Vol. 203, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1994 Pages 532-539

LOSS OF CYP2E1 AND CYP1A2 ACTIVITY AS A FUNCTION OF ACETAMINOPHEN DOSE: RELATION TO TOXICITY

John E. Snawder[§1], Amy L. Roe[†], R.Wayne Benson[§], and Dean W. Roberts[§2]

[§]Division of Biochemical Toxicology, National Center for Toxicological Research, FDA, Jefferson, AR 72079

[†]Graduate Center for Toxicology, University of Kentucky, Lexington, KY 40536

Received July 13, 1994

The effect of acetaminophen (APAP) dose on the cytochrome P450s responsible for its bioactivation was examined in control mice and mice treated with acetone to induce CYP2E1, or β-napthaflavone to induce CYP1A2. In non-induced mice, 150 mg/kg APAP caused minimal hepatotoxicity and loss of CYP2E1- but not CYP1A2-dependent activity. In contrast, 400 mg/kg APAP was hepatotoxic and diminished both CYP2E1 and CYP1A2 activities. In acetone-pretreated mice, the 150 and 400 mg/kg APAP doses caused similar depletion of CYP2E1 activity and similar levels of covalent binding of APAP to liver proteins. In β-napthaflavone-pretreated mice, CYP1A2 activity was decreased only by the high dose of APAP, and covalent binding was >2-fold higher at the high APAP dose. The data indicate CYP2E1 is important in the bioactivation of APAP at the low dose with little additional contribution at the high dose, whereas CYP1A2 contributes more to the bioactivation and toxicity APAP at high doses.

Acetaminophen (APAP) hepatotoxicity is associated with its P450-dependent biotransformation to the reactive metabolite, N-acetyl-*p*-benzoquinone imine (NAPQI), that is normally eliminated by conjugation with glutathione (GSH). When reserves of GSH are depleted, NAPQI may react with cellular protein thiols forming either 3-(cystein-S-yl) APAP protein adduct (3-Cys-A) or oxidized protein thiol and the parent compound (1). APAP

[1] Current address:CDC/NIOSH, Taft Laboratories, MSC23, 4676 Columbia Parkway, Cincinnati, OH 45226.

[2] To whom correspondence should be addressed. Fax: (501) 543-7220.

Abbreviations: ALT, alanine aminotransferase; APAP, N-acetyl-p-aminophenol, acetaminophen, paracetamol; ßNF, β-napthaflavone; 3-Cys-A, 3-(cystein-*S*-yl)APAP; DMN, N-nitrosodimethylamine-*N*-demethylase activity; EROD, ethoxyresorufin O-deethylase activity; GSH, glutathione; NAPQI, *N*-acetyl-*p*-benzoquinone imine.

0006-291X/94 $5.00

overdose alters proteins after GSH depletion (2,3) and also causes changes in microsomal membranes (4).

CYP2E1, CYP1A2 and CYP3A4 are reported to be responsible for APAP bioactivation in rodents and man (5-8). Previous work in our laboratory, using human lymphoblastoid cells transfected with cDNA for the expression of specific human P450 forms demonstrated that CYP2E1 has a low Km for APAP bioactivation (Km 0.18 mM, Vmax 1.14 pmol 3-Cys-APAP mg^{-1} cell protein/min). In contrast, CYP1A2 has a higher Km and approximately 40% higher capacity for total APAP bioactivation (Km 2.5 mM, Vmax 1.59 pmol 3-Cys-APAP mg^{-1} cell protein/min) (9). These results prompted us to investigate the effect of minimally and severely hepatotoxic APAP doses in control, CYP2E1-, and CYP1A1/1A2-induced mice. Biochemical and morphological assessment of liver toxicity, total P450, and CYP2E1- and CYP1A2-specific metabolism were determined.

METHODS

Animals and Dosing: Male B6C3F1 mice from the National Center for Toxicological Research breeding colony, 10 weeks old and having an average weight of 22 g, were fasted by removing food at 4:00 pm the day before dosing (18 h prior to dosing). APAP was dissolved in pyrogen-free saline at 40° C, at concentrations such that 25 µl/gram body weight injected i.p. delivered the desired dose, i.e. 0, 150 or 400 mg/kg APAP. P450 was induced using methods previously described by Jeffery (5). Briefly, CYP2E1-dependent metabolism was induced by 0.1% acetone in the drinking water for 10 days. Acetone was removed 24 h prior to APAP treatment. CYP1A1/1A2-dependent metabolism was induced with β-napthaflavone (ßNF, 80 mg/kg) in corn oil, administered i.p. for three days. The last injection of ßNF was 24 h prior to APAP treatment. Control mice received either corn oil or tap water. After 4 h mice were bled under CO_2 anesthesia, killed by cervical dislocation, and tissues examined for evidence of hepatotoxicity.

Sample Preparation: Blood was allowed to clot at room temperature and serum was stored at -70°C. Livers were homogenized with a Teflon-coated tissue grinder using a 5:1 v/w ratio of 100 mM Tris-HCl (pH 7.2) containing 50 mM KCl. Homogenates were centrifuged at 10,000 g for 10 min at 4°C and two 1 ml aliquots of the supernate were removed and stored at -70°C for later estimation of GSH content. Microsomes were prepared from the remaining supernates (10). Microsomal protein was determined (11) with bovine serum albumin as a standard.

Hepatotoxicity: Serum ALT was determined using a Baker Encore autoanalyzer and Baker CentrifiChem ALT optimized reagents (Baker Instrument Co., Allentown, PA; 12). Total hepatic GSH levels were determined by adding 50 µl of ice cold 50 % sulfosalicylic acid (to denature and precipitate proteins and prevent autoxidation of the GSH to GSSG) to 1 ml of tissue homogenate. Samples were centrifuged (10,000g at 4°C for 20 min) and the supernates assayed according to the method of Tietze (13). Changes in relative liver weight were measured as an index of treatment-associated hepatomegaly.

P450 Metabolism: Total cytochrome P450 in microsomes was determined using the differential spectrophotometric method of Omura and Sato (14). CYP2E1-specific activity was determined in microsomes by assessing N-nitrosodimethylamine-*N*-demethylase activity (DMN, 15). CYP1A1/1A2 specific activity was assessed by determining ethoxyresorufin O-deethylase (EROD) activity by the method of Burke *et al.* (16). APAP bioactivation was estimated by determining covalent binding of 3-(cystein-*S*-yl)APAP (3-Cys-A) to liver proteins in dialyzed (MW cut-off 6,000) liver homogenates. Covalent binding of 3-Cys-A was quantified using a competitive

ELISA based on antiserum specific for 3-Cys-APAP (9,17,18).

Statistical Analysis: Differences between experimental groups were evaluated using a SAS General Linear Models program (SAS Institute, Cary, NC). Results were evaluated by multiple analysis of variance with post-hoc evaluation of differences by Student-Newman-Keuls test ($P \leq 0.05$).

RESULTS

In mice that were not dosed with APAP, neither acetone-pretreatment nor βNF-pretreatment alone was associated with hepatotoxicity (Table 1). Acetone pretreatment induced CYP2E1 but not CYP1A2 activity (Table 2). Similarly, βNF pretreatment induced CYP1A2 but not CYP2E1 activity (Table 2).

In mice that were not pretreated with βNF or acetone, the 150 mg/kg APAP dose caused minimal hepatotoxicity as reflected by a 25% drop in hepatic GSH, and insignificant increases in liver/body weight ratios and ALT (Table 1). The 400 mg/kg APAP dose caused severe hepatotoxicity as indicated by a 56% increase in relative liver weight, a >95% decrease in hepatic GSH, and a >100-fold increase in ALT (Table 1). Both acetone pretreatment and βNF pretreatment increased APAP hepatotoxicity; this effect was most pronounced at the 150 mg/kg dose (Table 1).

Table 1. Effect of Acetone or ß-Napthaflavone Pretreatment on APAP Toxicity in Mice at 4 h

Pre-treatment	APAP (mg/kg)	Liver/body Ratio[1]	Hepatic GSH[2]	Serum ALT[3]
None	0	3.54 ± 0.34 a[4]	3.86 ± 0.11 a,b	1.57 ± 0.03 a
None	150	3.96 ± 0.33 a	2.88 ± 0.19 c	1.67 ± 0.02 a
None	400	5.55 ± 0.59 b	0.04 ± 0.01 d	3.56 ± 0.11 b
ßNF	0	4.22 ± 0.53 a	4.15 ± 1.15 a	1.83 ± 0.04 a
ßNF	150	6.65 ± 1.44 c	3.44 ± 0.52 b,c	2.77 ± 0.13 c
ßNF	400	8.69 ± 0.70 d	0.07 ± 0.22 d	3.67 ± 0.24 b
Acetone	0	3.84 ± 0.26 a	3.44 ± 0.46 b,c	1.79 ± 0.03 a
Acetone	150	4.15 ± 0.28 a	2.98 ± 0.22 c	2.06 ± 0.65 d
Acetone	400	5.63 ± 0.46 b	0.02 ± 0.01 d	3.52 ± 0.15 b

[1] Liver weight/ body weight ratio times 100, mean ± S.D.
[2] Total GSH = nmol/g liver, mean ± S.D.
[3] Log serum ALT, IU/L serum, mean ± S.D.
[4] Data based on four animals per point. Values followed by different letters are significantly different ($p \leq 0.05$).

Table 2. Effect of APAP on P450 Activities in Acetone or ß-Napthaflavone Pretreated Mice at 4 h

Pre-treatment	APAP (mg/kg)	Total P450[1]	CYP1A1/1A2 Activity (EROD)[2]	CYP2E1 Activity (DMN)[3]
None	0	0.46 ± 0.10 b,c[4]	6.82 ± 0.88 d	0.99 ± 0.20 a
None	150	0.33 ± 0.11 b,c,d	7.05 ± 1.59 d	0.77 ± 0.04 a,b
None	400	0.22 ± 0.03 d	4.70 ± 0.53 d	0.77 ± 0.07 a,b
ßNF	0	0.95 ± 0.08 a	14.79 ± 0.88 a	1.16 ± 0.32 a
ßNF	150	1.01 ± 0.26 a	19.37 ± 3.99 b	0.56 ± 0.04 b
ßNF	400	0.49 ± 0.17 b	11.52 ± 0.94 c	0.41 ± 0.10 b
Acetone	0	0.42 ± 0.06 b,c	6.10 ± 1.56 d	1.91 ± 0.11 c
Acetone	150	0.29 ± 0.08 c,d	5.30 ± 0.50 d	1.27 ± 0.23 a
Acetone	400	0.20 ± 0.06 d	5.23 ± 0.40 d	1.16 ± 0.15 a

[1] Total P450 = nmol/mg microsomal protein, mean ± S.D.
[2] DMN = nmol/mg microsome protein/min, mean ± S.D.
[3] EROD = pmol/mg microsome protein/min, mean ± S.D.
[4] Data based on four animals per point. Values followed by different letters are significantly different ($p \leq 0.05$).

APAP alone caused a dose responsive decrease in total P450; 28% at the 150 mg/kg dose and 52% at the 400 mg/kg dose (Table 2). There was a 22% loss of CYP2E1 activity at both the 150 mg/kg and 400 mg/kg APAP dose. In contrast, there was no loss of CYP1A1/1A2 activity at the 150 mg/kg dose, but a 31% loss at the 400 mg/kg APAP dose (Table 2).

Among acetone-pretreated mice, there was a decrease in CYP2E1 activity that was similar 34 - 39% at the 150 and 400 mg/kg APAP doses (Table 2). Among βNF-pretreated mice, there was a decrease in both total P450 and CYP1A1/1A2 activity at the 400 mg/kg dose (Table 2).

In mice that received the low dose of APAP the relative amounts of covalent binding to hepatic protein as 3-Cys-A protein adduct 4 h after dosing were; acetone-pretreated >> βNF pretreated >> control. At the high APAP dose, βNF pretreated mice formed more adduct than either acetone-pretreated or control mice; βNF > acetone pretreated = control (Table 3).

DISCUSSION

Acetaminophen decreased total P450 and P450-dependent metabolism consistent with previous reports (19, 20). Although CYP2E1, CYP1A2 and CYP3A4 have been reported to be responsible for APAP bioactivation (5-8), the possible effect of APAP on 3A4 metabolism was

Table 3. Effect of Acetone or ß-Napthaflavone Pre-treatment on the Covalent Binding of APAP in Liver at 4 h

Pre-treatment	APAP (mg/kg)	Covalent Binding[a]
None	0	0.00
None	150	0.73 ± 0.23
None	400	2.38 ± 0.54
ßNF	0	0.00
ßNF	150	1.47 ± 0.52*
ßNF	400	3.54 ± 0.61**
Acetone	0	0.00
Acetone	150	2.25 ± 0.69***
Acetone	400	2.85 ± 0.24

[a] Values are APAP bound as 3-Cys-A adduct, nmol/mg protein, duplicate determinations, mean ± S.D. of 3 animals/point.
* Significantly different than control mice at same dose ($P \leq 0.001$).
** Significantly different than control and acetone mice at same dose ($P \leq 0.001$).
*** Significantly different than control and ßNF mice at same dose ($P \leq 0.001$).

not examined in the work reported here as narigenin, a 3A4-specific inhibitor (21) does not inhibit the hepatotoxicity of APAP in the B6C3F1 male mouse (unpublished observation, Snawder & Roberts). The total P450 levels and substrate-specific activities in Table 2 are down-modulated by APAP treatment and up-modulated by pretreatments to induce CYP2E1 or CYP1A2. However, the affect of APAP dose on the pattern of substrate-specific activities (i.e., similar loss of CYP2E1 activity at the low and high APAP doses, but greater loss of CYP1A1/1A2 activity at the high APAP dose) demonstrates a loss of activity by the P450 forms participating in the oxidation of APAP (Tables 2 & 3). DMN activity, a marker of CYP2E1 metabolism (15), was inhibited by 150 mg/kg APAP; but 400 mg/kg APAP did not further lower the activity of this enzyme. EROD activity, a CYP1A1/1A2-dependent metabolism (16), was not affected by the low dose of APAP but was lowered by 400 mg/kg APAP (Table 2).

Recently we demonstrated *in vitro* a relatively high Km for human CYP1A2 and a low Km for CYP2E1 for bioactivation of APAP (9). The 0.18 mM Km for human CYP2E1 is in the proximity of the 0.14 mM peak serum APAP concentration expected 2 h after a human therapeutic dose of two 500 mg APAP tablets (Figure 1, 22). The 2.5 mM Km for human CYP1A2 is relevant to drug levels seen in patients at high risk of severe hepatotoxicity. These at risk patients characteristically have serum APAP concentrations above 1.3 mM 4 h after an

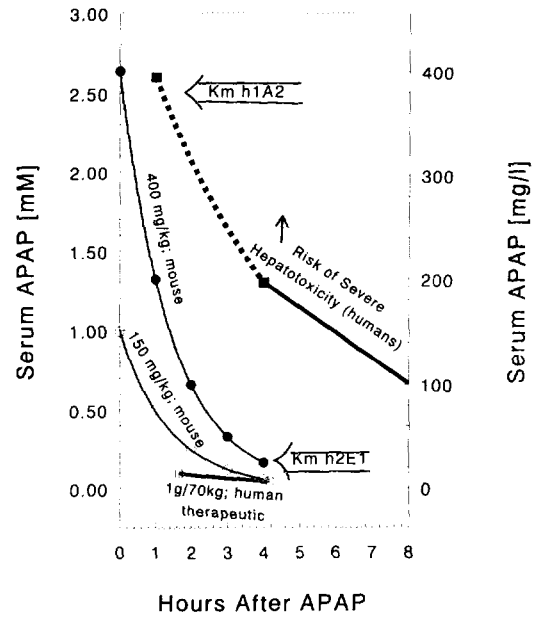

Figure 1. Relationship between low (150 mg/kg; 0.99 mmol/kg) and high (400 mg/kg; 2.65 mmol/kg) doses of APAP used in mice extrapolated to 4 h based on a half-life of 1 h and serum concentrations of APAP associated with therapeutic doses and risk of severe hepatotoxicity in humans extrapolated back to 1 h based on a half-life of 3 h in humans (21,22). The Km for bioactivation of APAP by human CYP1A2 (2.5 mM) and human CYP2E1 (0.18 mM) were determined *in vitro* using human lymphoblastoid cells expressing cDNA for human CYP2E1 or CYP1A2 with detection of covalently bound APAP by immunoassay (9).

acute overdose (23). Assuming a 3 h half-life for APAP in humans (22,23), serum values above 1.3 mM at 4 h would correspond to serum values above 2.6 mM at 1 h after overdose.

In the animal model used for this work, overnight fasted B6C3F1 male mice, APAP has a half-life of 1 h, and maximal immunohistochemically demonstrable hepatic centrilobular 3-Cys-A adducts occur within the first 2 h after APAP dosing, prior to significant depletion of total hepatic GSH (24). The low dose of APAP (150 mg/kg; 0.99 mmol/kg) was selected to produce *in vivo* APAP concentrations that would favor metabolism by CYP2E1 during the 4 h period of the experiment. Similarly, the high dose (400 mg/kg APAP; 2.65 mmol/kg) was selected to favor metabolism by CYP1A2. The relationship of the high and low doses of APAP used in mice to human therapeutic and hepatotoxic doses is shown in Figure 1.

APAP overdose has been reported to cause alterations in the microsomal membrane of rats. Araya *et al.* (4) found that APAP altered the fluorescence polarization of dansylchloride-labelled microsomes. Concurrent with the change in polarization, was a loss of P450-dependent ethoxybenzamide deethylation. The authors concluded that APAP-overdose perturbs the association of the P450-membrane complex. Placke *et al.* (25) also reported morphological changes in endoplasmic reticulum that were evident in electron micrographs of APAP-dosed mice.

The observed loss of total P450 reported in Table 2, is consistent with such changes in morphology. Comparison of absorbance spectra of reduced, CO-treated microsomes from control and APAP-dosed mice demonstrated a relative loss of P450 chromophore and an increase in P420 chromophore as a consequence of *in vivo* APAP treatment (data not shown). Absorbance at ~420 nm is typical of heme proteins lacking a cysteinyl axial ligand and could be attributable either to modification of cysteine in the active site by NAPQI or to configurational changes that would displace the thiolate ligand (26).

Birge *et al.* (27) reported that while APAP did not decrease total P450, it did cause a decrease in NADPH:P450 reductase. Patten *et al.* (28) reported that addition of NADPH:P450 reductase and cytochrome b5 is required for maximal DMN activity from TK 143 microsomes with vaccinia-expressed CYP2E1. Collectively, these observations suggest that the decrease in CYP2E1-dependent DMN activity may be in part attributable to effects of APAP on the NADPH:P450 reductase.

When CYP2E1 was induced by acetone, the low (150 mg/kg) dose of APAP resulted in high levels of hepatic adducts (2.25 nmol 3-Cys-A/mg protein) at a time when GSH was depleted only 13% relative to controls (Tables 1&3). This level of adduct formation is 95% of the (2.38 nmol/mg) adduct level observed in non-induced controls that received the high (400 mg/kg) APAP dose causing 99% GSH depletion (Tables 1&3). These findings are consistent with the reported low Km for human CYP2E1 (9) and with reports of increased susceptibility to the hepatotoxic effects of APAP in individuals with poor nutrition and compromised GSH reserves and induced CYP2E1 such as alcoholics (29), and in individuals taking drugs known to induce CYP2E1 such as patients receiving isoniazid to control active tuberculosis (30).

This work reports that APAP causes a dose-dependent loss of form-specific P450 activities. This observation reflects the enzyme kinetics of the two major P450's metabolizing APAP in this animal model, CYP2E1 and CYP1A2. These data indicate that CYP2E1 may be chiefly responsible for APAP bioactivation at therapeutic concentrations whereas CYP1A2 may play a greater role in the bioactivation and toxicity at high APAP doses. Further, the data suggest that APAP may influence the efficacy or toxicity of drugs metabolized by CYP2E1 and CYP1A2.

REFERENCES

1. Nelson, S.D. (1990) Seminars in Liver Disease Vol. 10 pp. 267-278, Thieme Medical Publishers Inc. New York, NY.
2. Jollow, D.J., Mitchell, J.R., Potter, W.Z., Davis, D.C., Gillette, J.R. and Brodie, B.B. (1973) J. Pharmacol. Exp. Ther. 187,195-202.
3. Tirmenstein, M.A. and Nelson, S.D. (1990). J. Biol. Chem. 265,3059-3065.
4. Araya, H., Horie, T., Hayashi, M. and Awazu, S. (1987) J. Pharm. Pharmacol. 39,1047-1049.
5. Jeffery, E.H., Arndt, K. and Haschek, W.M. (1990). In Biological Reactive Intermediates IV, Edited by C.M. Witmer *et al.*, Plenum Press, New York, 249-251.

6. Raucy, J.L., Lasker, J.M., Lieber, C.S. and Black, M. (1989) Arch. Biochem. Biophys. 271,270-283.
7. Thummel, K.E., Lee, C.A., Kunze, K.L., Nelson, S.D., and Slattery, J.T. (1993) Biochem. Pharmacol. 45:1563-1569.
8. Patten, C.J., Thomas, P.E., Guy, R.L., Lee, M., Gonzalez, F.J., Guengerich, F. P., and Yang, C.S. (1993) Chem. Res. Toxicol. 6:511-518.
9. Snawder, J.E., Roe A.L, Benson, R.W., Casciano, D.A., and Roberts, D.W. (1994) Pharmacogenetics 4:43-46.
10. Leakey, J.E.A., Cunny, H.C., Bazare, J.J. Jr., Webb, P.J., Feuers, R.J., Duffy, P.H. and Hart, R.W. (1989) Mech. Aging Devel. 48,145-155.
11. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, J (1951). J. Biol. Chem. 193, 265-275.
12. Bergmeyer, H.U., Scheibe, P., and Wahlefeld, A.W. (1978). Clin Chem. 4,58-73.
13. Tietze, F. (1969) Analyt. Biochem. 27,502-522.
14. Omura, T., and Sato, R.J. (1964) Biol. Chem. 239,2370-2378.
15. Peng, R., Tu Y.Y., and Yang, C.S. (1982) Carc. 3,1457-1461.
16. Burke, M.D., Thompson, S., Elcombe, C.R., Halpert, J., Haaparanta, T. and Mayerm, R.T. (1985) Biochem. Pharmac. 34,3337-3345.
17. Roberts, D.W., Pumford, N.R., Potter, D.W., Benson, R.W. and Hinson, J.A. (1987) J. Pharmacol. Exp. Ther. 241,527-533.
18. Potter, D.W., Pumford, N.R., Hinson, J.A., Benson, R.W., and Roberts, D.W. (1989) J. Pharmacol. Exp. Ther. 248,182-189.
19. Tuntataterdtum, S., Chaudhary, I.P., Cibull, M., Robertson, L.W., and Blouin, R.A. (1993) Tox. Appl. Pharmac. 123:219-225.
20. Thorgeirsson, S.S., Sasame, H.A., Mitchell, M.R., Jollow, D.J., and Potter, W.Z. (1976) Pharmacology 14:205-217.
21. Guengerich, F.P., and Kim, D.H.(1990) Carcinogenesis, 11:2275-2279.
22. Savides, M.C., and Oehme, F.W. (1983) J. Appl. Tox. 3:96-111.
23. Prescott, L.F., Wright, N., Roscoe, P., Brown, S.S. (1971) Lancet 1:519-522.
24. Roberts, D.W., Bucci, T.J., Benson, R.W., Warbritton, A.R., McRae, T.A., Pumford, N.R. and Hinson, J.A. (1991) Am. J. Path. 138,359-371.
25. Placke, M.E., Ginsgerg, G.L., Wyand, D.S. and Cohen, S.D. (1987) Toxicol. Pathol. 15,431-438.
26. Lipscomb, J.D. (1980) Biochemistry 19:3590-3599.
27. Birge, R.B., Bulera, S.J., Bartolone, J.B., Ginsberg, G.L., Cohen, S.D. and Khairallah, E.A. (1991) Tox. Appl. Pharmac. 109,443-454.
28. Patten, C.J., Ishizaki, H., Aoyama, T., Lee, M., Ning, S.M., Huang, W., Gonzalez, R.J., and Yang, C.S. (1992) Arch. of Biochem. and Biophys. 299:163-171.
29. Seifert, C.F., Lucas, D.S., Vondraced, T.G., Kastens, D.J., McCarty, D.L., and Bui, B. (1993) Pharmacotherapy 13:391-395.
30. Murphy, R., Scartz, R., and Watkins, P.B. (1990) Ann. Intern. Med. 113:799-800.