamazepine, either singly or in combination, and all three of these anticonvulsant drugs are recognized inducers of CYP3A4 [21, 22].

Our findings may be of particular relevance to drug interactions affecting the formation and disposition of NAPQI. Potentiation of APAP toxicity is of particular interest in the prophylaxis of tuberculosis with the CYP2E1 inducer isoniazid, itself a hepatotoxin [23]. Rifampin, a potent inducer of CYP3A4 [24,25], is also used for the treatment and prophylaxis of tuberculosis (sometimes in combination with isoniazid) and may potentiate APAP hepatotoxicity. The proximity of the K_m of CYP3A4 for APAP to concentrations that can be achieved after therapeutic doses of the drug suggests that acetaminophen may be a previously unrecognized clinically significant inhibitor of CYP3A4. APAP concentrations *in vivo* are high, and *in vitro* screens for inhibition of CYP3A4-catalyzed biotransformation by APAP may be missed if low concentrations are used. APAP has been reported not to affect the rate of cyclosporin oxidation in human tissues, but apparently was studied only as a potential inducer of CYP3A4 [22]. The metabolism of numerous therapeutically important drugs has been shown to be catalyzed by this P450 isoform [26]. Since CYP3A4 is concentrated in human enterocytes [27, 28], inhibition of gut-wall-mediated first pass metabolism of CYP3A4 substrates by high concentrations of APAP in the intestine following oral doses deserves investigation.

Acknowledgements - This research was supported by Grant GM32165 from the National Institutes of Health.

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