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CYP2A13-catalysed coumarin metabolism: comparison with CYP2A5 and CYP2A6

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1. We investigated the total metabolism of coumarin by baculovirus (BV)-expressed CYP2A13 and compared it with metabolism by BV-expressed CYP2A6. The major coumarin metabolite formed by CYP2A13 was 7-hydroxycoumarin, which accounted for 43% of the total metabolism. The product of 3,4-epoxidation, *o*-hydroxyphenylacetaldehyde (*o*-HPA), accounted for 30% of the total metabolites.

2. The K_m and V_{max} for CYP2A13-mediated coumarin 7-hydroxylation were $0.48 \pm 0.07\,\mu M$ and $0.15 \pm 0.006\,nmol\,min^{-1}\,nmol^{-1}$ CYP, respectively. The V_{max} of coumarin 7-hydroxylation by CYP2A13 was about 16-fold lower than that of CYP2A6, whereas the K_m was 10-fold lower.

3. In the mouse, there were two orthologues for CYP2A6: CYP2A4 and CYP2A5, which differed by only 11 amino acids. However, CYP2A5 is an efficient coumarin 7-hydroxylase, where as CYP2A4 is not. We report here that BV-expressed CYP2A4 metabolizes coumarin by 3,4-epoxidation. Two products of the 3,4-epoxidation pathway, *o*-HPA and *o*-hydroxyphenylacetic acid (*o*-HPAA), were detected by radioflow HPLC.

4. The K_m and V_{max} for the coumarin 3,4-epoxidation by CYP2A4 were $8.7 \pm 3.6\,\mu M$ and $0.20 \pm 0.04\,nmol\,min^{-1}\,nmol^{-1}$ CYP, respectively. Coumarin 7-hydroxylation by CYP2A5 was more than 200 times more efficient than 3,4 epoxidation by CYP2A4.

Introduction

CYP2A13 is one of three members of the human CYP2A gene family (Fernandez-Salguero *et al*. 1995). The other members are CYP2A6, a hepatic coumarin 7-hydroxylase, and CYP2A7, reported to be a non-functional enzyme (Yamano *et al*. 1990). A number of regions in the CYP2A13 sequence are similar to CYP2A7, therefore CYP2A13 was also predicted to be non-functional (Ding *et al*. 1995). However, Su *et al*. (2000) recently expressed CYP2A13 in *Spodoptera frugiperda* (Sf9) insect cells and reported that it is in fact a functional enzyme that metabolizes several compounds which are substrates for CYP2A6. The coumarin 7-hydroxylase activity by CYP2A13 was previously measured at a single coumarin concentration. At 100 μM NNK, CYP2A13 catalysed the 7-hydroxylation of coumarin at about 10% of the rate of CYP2A6 (Su *et al*. 2000).

The highest level of CYP2A13 expression was found in the nasal mucosa; however, 2A13 is also expressed in significant levels in human lung and trachea (Su *et al*. 2000). CYP2A6 is primarily hepatic, constituting 1–10% of total CYP in the human liver (Yun *et al*. 1991, Shimada *et al*. 1994). CYP2A6 has been detected in small amounts in nasal mucosa and there are conflicting results about whether or not CYP2A6 is present in the human lung (Crawford *et al*. 1998, Koskela *et al*.

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1999, Su *et al*. 2000). Koskela *et al*. did not detect any CYP2A6 transcripts in human lung using RT-PCR. In contrast, Crawford *et al*. detected CYP2A6 mRNA in human bronchial epithelial cells. The discrepancy observed might be due to the detection of CYP2A13 with the primers used by Crawford *et al*. The recognition site for these primers in CYP2A13 differs from 2A6 by only one nucleotide. Using sequence-specific primers and competitive RT-PCR, Su *et al*. (2000) reported that the amount of CYP2A13 mRNA in the lung is eight times higher than that of CYP2A6.

CYP2A6 metabolizes the carcinogens 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*-nitrosonornicotine (NNN), which are tobacco-specific nitrosamines (Patten *et al*. 1996, 1997). NNK is a potent lung carcinogen in laboratory animals and a likely human lung carcinogen (Hecht 1998). NNN primarily induces oesophageal tumours in animals and is believed to play a role in tobacco-induced oral and oesophageal cancer in humans. The metabolism of NNN by CYP2A6 is both specific and efficient (Patten *et al*. 1997); however, CYP2A6-catalysed NNK metabolism is much less efficient (Patten *et al*. 1996). CYP2A13 catalyses the activation of NNK by α-hydroxylation at a significantly higher rate than does 2A6 (Su *et al*. 2000). The V_{max}/K_m for NNK methylene hydroxylation by 2A6 is 0.008 compared with 0.36 for 2A13 (Patten *et al*. 1996, Su *et al*. 2000). Since CYP2A13 is present in human lung, it is likely to catalyse NNK activation in this tissue and therefore play an important role in tobacco related lung cancers.

In the mouse there are two orthologues of CYP2A6: CYP2A4 and 2A5 (Honkakoski and Negishi 1997). These two enzymes are 98% homologous, differing by only 11 amino acids; however, they catalyse NNK metabolism with strikingly different efficiencies (Felicia *et al*. 2000). CYP2A4 catalyses NNK α-hydroxylation with a 54-fold lower efficiency (V_{max}/K_m) than does CYP2A5. However, the rate of CYP2A5-catalysed NNK α-hydroxylation is 1000-fold greater than that of CYP2A6 at 1 μM NNK (Felicia *et al*. 2000).

CYP2A5 and 2A6 are well-characterized coumarin 7-hydroxylases. However, CYP2A4 has been reported to have little or no coumarin 7-hydroxylase activity (Lindberg and Negishi 1989, Negishi *et al*. 1989, Felicia *et al*. 2000). Coumarin 7-hydroxylation is one of several pathways of coumarin metabolism (figure 1). In all species studied to date, the predominant pathway is either 7-hydroxylation or the formation of the ring-opened products *o*-hydroxyphenylacetaldehyde (*o*-HPA) and *o*-hydroxyphenylacetic acid (*o*-HPAA) (Shilling *et al*. 1969, Egan and O'Kennedy 1992, Fentem and Fry 1993). *o*-HPA is generated non-enzymatically from the unstable coumarin 3,4-epoxide, whereas *o*-HPAA is an oxidation product of *o*-HPA (figure 1) (Born *et al*. 1997, 2000). 7-Hydroxylation is the predominant coumarin metabolism pathway in humans, due to the presence of CYP2A6 in the liver (Miles *et al*. 1990). However, other human CYPs metabolize coumarin by pathways other than 7-hydroxylation. *o*-HPA is the major metabolite formed by CYP1A1, 1A2, 2B6, 2E1 and 3A4, although the rate of metabolism is quite low (Zhuo *et al*. 1999).

In the current study, the coumarin 7-hydroxylase activity of CYP2A13 was compared with that of CYP2A6. The kinetic parameters for CYP2A13-mediated coumarin 7-hydroxylation were determined and other CYP2A13-catalysed pathways of coumarin metabolism were quantified. Total coumarin metabolism by the mouse CYP, 2A4, was also investigated and compared with the metabolism of

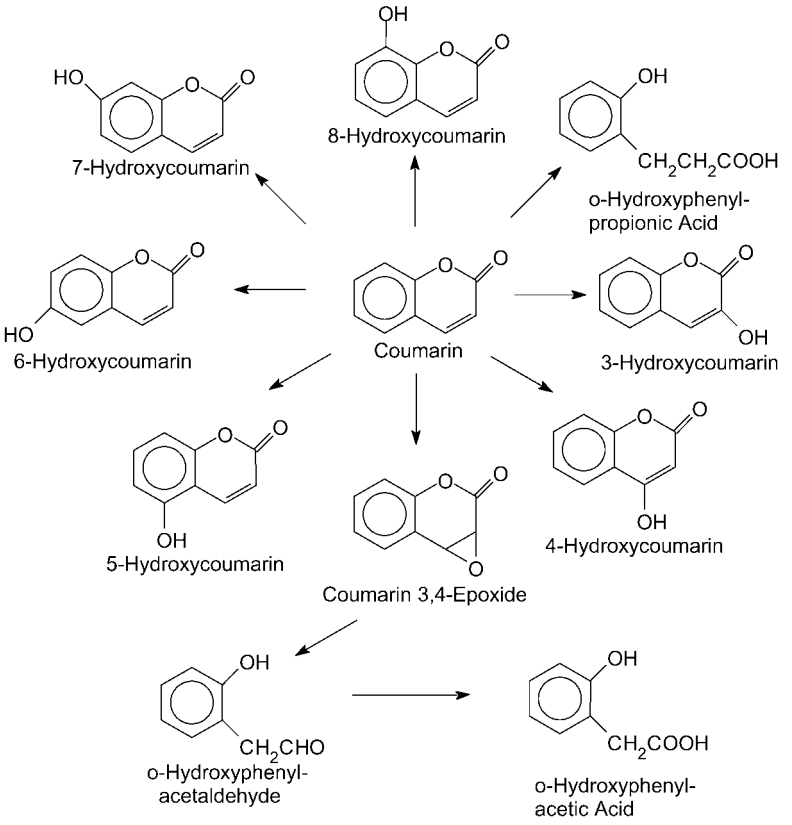

Figure 1. Pathways of coumarin metabolism.

coumarin by the highly homologous CYP, 2A5, as well as the human CYPs, 2A6 and 2A13.

Materials and methods

Chemicals

[U-^{14}C-benzyl] coumarin (58 mCi mmol^{-1}), purity > 99.5% by HPLC analysis, was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). 4-Hydroxycoumarin, 2-hydroxyphenylacetic acid, *o*-coumaric acid and 6,7-dihydroxycoumarin were purchased from Fluka Chemical Co. (Ronkonkoma, NY, USA). 3-Hydroxycoumarin was obtained from Indofine Chemical Co. (Sommerville, NJ, USA). *o*-Hydroxyphenylacetaldehyde was a gift from Dr Lois D. Lehman-McKeeman of Procter & Gamble (Cincinnati, OH, USA) and 5-hydroxycoumarin was a gift from Dr Takashi Harayama (Okayama University, Japan). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) or Aldrich Chemical Co. (Milwaukee, WI, USA).

Metabolism of ^{14}C-coumarin

Homogenate of baculovirus (BV)-expressed CYP2A13 (Su *et al.* 2000) was provided by Dr Xinxin Ding (Wadsworth Center, Albany, NY, USA). CYP2A4, CYP2A5 and CYP2A6 were expressed in Sf9 cells using the BV-expression system (Felicia *et al.* 2000), and microsomes were prepared as described. Microsomes from BV-expressed CYP2A4 (33.9 pmol), CYP2A5 (1.8 pmol), CYP2A6 (1.8 pmol) and CYP2A13 cell homogenate (7.2 pmol) were incubated with BV-expressed human P450:NADPH oxidoreductase, 5 μM [U-^{14}C-benzyl]-coumarin, and an NADPH-generating system (0.4 mM NADP$^+$, 100 mM glucose 6-phosphate, and 0.4 units ml^{-1} glucose 6-phosphate dehydrogenase) in a 200 μl total volume of 50 mM Tris buffer (pH 7.4) for 30 min at 37°C. A ratio of 1:5, CYP to

oxidoreductase, was used in all experiments. All analyses were performed in duplicate. The reactions were terminated by the addition of 20 µl 15% trichloroacetic acid and then centrifuged. The supernatant was mixed with standards and analysed by reverse-phase HPLC with radioflow detection. The standards added were *o*-HPA, *o*-HPAA, *o*-hydroxyphenypropionic acid, *o*-coumaric acid, 5-, 6-, 7-, and 8-hydroxycoumarin, coumarin, and 6,7-dihydroxycoumarin. A Phenomenex (Torrance, CA, USA) Bondclone C_{18} column (3.9 × 300 mm, 10 µm) was used for the analysis. The mobile phase consisted of 1% acetic acid in water (A) and 1% acetic acid in methanol (B). The metabolites were eluted isocratically with 29% B and 71% A. The flow rate was 1 ml min^{-1}. The metabolism of coumarin was linear with time and protein for all CYP enzymes. For the determination of kinetic parameters, CYP2A4 (18.4 pmol) was incubated with 0.5–20 µM ^{14}C-coumarin as described above.

Coumarin 7-hydroxylation by CYP2A13

CYP2A13 (4.15 pmol) was incubated with 0.1–20 µM coumarin (non-radioactive) for 10 min at 37°C as described above. The 7-hydroxycoumarin formed was analysed by HPLC with fluorescence detection (excitation wavelength 350 nm, emission wavelength 453 nm) as described by von Weymarn *et al.* (1999). Quantification was obtained by comparison to a standard curve of 7-hydroxycoumarin versus peak area.

Kinetics and statistical analysi

K_m and V_{max} for coumarin metabolism by CYP2A13 and 2A4 were determined using the EZ-Fit 5 kinetics program from Perrella Scientific (Amherst, NH, USA). It uses a non-linear regression method of curve fitting and the Runs test of residuals to determine statistically whether experimental data are randomly distributed around the curve with 95% confidence (Perrella 1988).

Results and discussion

The metabolism of ^{14}C-coumarin by CYP2A4, CYP2A5, CYP2A6 and CYP2A13 was analysed by reverse-phase HPLC with radioflow detection (figures 2 and 4). The use of coumarin labelled with ^{14}C in the benzene ring allows the detection of other metabolites, as well as 7-hydroxycoumarin, and we previously developed an HPLC system to separate most coumarin metabolites (von Weymarn and Murphy 2001). The major metabolite of CYP2A6-catalysed coumarin metabolism eluted at 20.3 min (figure 2A), namely 7-hydroxycoumarin. As expected, this is the only metabolite detected (table 1) (Miles *et al.* 1990, Yamano *et al.* 1990). However, in contrast to CYP2A6, several radioactive metabolites of CYP2A13-catalysed coumarin metabolism were detected (figure 2B). The major metabolite was 7-hydroxycoumarin (20.3 min), accounting for 43% of the total metabolism (table 1). However, a product of 3,4-epoxidation, *o*-HPA, (14.2 min), was also formed at a significant rate accounting for 30% of the total metabolism. Two hydroxylated coumarins, 6-hydroxy (6-OH, 15.6 min) and 8-hydroxycoumarin (8-OH, 16.4 min), were also detected. The formation of all of these products was linear with enzyme and time under the conditions studied (data not shown). The early eluting peak (5 min) was present in control samples and dependent on the presence of Sf9 microsomes (figure 2C). The metabolism of coumarin by CYP2A13 is clearly not as specific as metabolism by CYP2A6 despite the 94% sequence homology between the two enzymes.

The kinetic parameters for 7-hydroxycoumarin formation by CYP2A13 were determined using reverse-phase HPLC with fluorescence detection. The formation of 7-hydroxycoumarin followed simple Michaelis–Menten kinetics (figure 3). The K_m for coumarin 7-hydroxylation was 0.48 ± 0.07 µM, the V_{max} 0.15 ± 0.006 nmol min^{-1} $nmol^{-1}$ CYP. This K_m is similar to that of BV-expressed CYP2A5 (1.2 µM; Felicia *et al.* 2000) and about 10-fold lower than that of BV-expressed CYP2A6 (6 µM; Zhuo *et al.* 1999). The V_{max} for CYP2A13-catalysed

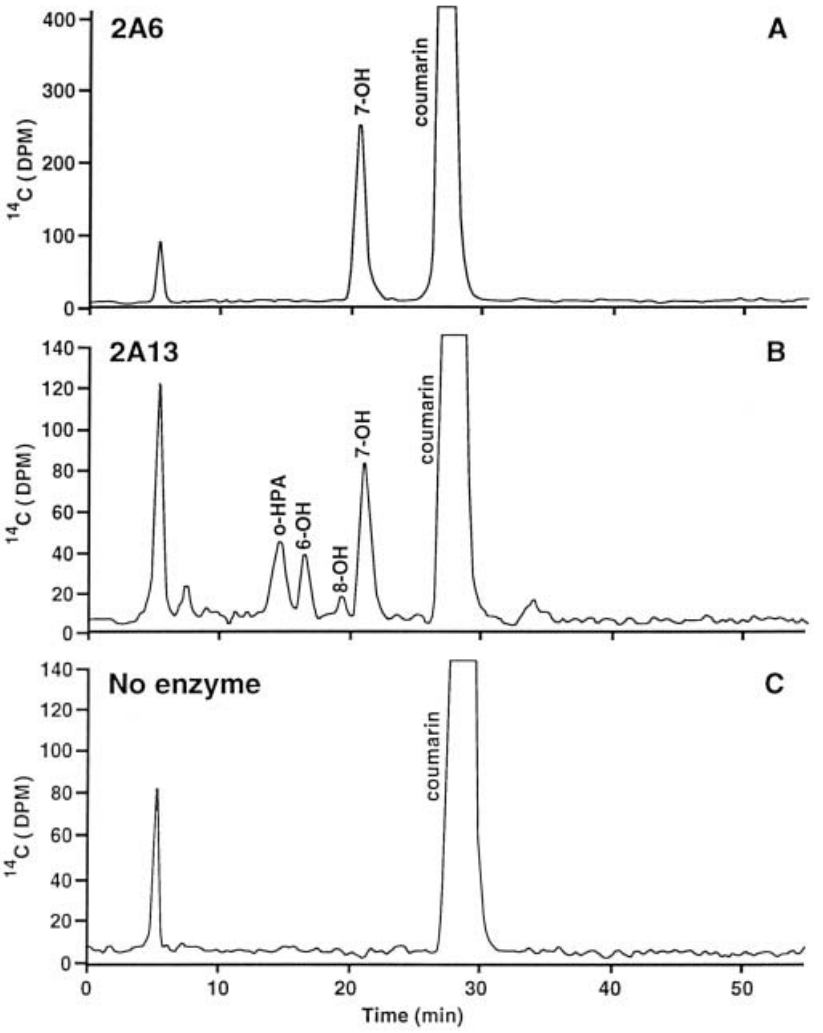

Figure 2. Radioflow HPLC analysis of coumarin metabolites. ^{14}C-coumarin (5 μM was incubated for 30 min with 1.8 pmol CYP2A6 (A), 7.2 pmol CYP2A13 (B) or without enzyme (C) as described in the Materials and methods. The products of the reaction were co-injected with standards on a C_{18} HPLC column.

Table 1. Relative metabolism of coumarin by two major pathways.[a]

	Metabolites (% of total)[b]			
	Mouse		Human	
	CYP2A4	CYP2A5	CYP2A6	CYP2A13
7-Hydroxylation	0	79	100	43
3,4-Epoxidation[c]	98	7	0	30

[a] CYP2A4 (33.9 pmol), CYP2A5 (1.8 pmol), CYP2A6 (1.8 pmol) or CYP2A13 (7.2 pmol) were incubated for 30 min with 5 μM coumarin as described in the Materials and methods. The products were analysed by radioflow HPLC.

[b] Averages of duplicate determinations.

[c] Values determined for the 3,4-epoxide pathway are the sum of *o*-HPA and *o*-HPAA.

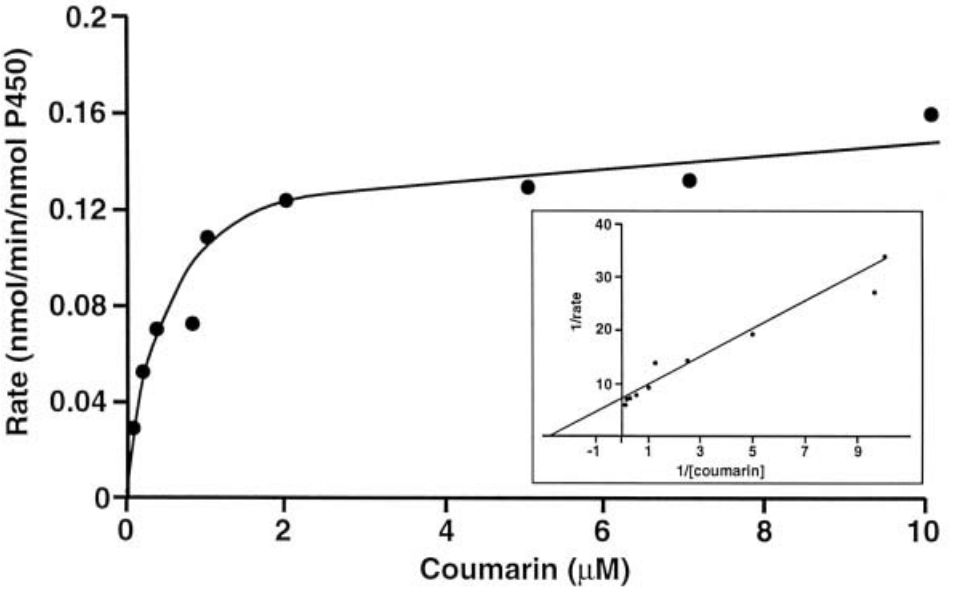

Figure 3. Kinetic analysis of 7-hydroxycoumarin formation by CYP2A13. Coumarin (0.5–10 μM) was incubated for 10 min with 4.15 pmol CYP2A13 as described in the Materials and methods. The formation of 7-hydroxycoumarin was determined by HPLC analysis with fluorescence detection. Curves were graphed by non-linear regression kinetic parameters determined using EZ-Fit 5. The insert is a Lineweaver–Burk plot of the kinetic analysis. The data points are the average of two sets of duplicate determinations.

coumarin 7-hydroxylation is about 16-fold less than that of CYP2A5 and CYP2A6 (Zhuo *et al*. 1999, Felicia *et al*. 2000).

Coumarin metabolism by CYP2A4 and 2A5, two mouse orthologues of CYP2A6, was next investigated using ^{14}C-coumarin (figure 4). The major metabolites of CYP2A4-mediated coumarin metabolism were *o*-HPA (13.5 min, figure 4B) and *o*-HPAA (12.1 min), products of the coumarin 3,4-epoxide pathway. 7-Hydroxycoumarin was not detected (limit of detection < 0.1 pmol) (figure 4A and table 1). The formation of products from the 3,4-epoxide pathway accounted for 98% of the coumarin metabolism by CYP2A4 (table 1). An unknown metabolite, eluting at 7.5 min, accounted for the remaining 2%. The radioactive peak eluting at 5 min, as noted above, is dependent on the presence Sf9 membranes. This peak is greater in the CYP2A4 than the CYP2A5 sample illustrated in figure 4 since more CYP2A4 was used, and therefore more Sf9 membrane proteins are present. The kinetic parameters for the 3,4-epoxidation of coumarin by CYP2A4 were determined. The K_m was 8.7 ± 3.6 μM, the V_{max} 0.20 ± 0.04 nmol min^{-1} nmol^{-1} CYP. The product distribution did not change with coumarin concentration.

The major product of CYP2A5-catalysed coumarin metabolism detected was 7-hydroxycoumarin (figure 4B), consistent with the previous characterizations of 2A5 as a coumarin 7-hydroxylase (Negishi *et al*. 1989, Felicia *et al*. 2000). Small amounts of three other metabolites, *o*-HPA, 6-hydroxy and 8-hydroxy coumarin, were also detected (figure 4B). Compared with the kinetic parameters of coumarin 7-hydroxylation by CYP2A5, CYP2A4-catalysed coumarin 3,4-epoxidation has a ninefold higher K_m and a 40-fold lower V_{max} (Felicia *et al*. 2000). Relative to

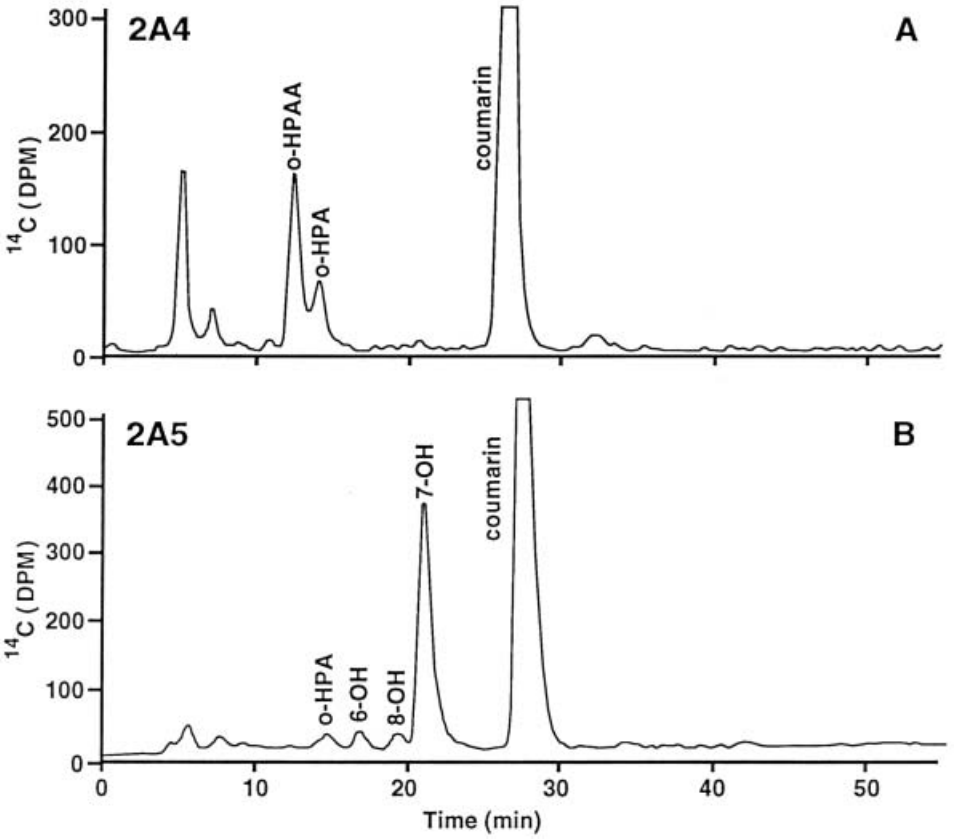

Figure 4. Radioflow HPLC analysis of coumarin metabolites. ^{14}C-coumarin (5 μM) was incubated for 30 min with 33.9 pmol CYP2A4 (A) or 1.8 pmol CYP2A5 (B) as described in the Materials and methods. The products of the reaction were co-injected with standards on a C_{18} HPLC column.

CYP2A5-catalysed coumarin 7-hydroxylation, CYP2A4 is not a very efficient catalyst of coumarin 3,4-epoxidation. This is also true compared to the rates of coumarin 3,4-epoxidation by the rat and human P450s CYP1A1, CYP1A2 and CYP2E2 (Born *et al*. 2002). Recombinant human CYP1A1 and rat CYP1A2 catalyse coumarin 3,4-epoxidation at a rate of 6.26 and 22.2 nmol min^{-1} nmol^{-1} CYP, respectively (Born *et al*. 2002). These rates are 31- and 111-fold higher than the rate of CYP2A4-mediated coumarin 3,4-epoxidation.

Coumarin 3,4-epoxide is reactive and could bind to cellular protein. We determined here that 3,4-epoxidation is the major pathway of coumarin metabolism catalysed by CYP2A4. Negishi *et al*. (1989) reported that even though CYP2A4 did not metabolize coumarin by 7-hydroxylation, coumarin did inhibit CYP2A4-mediated testosterone 15α-hydroxylation. Both enzyme modification by coumarin 3,4-epoxide and competitive inhibition may account for the inhibition observed by Negishi *et al*. (1989).

The striking difference in the efficiency of coumarin 7-hydroxylation by CYP2A4 and CYP2A5 has been extensively studied by Honkakoski and Negishi (1997), Lindberg and Negishi (1989) and Negishi *et al*. (1989). By using site-directed mutagenesis, they changed three amino acids in CYP2A4 to their

counterparts in CYP2A5. The resulting enzyme had coumarin 7-hydroxylase activity of equal efficiency to that of CYP2A5 (Lindberg and Negishi 1989). The mutated amino acids were 117, 209 and 365. These three amino acids have been indicated to play a role in coumarin metabolism by several CYP2A enzymes (Honkakoski and Negishi 1997). The CYP2A enzymes capable of catalysing the 7-hydroxylation of coumarin all have a phenylalanine at position 209. CYP2A4, which is not a coumarin 7-hydroxylase, has a leucine at position 209. CYP2A13 and CYP2A4 both have an alanine at position 117 compared with valine in 2A5 and 2A6, which may contribute to the low coumarin metabolism observed with 2A4 and 2A13. With respect to amino acids 117, 209 and 365, CYP2A13 is identical to rat CYP2A3. CYP2A3 is an efficient coumarin 7-hydroxylase (von Weymarn *et al*. 1999). However, CYP2A3, like CYP2A13, also catalyses the 3,4-epoxidation of coumarin.

A number of CYP2A enzymes in different species have sequence homology $>85\%$ (Honkakoski and Negishi 1997), however their substrate specificities differ significantly. This is clearly illustrated by the differences observed in coumarin and NNK metabolism by CYP2A4, CYP2A5, CYP2A6 and CYP2A13. CYP2A6 and CYP2A13 are 94% homologous, they differ by only 32 amino acids (Su *et al*. 2000). We report here that compared with CYP2A6, CYP2A13 is not a specific coumarin 7-hydroxylase. Su *et al*. (2000) reported that CYP2A13 efficiently metabolizes NNK, however 2A6 is significantly less efficient at catalysing NNK metabolism (Patten *et al*. 1996). A different relationship exists between the closely related CYP2A enzymes in the mouse. CYP2A4 is not a catalyst of coumarin 7-hydroxylation and is a poor catalyst of NNK hydroxylation while CYP2A5 is a very efficient catalyst of both reactions.

While amino acid residues 117, 309 and 365 are clearly important in determining the substrate specificity of CYPs 2A4 and 2A5, other amino acids must play a role in CYP2A13 and other CYP2A enzymes. Complete characterization of coumarin metabolism by CYP2A enzymes should help to identify amino acid residues important in defining the active site of these enzymes. This information, in turn, should contribute to our understanding of the role specific amino acids play in determining the specificity of nitrosamine metabolism by these enzymes.

Acknowledgements

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References

Born, S. L., Caudill, D., Fliter, K. L. and Purdon, M. P., 2002, Identification of the cytochromes p450 that catalyze coumarin 3,4-epoxidation and 3-hydroxylation. *Drug Metabolism and Disposition*, **30**, 483–487.

Born, S. L., Hu, J. K. and Lehman-McKeeman, L. D., 2000, *o*-Hydroxyphenylacetaldehyde is a hepatotoxic metabolite of coumarin. *Drug Metabolism and Disposition*, **28**, 218–223.

Born, S. L., Rodriguez, P. A., Eddy, C. L. and Lehmann-McKeeman, L. D., 1997, Synthesis and reactivity of coumarin 3,4-epoxide. *Drug Metabolism and Disposition*, **25**, 1318–1323.

Crawford, E. L., Weaver, D. A., Demuth, J. P., Jackson, D. M., Khuder, S. A., Frampton, M. W., Utell, M. J., Thilly, W. G. and Willey, J. C., 1998, Measurement of cytochrome P450 2A6 and 2E1 gene expression in primary human bronchial epithelial cells. *Carcinogenesis*, **19**, 1867–1871.

DING, S., LAKE, B. G., FRIEDBERG, T. and WOLF, C. R., 1995, Expression and alternative splicing of the cytochrome P450 CYP2A6. *Biochemical Journal*, **306**, 161–166.

EGAN, D. A. and O'KENNEDY, R., 1992, Rapid and sensitive determination of coumarin and 7-hydroxycoumarin and its glucuronide conjugate in urine and plasma by high-performance liquid chromatography. *Journal of Chromatographic and Biomedical Applications*, **582**, 137–143.

FELICIA, N. D., REKHA, G. K. and MURPHY, S. E., 2000, Characterization of cytochrome P450 2A4 and 2A5 catalyzed 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) metabolism. *Archives of Biochemistry and Biophysics*, **384**, 418–424.

FENTEM, J. H. and FRY, J. R., 1993, Species differences in the metabolism and hepatotoxicity of coumarin. *Comparative Biochemistry and Physiology*, **104C**, 1–8.

FERNANDEZ-SALGUERO, P., HOFFMAN, S., CHOLERTON, S., MOHRENWEISER, H., RAUNIO, H., RAUTIO, H., PELKONEN, O., HUANG, J. D., EVANS, W., IDLE, J. and GONZALEZ, F., 1995, A genetic polymorphism in coumarin 7-hydroxylation: sequence of the human CYP2A genes and identification of variant CYP2A6 alleles. *Human Genetics*, **57**, 651–660.

HECHT, S. S., 1998, Biochemistry, biology, and carcinogenicity of tobacco-specific *N*-nitrosamines. *Chemical Research in Toxicology*, **11**, 559–603.

HONKAKOSKI, P. and NEGISHI, M., 1997, The structure, function, and regulation of cytochrome P450 2A enzymes. *Drug Metabolism Reviews*, **29**, 977–996.

KOSKELA, S., HAKKOLA, J., HUKKANEN, J., PELKONEN, O., SORRI, M., SARANEN, A., ANTTILA, S., FERNANDEZ-SALGUERO, P., GONZALEZ, F. and RAUNIO, H., 1999, Expression of CYP2A genes in human liver and extrahepatic tissues. *Biochemical Pharmacology*, **57**, 1407–1413.

LINDBERG, R. L. P. and NEGISHI, M., 1989, Alteration of mouse cytochrome P450(coh) substrate specificity by mutation of a single amino-acid residue. *Nature*, **339**, 632–634.

MILES, J. S., MCLAREN, A. W., FORRESTER, L. M., GLANCEY, M. J., LANG, M. A. and WOLF, C. R., 1990, Identification of the human liver cytochrome P-450 responsible for coumarin 7-hydroxylase activity. *Biochemical Journal*, **267**, 365–371.

NEGISHI, M., LINDBERG, R., BURKHART, B., ICHIKAWA, T., HONKAKOSKI, P. and LANG, M., 1989, Mouse steroid 15alpha-hydroxylase gene family: identification of type II P-450 15alpha as coumarin 7-hydroxylase. *Biochemistry*, **28**, 4169–4172.

PATTEN, C. J., SMITH, T. J., MURPHY, S. E., WANG, M. H., LEE, J., TYNES, R. E., KOCH, P. and YANG, C. S., 1996, Kinetic analysis of activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone by heterologously expressed human P450 enzymes, and the effect of P450 specific chemical inhibitors on this activation in human liver microsomes. *Archives of Biochemistry and Biophysics*, **333**, 127–138.

PATTEN, C. J., SMITH, T. J., TYNES, R., FRIESEN, M., LEE, J., YANG, C. S. and MURPHY, S. E., 1997, Evidence for cytochrome P450 2A6 and 3A4 as major catalysts for *N*'-nitrosonornicotine α-hydroxylation in human liver microsomes. *Carcinogenesis*, **18**, 1623–1628.

PERRELLA, F. W., 1988, EZ-FIT: a practical curve fitting microcomputer program for the analysis of enzyme kinetics on IBM-PC computers. *Analytical Biochemistry*, **174**, 437–447.

SHILLING, W. H., CRAMPTON, R. F. and LONGLAND, R. C., 1969, Metabolism of coumarin in man. *Nature*, **221**, 664–665.

SHIMADA, T., YAMAZAKI, H., MIMURA, M., INUI, Y. and GUENGERICH, F. P., 1994, Interindividual variations in human liver cytochrome p450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *Journal of Pharmacology and Experimental Therapeutics*, **270**, 414–423.

SU, T., BAO, Z., ZHANG, Q. Y., SMITH, T. J., HONG, J. Y. and DING, X., 2000, Human cytochrome P 450 CYP2A13: Predominant expression in the respiratory tract and in high efficiency metabolic activation of a tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *Cancer Research*, **60**, 5074–5079.

VON WEYMARN, L. B., FELICIA, N. D., DING, X. and MURPHY, S. E., 1999, *N*-Nitrosobenzylmethylamine α-hydroxylation and coumarin 7-hydroxylation by rat esophageal microsomes and cytochrome P450 2A3 and 2A6 enzymes. *Chemical Research in Toxicology*, **12**, 1254–1261.

VON WEYMARN, L. B. and MURPHY, S. E., 2001, Coumarin metabolism by rat esophageal microsomes and cytochrome P450 2A3. *Chemical Research in Toxicology*, **14**, 1386–1392.

YAMANO, S., TATSUNO, J. and GONZALEZ, F. J., 1990, The CYP2A3 gene product catalyzes coumarin 7-hydroxylation in human liver microsomes. *Biochemistry*, **29**, 1322–1329.

YUN, C. H., SHIMADA, T. and GUENGERICH, F. P., 1991, Purification and characterization of human liver microsomal cytochrome P-450 2A6. *Molecular Pharmacology*, **40**, 679–685.

ZHUO, X., GU, J., ZHANG, Q. Y., SPINK, D. C., KAMINSKY, L. S. and DING, X., 1999, Biotransformation of coumarin by rodent and human cytochrome P-450: metabolic basis of tissue-selective toxicity in olfactory mucosa of rats and mice. *Journal of Pharmacology and Experimental Therapeutics*, **288**, 463–471.