J. Pharm. Pharmacol. 1997, 49: 446–449
Received September 27, 1996
Accepted December 17, 1996

© 1997 J. Pharm. Pharmacol.

Biochemical Basis for Deficient Paracetamol Glucuronidation in Cats: an Interspecies Comparison of Enzyme Constraint in Liver Microsomes

MICHAEL H. COURT AND DAVID J. GREENBLATT

Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, 136 Harrison Avenue, Boston MA 02111, USA

Abstract

Unlike most other mammalian species, domestic cats glucuronidate phenolic compounds poorly and are therefore highly susceptible to the toxic side effects of many drugs, including paracetamol. In this study, we evaluated the role of enzyme constraint, a characteristic that limits the activity of all uridine 5′-diphosphoglucuronosyltransferase (UGT) enzymes, in the aetiology of this species-dependent defect of drug metabolism.

Detergent activation experiments were performed using hepatic microsomes from cats (4), dogs (4), man (4), and 6 other mammalian species (1 liver each). In addition, we used microsomes from Gunn rats which are sensitive to paracetamol toxicity because of a genetic defect affecting all family 1 UGTs. Increase in paracetamol-UGT activity at optimum concentrations of detergent was used as an index of enzyme constraint. Native activity (measured in the absence of detergent) was less than one-sixth in cats compared with other species. Optimum detergent treatment tended to enhance rather than abolish this difference, however, indicating relatively lower levels of constraint of paracetamol-UGT in cats compared with other species. Similarly, detergent treatment failed to reduce the native activity difference between homozygous mutant and normal Gunn rats. Initially CHAPS (3-(3-cholamidopropyl)-dimethylammonio-1-propanesulphonic acid) was used as the detergent activator; in 3 of 4 microsomal preparations from man, however, inhibition rather than activation was observed at all detergent concentrations used. Studies were repeated using the non-ionic detergent, Brij 58 (polyoxyethylene 20-cetyl ether), which resulted in similar although more profound activation and no inhibition.

We conclude that deficient paracetamol glucuronidation in cats does not result from increased paracetamol-UGT constraint in this species compared with other mammalian species. Other causes, such as differences in enzyme protein concentration or substrate affinity might be responsible.

The catalytic function of the uridine 5′-diphosphoglucuronosyltransferase (UGT) enzymes in microsomal preparations (and possibly in-vivo) is considered to be constrained, with full activity becoming apparent only after treatment with some form of membrane perturbant, such as a detergent (Bock & White 1974; Zakim & Dannenberg 1992). Although the mechanistic basis of this phenomenon is currently unknown, a current theory proposes that the lipid environment of the membrane regulates the conformational state and function of the enzyme (Zakim & Dannenberg 1992). In support of this, recent studies have shown that the degree of constraint of a particular UGT (as determined by measuring the maximum increase in activity with detergent treatment) is affected by changes in the physicochemical properties of the microsomal membrane caused by dietary manipulation (Catsuma & Brenner 1986) and disease (Morrisson & Hawksworth 1984; Vega et al 1986). Gender differences in UGT constraint have also been related to differences in membrane composition (Catania et al 1995). Detergent activation studies are therefore used to determine whether differences in UGT activity relate to differences in enzyme protein level or are caused by variations in the enzyme membrane environment (Lett et al 1992).

Correspondence: M. H. Court, Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, 136 Harrison Avenue, Boston MA 02111, USA.

Cats are highly susceptible to the toxic effects of paracetamol, a commonly used analgesic drug (Jernigan 1988). Previous in-vivo studies have indicated that this species-dependent sensitivity results from a relative deficiency of phenol glucuronidation as a pathway for the conjugation and elimination of paracetamol (Savides et al 1984). Although the in-vitro metabolism of paracetamol in the cat has not been reported, a number of studies using structurally related phenolic compounds as substrates has shown substantially reduced hepatic microsomal UGT activity in cats compared with other species of animal (Jansen & Henderson 1972; Gregus et al 1983; Watkins & Klaassen 1986). Factors possibly contributing to this anomaly include species differences in constraint or enzyme protein concentration, or both.

In this study we investigated the contribution of constraint to limiting the activity of paracetamol-UGT in cats. Specifically, we measured the increase in paracetamol glucuronidation in the presence of optimally activating concentrations of detergent (as an index of constraint) using liver microsomes from cats in comparison with those from species less prone to paracetamol intoxication, including man, dogs and six other mammalian species. We also measured constraint in liver microsomes from Gunn rats, which are also sensitive to paracetamol intoxication because of a genetic defect of all UGT family 1 isoenzymes. Initially we used CHAPS (3-(3-cholamidopropyl)-dimethylammonio-1-propane-

sulphonic acid) as the detergent activator. Because of excessive inhibition of UGT activity, however, these studies were repeated using Brij 58 (polyoxyethylene 20-cetyl ether).

Materials and Methods

Reagents

Brij 58, CHAPS, UDP-glucuronic acid (UDPGA, sodium salt) and paracetamol were purchased from Sigma (St Louis, MO) and 2-acetamidophenol from Aldrich (Milwaukee, WI). Paracetamol glucuronide was a gift from McNeil Consumer Products Company (Fort Washington, PA).

Liver microsomes

Liver tissues were acquired from four male domestic short-hair cats (Harlan Sprague Dawley, Indianapolis, IN), two male and two female mixed-breed dogs (Team Associates, Dayville, CT), one crab-eating Macaque monkey (unknown source), four male CD-1 mice (Charles River Laboratories, Wilmington, MA), one female New Zealand White rabbit (Hazleton Research Laboratories (Denver, PA), three male Gunn rats (one each of J/J homozygous mutant, J/+ heterozygous mutant and +/+ congenic normal; Harlan Sprague Dawley); one Holstein cow, one male quarter horse and one Yorkshire pig (all from Tufts University School of Veterinary Medicine, North Grafton, MA). Animals were either procured specifically for these studies, or were untreated control animals from studies in which tissue other than liver was being collected. Tissue collection was approved by the Tufts University Animal Research Committee. Liver tissues from man (three male and one female Caucasians) were obtained from the International Institute for the Advancement of Science (Exton, PA). Donors had no history of liver disease but had failed tissue match to possible transplant recipients. All livers were stored at −80°C.

Microsomes were prepared by differential centrifugation using a technique described previously (von Moltke et al 1993). The resulting pellet was reconstituted in 20% glycerol-phosphate buffer, divided into portions and stored at −80°C. Microsomes prepared by this method in this laboratory have been shown to be stable for at least 12 months. Mouse-liver microsomes were prepared from the pooled livers of four animals. All other microsomes were prepared from separate livers. Microsomal protein concentrations were assayed using the bicinchoninic acid method (Pierce, Rockford, IL).

Paracetamol glucuronidation

The assay method used was similar to that described previously (von Moltke et al 1993) with minor modifications. Briefly, microsomes (200 μg protein), detergent (CHAPS, 0–0·32% final concentration, or Brij 58, 0–0·16% final concentration), $MgCl_2$ (5 mM final concentration), and paracetamol (5 mM final concentration), and phosphate buffer (50 mM final concentration, pH 7·5) to a volume of 150 μL were mixed in disposable 10 mL glass tubes, cooled on ice. These solutions were then equilibrated in an agitated water bath at 37°C for 3 min. Reactions were initiated by addition of 100 μL UDPGA (5 mM final concentration) and left to proceed for 30 min (180 min for cat liver microsomes), at which time the reaction was stopped by adding a mixture of HCl (1 N; 25 μL) and 2-acetamidophenol (10 μg; the internal standard) in methanol (25 μL), vortex mixing, and then immediate cooling on ice. This mixture was transferred to microcentrifuge tubes and spun at 14 000 rev min^{-1} for 5 min. The supernatant was then transferred to vials for determination of product concentration by high performance liquid chromatography (HPLC).

Chromatography was performed with a 300 mm × 3·9 mm i.d. C_{18} μBondapack column (Waters, Milford, MA) with 100 mM KH_2PO_4 in water–glacial acetic acid–acetonitrile, 96 : 1 : 3 (v/v) as mobile phase at a flow rate of 1·4 mL min^{-1}. The eluate was monitored by ultraviolet absorption at 254 nm (Lambda-Max Model 481, Waters, Milford, MA). Product identity was verified by showing co-elution with known standard and by disappearance of product after treatment with β-glucuronidase. A calibration curve was prepared for each run using a series of known concentrations of pure paracetamol glucuronide. Metabolite concentrations were calculated by linear regression of standard curve data using measured metabolite/internal standard peak height ratios. Intra- and inter-assay coefficients of variation were less than 8% and 12%, respectively. Enzyme activity was calculated by dividing the amount of product formed by the incubation time and microsomal protein content (nmol min^{-1} mg^{-1}). Linear dependencies of enzyme activity on incubation time (up to 240 min) and microsomal protein concentration (up to 1 mg mL^{-1}) were established in initial experiments both with and without detergents added. These were also substantiated in each experiment by demonstrating proportionally increased product formation when reaction mixtures contained 50% more microsomal protein or were incubated for 50% longer than usual at the lowest substrate concentration.

Data analysis

Derived parameters included: glucuronidation activity in the absence of detergent (native activity); maximum observed activity with detergent activation (optimum activity); maximum percentage increase in activity from native activity (optimum activation); and detergent concentration at maximum (optimum detergent concentration, % w/v). Data from hepatic microsomes from cat, dog and man are given as mean ± s.e.m. For other species in which individual livers (per species) were used, results are given as mean values of triplicate determinations. For statistical comparison purposes data from cow, horse, monkey, mouse, pig, rabbit and rat (homozygous normal Gunn rat) hepatic microsomes are also summarized as mean ± s.e.m. (denoted as 'other species' group). These data were subsequently analysed by analysis of variance using the rank transformation approach with post hoc multiple comparisons testing using the Student-Neuman-Keuls method (Iman 1982). The significance of differences between detergent treatments (CHAPS compared with Brij 58) was determined by use of the paired Student's *t*-test. A significance level of $P < 0.05$ was used for all analyses. Use of the heterogeneous 'other species' group, enabled us to determine which of the homogenous groups (cat, dog or man) most probably represented the species outlier and which were closer to the species norm when differences between these homogenous groups were detected.

Results

Detergent activation parameters are given in Table 1. Confirming previous observations of deficient glucuronidation of paracetamol in-vivo, paracetamol-UGT activity in native microsomes was over sixfold less in cats than in other species

Table 1. Comparative detergent activation of hepatic microsomal paracetamol-UGT activity.

Detergent	Livers	Native activity (nmol min^{-1} mg^{-1})	Optimum activity (nmol min^{-1} mg^{-1})	Optimum activation (% increase)	Optimum detergent concentration (% w/v)	n*
Brij 58	Cat	$0.13 \pm 0.02^{d,h,o}$	$0.37 \pm 0.08^{a,d,o}$	$174 \pm 16^{a,d,o}$	0.012 ± 0.000^{a}	4
	Dog	0.96 ± 0.13^{c}	$3.77 \pm 0.62^{a,c,h}$	$292 \pm 31^{a,c,h}$	0.014 ± 0.001^{a}	4
	Man	1.16 ± 0.38^{c}	$1.79 \pm 0.51^{a,d,o}$	$66 \pm 40^{a,d,o}$	0.012 ± 0.002^{a}	4
	Other species†	1.79 ± 0.55^{c}	$8.26 \pm 2.74^{a,c,h}$	$352 \pm 39^{a,c,h}$	0.018 ± 0.002^{a}	7
	Cow	1·.22	4·58	275	0·024	1
	Horse	1·74	6·12	251	0·024	1
	Monkey	1·36	5·56	310	0·016	1
	Mouse	1·44	9·57	563	0·012	1
	Pig	1·01	4·22	317	0·012	1
	Rabbit	4·99	24·08	382	0·020	1
	Gunn rat (+/+)	0·80	3·70	365	0·016	1
	Gunn rat (J/+)	0·61	2·54	319	0·020	1
	Gunn rat (J/J)	0·49	2·08	326	0·016	1
CHAPS	Cat	$0.12 \pm 0.02^{d,h,o}$	$0.22 \pm 0.04^{a,d,o}$	90 ± 20^{a}	$0.068 \pm 0.004^{a,d}$	4
	Dog	$0.70 \pm 0.10^{c,o}$	$2.12 \pm 0.24^{a,c,h,o}$	$209 \pm 16^{a,h}$	$0.110 \pm 0.010^{a,c,o}$	4
	Man	1.03 ± 0.33^{c}	$1.05 \pm 0.28^{a,d,o}$	$9 \pm 30^{a,d,o}$	0.072 ± 0.008^{a}	4
	Other species†	$1.48 \pm 0.35^{c,d}$	$6.12 \pm 2.37^{a,c,d,h}$	$255 \pm 57^{a,h}$	$0.081 \pm 0.007^{a,d}$	7
	Cow	1·05	2·98	184	0·064	1
	Horse	1·35	3·74	178	0·080	1
	Monkey	0·95	1·30	37	0·064	1
	Mouse	1·61	8·69	439	0·080	1
	Pig	0·94	2·78	197	0·080	1
	Rabbit	3·49	19·36	454	0·080	1
	Gunn rat (+/+)	1·00	3·96	295	0·120	1
	Gunn rat (J/+)	0·82	2·73	231	0·120	1
	Gunn rat (J/J)	0·45	1·12	149	0·080	1

Values are given as mean ± s.e.m. Activities were measured using 5 mM paracetamol, 5 mM UDPGA and 200 μg microsomal protein. *Number of individual livers. †The other species group includes data from cow, horse, monkey, mouse, pig, rabbit, and homozygous normal Gunn rat (+/+). [a]Significantly different ($P < 0.05$) compared with same species group treated with other detergent (paired *t*-test). [c,d,h,o]Significantly different ($P < 0.05$) compared with cat (c), dog (d), human (h) or 'other species' group (o), treated with same detergent (analysis of variance on ranks with Student-Newman-Keuls post hoc multiple comparisons).

($P < 0.01$, analysis of variance). Native activity was also lower in homozygous mutant compared with homozygous normal Gunn rat microsomes (mean of n = 6 determinations; 0·47 compared with 0·9 nmol min^{-1} mg^{-1}, respectively). Species differences in detergent activation were observed, with significantly less activation measured with microsomes from cat and man compared with those from dogs and the other species group ($P < 0.01$, analysis of variance). This species-associated pattern of activation was generally similar for both CHAPS- and Brij 58-induced activation, except that optimum activity and optimum activation were both consistently lower in CHAPS-treated microsomes than in Brij 58-treated microsomes ($P < 0.05$, paired *t*-tests). In addition, microsomes from man differed from those from cats, dogs and other species in the response to CHAPS in that as detergent concentration increased, a decrease in paracetamol-UGT activity preceded an increase to a peak value (Fig. 1). In three of the four microsome samples from man examined, the relative activity value at this peak was less than unity (no activation was observed).

Discussion

We had hypothesized that deficient paracetamol glucuronidation in cats resulted from increased levels of constraint in this species compared with other mammalian species. Rather than abolish UGT activity differences between cats and other species, however, optimum detergent treatment enhanced these differences, indicating that cats might, in fact, have relatively lower levels of enzyme constraint. Even less activation was observed with microsomes from man (66% increase with Brij 58). Similar results have been reported for Brij 58-activation of microsomal paracetamol-UGT from man (52% increase), but contrasts with activation values of over 300% reported by the same investigators for morphine-UGT and 4-methylumbelliferone-UGT from man (Miners et al 1990). Poor activation appears, therefore, to be a unique characteristic of the isoforms of paracetamol-UGT from man and possibly from the cat.

Species and UGT isoform differences in UGT constraint most likely relate to differences in membrane lipid composition or enzyme protein structure, or both, affecting lipid-protein interactions. Differences between protein-protein interactions might also occur because UGTs exist as oligomers (Vessey & Kempner 1989). Determination of which of these factors is responsible for the observed differences in paracetamol-UGT constraint among man, cats and other species will require further comparative analysis of microsomal membrane composition and enzyme protein structure.

Gunn rats have a genetic mutation that causes functional loss of all UGT family 1 isoenzymes (Chowdhury et al 1993), including UGT1·6 which has significant paracetamol-UGT activity (Bock et al 1993). The lower native activity in the homozygous mutant compared with the homozygous normal animal in this study is, therefore, most likely attributable to a lower effective enzyme protein concentration and not to increased constraint. This was confirmed by activation studies in that constraint was not higher in the mutant compared with the normal animal (it should be noted that this is based on multiple determinations using livers from individual rats).

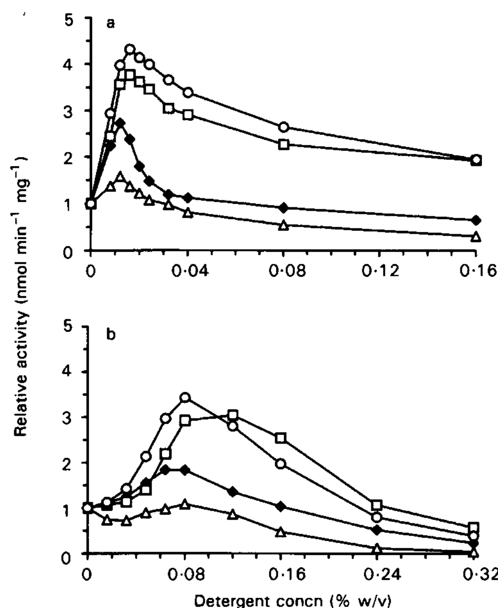

FIG. 1. Effect of increasing a. Brij 58 or b. CHAPS concentration on paracetamol-UGT activity (relative to activity without detergent) in hepatic microsomes obtained from four cats (◆), four dogs (□), four humans (△) and seven other mammalian species (○). Activities were measured at 5 mM paracetamol and 5 mM UDPGA in 250 μL incubation volume using 200 μg hepatic microsomes. Data points represent mean values. Error bars are omitted for clarity.

On the basis of studies using rat microsomes, both CHAPS and Brij 58 have been proposed as suitable agents for detergent activation of UGTs because of minimum inhibition and a broad activation profile (Lett et al 1992). We initially used CHAPS for UGT activation, because of ready availability in this laboratory. In all species, however, CHAPS-treated microsomes consistently showed less activation at optimum detergent concentrations when compared with Brij 58-treated microsomes. In microsomes from man CHAPS, even at low concentrations, appears to act predominantly as an inhibitor. Detergents might inhibit glucuronidation by a number of different mechanisms. Loss of activation (deactivation) at detergent concentrations greater than the optimum activation concentration might be related to solubilization of membrane proteins and associated loss of lipid–protein and protein–protein interactions. In support of this, the critical micelle concentration (CMC) of Brij 58 (0·0085% in water (Schick 1966), probably less in salt solutions) is less than detergent concentrations associated with deactivation as measured in this study (> 0·012–0·024%; Fig. 1) and in a previous study (> 0·03% (Miners et al 1990)). In contrast, deactivation with CHAPS occurred at detergent concentrations (> 0·064–0·12%; Fig. 1) 2 to 8 times less than the CMC (0·5% in water; 0·25% in 40 mM KCl (Partearroyo et al 1988)). Other detergent effects, such as enzyme denaturation which has been shown to occur with Triton X-100, might be responsible for the observed inhibitory effects of CHAPS. Direct catalytic inhibition is also possible, because CHAPS is a bile derivative and many bile salts are endogenous substrates for the UGT enzymes.

In conclusion, we have determined that deficient paracetamol glucuronidation in cats is not explained by species differences in UGT constraint. In future studies we plan to use kinetic analysis and Western blotting to investigate the possible role of species differences in paracetamol-UGT protein concentration.

Acknowledgements

M. H. Court is the recipient of a Special Emphasis Research Career Award (K01-RR-00104) from the National Institutes of Health. This work was also supported in part by grants MH-34223 and MH-19924 from the National Institutes of Health.

References

Bock, K., White, I. (1974) UDP-glucuronyltransferase in perfused rat liver and in microsomes: influence of phenobarbital and 3-methylcholanthrene. Eur. J. Biochem. 46: 451–459

Bock, K. W., Forster, A., Gschaidmeier, H., Brück, M., Munzel, P., Schareck, W., Fournel-Gigleux, S., Burchell, B. (1993) Paracetamol glucuronidation by recombinant rat and human phenol UDP-glucuronosyltransferases. Biochem. Pharmacol. 45: 1809–1814

Catania, V. A., Dannenberg, A. J., Luquita, M. G., Sanchez Pozzi, E. J., Tucker, J. K., Yang, E. K., Mottino, A. D. (1995) Gender-related differences in the amount and functional state of rat liver UDP-glucuronosyltransferase. Biochem. Pharmacol. 50: 509–514

Catsuma, C., Brenner, R. (1986) Effect of dietary cholesterol on microsomal membrane composition, dynamics and kinetic properties of UDP-glucuronosyltransferase. Biochim. Biophys. Acta 855: 231–242

Chowdhury, J. R., Kondapalli, R., Chowdhury, R. N. (1993) Gunn rat: a model for inherited deficiency of bilirubin glucuronidation. Adv. Vet. Sci. Comp. Med. 37: 149–173

Gregus, Z., Watkins, J. B., Thompson, T. N., Harvey, M. J., Rozman, K., Klaassen, C. D. (1983) Hepatic phase I and phase II biotransformations in quail and trout: comparison to other species commonly used in toxicity testing. Toxicol. Appl. Pharm. 67: 430–441

Iman, R. L. (1982) Some aspects of the rank transform in analysis of variance problems. Proc. 7th Annu. SAS Users Group, Int. Conf., San Francisco, CA, February 14–17, 1982, SAS Institute Inc., Cary, North Carolina, pp 676–680

Jansen, P. L. M., Henderson, P. T. (1972) Influence of phenobarbital treatment on *p*-nitrophenol and bilirubin glucuronidation in Wistar rat, Gunn rat and cat. Biochem. Pharmacol. 21: 2457–2462

Jernigan, A. D. (1988) Idiosyncrasies of feline drug metabolism. 12th Annu. Kal Kan Symp., pp 65–69

Lett, E., Kriszt, W., de Sandro, V., Ducrotoy, G., Richert, L. (1992) Optimal detergent activation of rat liver microsomal UDP-glucuronosyltransferases toward morphine and 1-naphthol: contribution to induction and latency studies. Biochem. Pharmacol. 43: 1649–1653

Miners, J. O., Lillywhite, K. J., Yoovathaworn, K., Pongmarutai, M., Birkett, D. J. (1990) Characterization of paracetamol UDP-glucuronosyltransferase activity in human liver microsomes. Biochem. Pharmacol. 40: 595–600

Morrisson, M., Hawksworth, G. (1984) Glucuronic acid conjugation by hepatic microsomal fractions isolated from streptozotocin-induced diabetic rats. Biochem. Pharmacol. 33: 3833–3838

Partearroyo, M., Goni, F., Katine, I. (1988) Micellar properties of the zwitterionic bile derivative CHAPS. Biochem. Int. 16: 259–265

Savides, M. C., Oehme, F. W., Nash, S. L., Leipold, H. W. (1984) The toxicity and biotransformation of single doses of acetaminophen in dogs and cats. Toxicol. Appl. Pharm. 74: 26–34

Schick, M. (1966) Nonionic Surfactants. Marcel Dekker, New York

Vega, P., Gaule, C., Sanchez, E., Villar, E. (1986) Inhibition and activation of UDP-glucuronosyltransferase in alloxanic-diabetic rats. Gen. Pharmacol. 17: 641–645

Vessey, D., Kempner, E. (1989) In situ structural analysis of microsomal UDP-glucuronosyltransferase determined with radiation-inactivation analysis. J. Biol. Chem. 264: 6334–6338

von Moltke, L. L., Manis, M., Harmatz, J. S., Poorman, R., Greenblatt, D. J. (1993) Inhibition of acetaminophen and lorazepam glucuronidation in vitro by probenecid. Biopharm. Drug Disp. 14: 119–130

Watkins III, J. B., Klaassen, C. D. (1986) Xenobiotic biotransformation in livestock: comparison to other species commonly used in toxicity testing. J. Anim. Sci. 63: 933–942

Zakim, D., Dannenberg, A. J. (1992) How does the microsomal membrane regulate UDP-glucuronosyltransferases? Biochem. Pharmacol. 43: 1385–1393