Pergamon

Biochemical Pharmacology, Vol. 48, No. 1, pp. 197–200, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0164-G

SHORT COMMUNICATIONS

Comparison of levels of aldehyde oxidase with cytochrome P450 activities in human liver *in vitro*

(*Received* 6 *January* 1994; *accepted* 10 *March* 1994)

Abstract—Microsomal suspensions and 9000 *g* supernatant (S-9) fractions were prepared from the liver tissue of six human multiorgan donors. The S-9 fractions were characterized for cytosolic aldehyde oxidase (AO) activity, using three different substrates [N^1-methylnicotinamide (NMN), benzaldehyde and 6-methylpurine]. In addition, human liver NMN oxidase activity was compared with that detected in rat, dog and monkey liver S-9 fractions. As expected, the rank order of NMN oxidase activity was monkey > rat > dog, and in five out of six subjects the activity was lowest in humans (<2.0 nmol/min/mg). The variation in AO activity among the various human livers was greater for NMN oxidase (>40 fold) than for 6-methylpurine and benzaldehyde oxidase (≤3.6 fold) activity. The corresponding microsomal preparations were characterized with respect to the levels of total cytochrome P450 (CYP) and six CYP-dependent mixed-function monooxygenase (MFO) activities. The variation was greatest for dextromethorphan *O*-demethylase (CYP2D6) and lowest for *N*,*N*-dimethylnitrosamine *N*-demethylase (CYP2E1) activity (147- vs 1.4-fold). The inter-sample variation for the total CYP, CYP3A (erythromycin *N*-demethylase), CYP1A2 (7-ethoxyresorufin *O*-deethylase), CYP2A6 (coumarin 7-hydroxylase) and CYP2C9/10 (tolbutamide 4-methyl hydroxylase) was 2.2- to 5.3-fold. Furthermore, the levels of AO activity did not correlate with total (spectrally detectable) CYP or any of the CYP form-selective MFO activities.

Key words: aldehyde oxidase (EC 1. 2. 3. 1); cytochrome P450 (EC 1. 14. 14. 1); human liver; *in vitro* comparison; subcellular fractions; inter-subject variability

Many drugs, carcinogens and steroids are known to undergo so-called "phase I" oxidative metabolism by liver microsomal CYP* (EC 1. 14. 14. 1), which has been shown to represent a "superfamily" of hemeproteins [1]. With the increasingly widespread use of human liver transplant and biopsy tissue, a large amount of information has been gained concerning the substrate selectivity, mode of regulation and inter-subject variability of the various CYP "family" or "subfamily" members [2, 3]. However, enzymes, such as cytosolic molybdenum-containing AO (EC 1. 2. 3. 1), are also known to mediate the oxidation of a large number of compounds [4, 5], but only a few studies have focused on human tissue [6, 7]. One problem has been the reported instability of the enzyme during tissue processing and storage, which may lead to marked inter-sample variability [6, 8]. A second problem has been the observation that there are marked species differences with respect to the ability of AO to oxidize a given substrate, a problem that is often exacerbated by the lack of a common analytical method [4, 5]. To date, there is no information concerning the variability of AO activity in subcellular fractions prepared from human liver tissue and how this relates to the levels of the various forms of CYP. This is of extreme importance, when one considers that CYP and AO are coordinately involved in the oxidation of a number of agents [3–5, 9–11]. Furthermore, AO has also been implicated in the reduction of *N*-oxides, the products of CYP and/or FMO (EC 1. 14. 13. 8) mediated oxidation, which may subsequently result in their redox cycling [5]. Therefore, in order to more fully predict the *in vivo* metabolic profile in humans and obtain a more accurate species comparison, *in vitro* metabolism studies with these types of compound necessitate the use of models (e.g. cultured hepatocytes, tissue slices, whole homogenate or S-9 fractions) that allow for their coupled (microsomal and cytosolic) oxidation. Obviously, this cannot be achieved with microsomal preparations. The purpose of this preliminary study was to investigate the variability of AO activity in conventionally prepared and stored human liver (S-9) fractions and to compare this with the levels of various CYP form-selective monooxygenase activities in the corresponding microsomal fractions.

Materials and Methods

Chemicals. 6-Methylpurine, benzaldehyde, N^1-methylnicotinamide, $NADP^+$, glucose 6-phosphate, glucose 6-phosphate dehydrogenase (Sigma Type VII, from baker's yeast), resorufin, 7-ethoxyresorufin, and *N*, *N*-dimethylnitrosamine were obtained from the Sigma Chemical Co. (St. Louis, MO). Coumarin and 7-hydroxycoumarin were obtained from the Aldrich Chemical Co. (Milwaukee, WI). [1,2-$^3H_2$]Tolbutamide was synthesized as previously described [12]. All other reagents were purchased commercially at the best obtainable grade.

Liver samples. Transplant quality human liver tissue (N = 6 human liver samples) was obtained from the International Institute for the Advancement of Medicine (IIAM, Exton, PA) and was received at Abbott Laboratories within 24 hr of removal from the donor. In all cases, the hepatic tissue was transported by commercial carrier in cold Belzer's solution. All of the subjects had received various combinations of dopamine, mannitol and/or furosemide. Only one subject (FRX) was known to have received a CYP inducer (dexa-

* Abbreviations: CYP, cytochrome P-450; AO, aldehyde oxidase; FMO, NADPH-dependent flavin-containing monoxygenase; S-9, 9000 *g* supernatant; and MFO, mixed-function monooxygenase.

Table 1. Aldehyde oxidase activity in human liver 9000 *g* supernatant (S-9) fractions

Subject ID	Age (years)	Sex	Activity* (nmol/min/mg S-9 protein) BA	6-MP	NMN
FGL	26	F	11.4	17.5	1.44
GDD	25	F	14.5	14.7	<0.5
GEQ	20	M	19.5	14.1	<0.5
GFE	50	F	40.7	32.8	20.4
GC4	29	M	26.3	23.1	<0.5
FRX	58	M	22.2	15.5	1.50
Mean ± SD			22.4 ± 10.4	19.6 ± 7.24	7.78 ± 10.9
Fold variation†			3.6	2.3	>40

* Data for each subject represent the mean of duplicate determinations. Abbreviations: BA, benzaldehyde; 6-MP, 6-methylpurine; and NMN, N^1-methylnicotinamide.
† Ratio of the highest/lowest activity.

Table 2. Cytochrome P450 (CYP) dependent monooxygenase activities in human liver microsomes

Subject ID	Total CYP*	Activity† (pmol product formed/min/mg microsomal protein) ERODase (CYP1A2)	ERNDase (CYP3A)	TOLase (CYP2C9/10)	DMNase (CYP2E1)	DEXase (CYP2D6)	COHase (CYP2A6)
FGL	410	54	1180	270	720	80	412
GDD	77	38	540	180	500	16	270
GEQ	138	67	780	90	590	1.5	97
GFE	352	115	1010	ND‡	710	66	313
GC4	270	89	580	270	620	87	472
FRX	314	262	810	350	620	220	113
Mean ± SD	260 ± 128	104 ± 82	817 ± 246	232 ± 100	626 ± 81	78 ± 76	280 ± 152
Fold variation§	5.3	4.9	2.2	3.9	1.4	147	4.9

† Data for each subject represent the mean of duplicate determinations. The various activities (CYP form selectivity shown in parentheses) are abbreviated as follows: ERODase, 7-ethoxyresorufin *O*-deethylase; ERNDase, erythromycin *N*-demethylase; TOLase, tolbutamide 4-methyl hydroxylase; DMNase, *N,N*-dimethylnitrosamine *N*-demethylase; DEXase, dextromethorphan *O*-demethylase; and COHase, coumarin 7-hydroxylase.
* Represents total (spectrally detectable) CYP (pmol CYP/mg microsomal protein).
§ Ratio of the highest/lowest activity.
‡ ND, not determined.

methasone). At present, the effects of these agents on human liver AO activities remain unknown. Upon arrival, the liver tissue was rapidly chopped into small (6.0 g) pieces and finely minced with a hand-held razor blade. Thereafter, all steps were performed at 4°. The chopped tissue was mixed with 10 mM potassium phosphate buffer (pH 7.4), containing 1.15% (w/v) potassium chloride (buffer A); the final tissue concentration was 0.20 to 0.25 g of wet tissue/mL. The tissue was subsequently homogenized with a Tissumizer® (Tekmar Co., Cincinnati, OH) and then centrifuged at 9000 *g* for 20 min. In each case, the supernatant (S-9 fraction) was stored at −70°, in buffer A, or was processed (105,000 *g* for 60 min) to yield a microsomal pellet. The pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.4), containing 20% (v/v) glycerol, and the resultant microsomal suspension was stored at −70°. All activity measurements were carried out with microsomes (CYP assays) or S-9 (AO assays) that had been stored frozen (at least 2 months) and thawed only once. The samples were discarded after use.

AO assays. All activity measurements were carried out at 37° in a final assay volume of 1.0 mL as previously described [13]. Assay mixtures contained potassium ferricyanide [$K_3Fe(CN_6]$ (1 mM) as the electron acceptor, EDTA (0.1 mM), S-9 protein (0.2 to 0.7 mg/mL) in 0.1 M potassium phosphate buffer (pH 7.8). Reactions were performed in the presence of N^1-methylnicotinamide (5.0 mM), benzaldehyde (0.1 mM) or 6-methylpurine (3.5 mM) [6, 7, 13]. Reduction of the electron acceptor was monitored at 420 nm ($E = 1.02\ mM^{-1}cm^{-1}$), and activities are expressed as nanomoles potassium ferricyanide reduced per minute per milligram of S-9 protein. All activities were corrected for non-substrate-dependent reduction of potassium ferricyanide. Menadione (10 μM), a well-documented inhibitor of AO [6, 7, 10, 13], decreased the oxidation of 6-methylpurine, benzaldehyde and N^1-methylnicotinamide by 57, 89 and 100%, respectively. The three activities were less sensitive (≤40% inhibition) to the effects of allopurinol (10 μM), a xanthine oxidase inhibitor [6, 10]. Inhibitors were dissolved in dimethyl sulfoxide (final assay concentration 0.2%, v/v), which had no effect on activity. Total protein was determined using a bicinchoninic acid (BCA) assay kit procedure (Pierce, Rockford, IL), with BSA as the standard [14].

CYP assays. Liver microsomal total CYP was determined by the method of Omura and Sato [15]. Unless otherwise indicated, all CYP monooxygenase assays were carried out at 37° in 0.1 M potassium phosphate buffer (pH 7.4), EDTA (15 mM), $NADP^+$ (4.0 mM), glucose 6-phosphate (10 mM), glucose 6-phosphate dehydrogenase (2.0 U/mL),

microsomal protein (0.1 to 1.0 mg/mL) and the appropriate concentration of substrate (see below). The formaldehyde formed during the N-demethylation of erythromycin (0.5 mM, CYP3A) and *N,N*-dimethylnitrosamine (0.2 mM, CYP2E1) was measured using the Nash reagent with a fluorimetric end point [16–18]. The methyl hydroxylation of [1,2-$^3H_2$]tolbutamide (1.0 mM, CYP2C9/10) was measured using a high performance liquid radiochromatographic method as previously described [12]. Coumarin 7-hydroxylase (0.2 mM, CYP2A6) and 7-ethoxyresorufin *O*-deethylase (2.5 μM, CYP1A2) activities were measured in the presence of NADPH (0.2 mM), and in both cases product formation was monitored fluorimetrically in a continuous manner [19. 20], except that the latter was carried out in 0.1 M Tris–HCl buffer (pH 7.8). The formation of [^{14}C]formaldehyde during the O-demethylation of [*O-methyl*-^{14}C]dextromethorphan (20 μM, CYP2D6) was measured radiometrically. [21]. In all cases, inter-subject variability (fold variation) is defined as the ratio of the highest/lowest activity in the panel of six human livers. Correlation coefficients (r) were determined graphically using CA-Cricket® Graph software (Computer® Associates, San Jose, CA).

Results and Discussion

Table 1 gives the AO activity in S-9 fractions derived from human liver. Aldehyde oxidase activity with N^1-methylnicotinamide as substrate was low (≤1.5 nmol/min/mg) in five out of the six subjects and was lower than that observed with S-9 fractions of rat, beagle dog and cynomolgus monkey: 16 ± 6.0, 4.1 ± 1.2 and 62 ± 5.4 nmol/min/mg, respectively (mean ± SD, N = 3). This is in agreement with previous studies, which have shown that AO activity is high in non-human primates and low in species such as the dog [4, 6, 10]. The levels of AO activity in human liver tissue are dependent on the substrate used, and it is known that the human AO activity is higher with substrates such as benzaldehyde than with charged compounds such as N^1-methylnicotinamide [7]. In this study, the inter-subject variability in AO activity was greatest for N^1-methylnicotinamide oxidase (>40-fold), when compared with 6-methylpurine and benzaldehyde oxidase activity (≤3.6-fold). Whether this reflects differences in the relative levels and/or stability of multiple AO forms (isoenzymes) remains to be determined. At present, there is only evidence for one form of the enzyme in human liver, as compared with two or more forms in rodent livers [5, 8, 22]. However, the conclusion that only one form of AO exists in humans is based on electrophoretic analyses of hepatic cytosol, with benzaldehyde as a stain [8]. Conclusive proof of multiple forms will require identification of AO genes, their sequencing and expression in *Escherichia coli*, yeast or some other heterologous expression system, and the development of form-selective substrates, inhibitors and/or antibodies (cf. cytochromes P450 [1–3]). Incidentally, 6-methylpurine and benzaldehyde oxidase activities were shown to be reasonably well correlated (r = 0.886), indicating that both activities might be reflective of the same form(s) of human AO. However, a larger sample size (N ≥ 20) is needed to address this issue unequivocally. The corresponding human liver microsomal preparations were characterized with respect to the variability of total (spectrally detectable) CYP and six CYP-dependent MFO activities. Each activity was chosen because of its known CYP form(s) selectivity [2, 3]. The observed inter-subject variability was similar to that already documented by a number of workers [16, 17, 23] and was greatest for dextromethorphan *O*-demethylase (CYP2D6) and lowest for *N,N*-dimethylnitrosamine *N*-demethylase (CYP2E1) activity (Table 2). The former can be partly explained by the fact that CYP2D6 is known to be polymorphically expressed in humans [1, 3]. The inter-subject variability of the remaining activities, total CYP, CYP3A (erythromycin *N*-demethylase), CYP2C9/10 (tolbutamide 4-methyl hydroxylase), CYP2A6 (coumarin 7-hydroxylase) and CYP1A2 (7-ethoxyresorufin *O*-deethylase), was 2.2- to 5.3-fold. Furthermore, AO activity (6-methylpurine or benzaldehyde as substrate) was not strongly correlated (r ≤0.587) with any of the CYP activities tested. The lack of correlation with the various CYP activities and the variability in AO activity with some substrates may have implications for studies involving the *in vitro* oxidative metabolism of agents such as quinidine [9], nicotine, azapetine, cyclophosphamide [4, 5], carbazeran [10], and prolintane [24]. These agents are known to undergo sequential or concomitant CYP and AO mediated oxidation. This is of particular importance when one realizes that many of these CYP-dependent oxidations are catalyzed by specific forms such as CYP2A6 (nicotine) and CYP3A (quinidine) [3, 11]. If the involvement of CYP and/or AO in the metabolism of a given compound is suspected from *in vivo* animal data, then *in vitro* species comparisons and subsequent predictions of human metabolism should be based on the use of models that allow for minimal tissue disruption (e.g. liver slices) and/or well-characterized subcellular fractions. The measurement of the relative levels of AO and CYP activities in a given sample(s) of human liver tissue is warranted, especially when it is often difficult to control the quality of the tissue that is obtained from various sources.

Drug Metabolism Department A. DAVID RODRIGUES*
Pharmaceutical Products Division
Abbott Laboratories
Abbott Park, IL 60064, U.S.A.

REFERENCES

1. Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R, Gonzalez FJ, Coon MJ, Gunsalus IC, Gotoh O, Okuda K and Nebert DW, The P450 superfamily: Update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol* **12**: 1–51, 1993.
2. Guengerich FP, Characterization of human cytochrome P450 enzymes. *FASEB J* **6**: 745–748, 1992.
3. Wrighton SA and Stevens JC, The human hepatic cytochromes P-450 involved in drug metabolism. *Crit Rev Toxicol* **22**: 1–21, 1992.
4. Beedham C, Molybdenum hydroxylases: Biological distribution and substrate–inhibitor specificity. In: *Progress in Medicinal Chemistry* (Eds. Ellis GP and West GB), Vol. 24, pp. 85–127. Elsevier, Amsterdam, 1987.
5. Beedham C, Molybdenum hydroxylases as drug-metabolizing enzymes. *Drug Metab Rev* **16**: 119–156, 1985.
6. Krenitsky TA, Tuttle JV, Cattau EL and Wang P, A comparison of the distribution and electron acceptor specificities of xanthine oxidase and aldehyde oxidase. *Comp Biochem Physiol* **49B**: 687–703, 1974.
7. Johns DG, Human liver aldehyde oxidase: Differential inhibition of oxidation of charged and uncharged substrates. *J Clin Invest* **46**:1492–1505, 1967.
8. Duley JA, Harris O and Holmes RS, Analysis of human alcohol- and aldehyde-metabolizing isozymes by electrophoresis and isoelectric focusing. *Alcohol Clin Exp Res* **9**: 263–271, 1985.
9. Beedham C, Al-Tayib Y and Smith JA, Role of guinea pig and rabbit hepatic aldehyde oxidase in oxidative *in*

* Correspondence: Dr. A. David Rodrigues, Drug Metabolism Department (Dept. 46V), Pharmaceutical Products Division (Building AP9), Abbott Laboratories, One Abbott Park Rd., Abbott Park, IL 60064. Tel. (708) 938-5835; FAX (708) 938-5193.

vitro metabolism of cinchona antimalarials. *Drug Metab Dispos* **20**: 889–895, 1992.
10. Kaye B, Rance DJ and Waring L, Oxidative metabolism of carbazeran *in vitro* by liver cytosol of baboon and man. *Xenobiotica* **15**: 237–242, 1985.
11. Cashman JR, Park SB, Yang Z-C, Wrighton SA, Jacob P and Benowitz NL, Metabolism of nicotine by human liver microsomes: Stereoselective formation of *trans*-nicotine *N'*-oxide. *Chem Res Toxicol* **5**: 639–646, 1992.
12. Srivastava PK, Yun C-H, Beaune PH, Ged C and Guengerich FP, Separation of human liver microsomal tolbutamide hydroxylase and (*S*)-mephenytoin 4'-hydroxylase cytochrome P-450 enzymes. *Mol Pharmacol* **40**: 69–79, 1991.
13. Rajagopalan KV and Handler P, Aldehyde oxidase. *Methods Enzymol* **9**: 364–368, 1966.
14. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC, Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**: 76–85, 1985.
15. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239**: 2370–2378, 1964.
16. Yoo JS, Guengerich FP and Yang CS, Metabolism of *N*-nitrosodialkylamines by human liver microsomes. *Cancer Res* **88**: 1499–1504, 1988.
17. Hunt CM, Westerkam WR and Stave GM, Effect of age and gender on the activity of hepatic CYP3A. *Biochem Pharmacol* **44**: 275–283, 1992.
18. Belman S, The fluorimetric determination of formaldehyde. *Anal Chim Acta* **29**: 120–126, 1963.
19. Maurice M, Emiliani S, Dalet-Beluche I, Derancourt J and Lange R, Isolation and characterization of a cytochrome P-450 of the IIA subfamily from human liver microsomes. *Eur J Biochem* **200**: 511–517, 1991.
20. Rodrigues AD and Prough RA, Induction of cytochromes P450IA1 and P450IA2 and measurement of catalytic activities. *Methods Enzymol* **206**: 423–431, 1991.
21. Rodrigues AD, Kukulka MJ, Surber BW, Rotert G, Uchic J, Michel G, Thome-Kromer B and Machinist J, Measurement of liver microsomal cytochrome P-450 (CYP2D6) activity using [*O-methyl*-^{14}C]dextromethorphan. *Anal Biochem*, in press.
22. Ohkubo M and Fujimura S, Rat liver N^1-methylnicotinamide oxidase I and II: Substrate-non specific enzyme "aldehyde oxidase" and -specific enzyme. *Biochem Int* **4**: 353–358, 1982.
23. Forrester LM, Henderson CJ, Glancey MJ, Back DJ, Park BK, Ball SE, Kitteringham NR, McLaren AW, Miles JS, Skett P and Wolf CR, Relative expression of cytochrome P-450 isoenzymes in human liver and association with the metabolism of drugs and xenobiotics. *Biochem J* **281**: 359–368, 1992.
24. Whittlesea CMC and Gorrod JW, The enzymology of the *in vitro* oxidation of prolintane to oxoprolintane. *J Clin Pharm Ther* **18**: 357–364, 1993.