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SHORT COMMUNICATION

Methoxyresorufin: An Inappropriate Substrate for CYP1A2 in the Mouse*

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ABSTRACT. Hepatic microsomes derived from *Cyp1a2*(−/−) knockout (KO) and parental strains of mice, C57BL/6N and 129Sv, were used to examine the specificity of methoxyresorufin and acetanilide as substrates for CYP1A2 activity. In addition, animals from each group were exposed to CYP1-inducing compounds. As expected, microsomes from untreated 1*a*2 KO mice did not have immunodetectable CYP1A2 protein; however, methoxyresorufin-*O*-demethylase (MROD, 25.5 ± 6.1 pmol/min/mg protein) and acetanilide-4-hydroxylation (ACOH, 0.64 ± 0.04 nmol/min/mg protein) activities were still present. Furthermore, induction of ethoxyresorufin-*O*-deethylase (EROD) by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in 1*a*2 KO mice was accompanied by a greater than 70-fold increase in MROD activity. In contrast, ACOH was only induced 2-fold by TCDD. As with 1*a*2 KO mice, the parental strains exposed to TCDD or 2,3,4,7,8-pentachlorodibenzofuran (4-PeCDF) showed substantial EROD and MROD induction, whereas ACOH activity was induced to a lesser degree. PCB153 (2,2′,4,4′,5,5′-hexachlorobiphenyl) resulted in low levels of both EROD and MROD induction. Results indicate that both substrates are subject to metabolism by non-CYP1A2 sources, and the apparent contribution of CYP1A1 activity to methoxyresorufin metabolism makes MROD unsuitable for differentiating CYP1A1 and CYP1A2 activities in the mouse. BIOCHEM PHARMACOL **56**;12:1657–1660, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. methoxyresorufin; CYP1A2; mouse; substrate specificity

Numerous substrates have been identified as selective markers for CYP isoforms based upon their preferential metabolism. For example, ethoxyresorufin was shown to be preferentially metabolized by microsomes from 3-methylcholanthrene-treated animals [1], and subsequent substitution of other alkyl groups has led to a series of resorufin ethers that are widely used as isoform specific markers [2]. Through the use of immunoinhibition as well as multiple enzyme inducers and inhibitors, methoxyresorufin was reported to be a selective substrate for CYP1A2 in S9 fractions from B6C3F1 and NIH/Swiss mice [3]. Alternatively, CYP1A2 activity can be measured by ACOH‖; an antibody capable of inhibiting CYP1A1 and 1A2 activities inhibited 80% of ACOH by expressed murine CYP1A1 and 1A2 cDNA, human CYP1A2 cDNA, and from murine hepatic microsomes [4].

CYP1 substrates are important in the differentiation and quantification of induced CYP enzyme activity following exposure to polycyclic aromatic hydrocarbons (PAHs). Studies conducted in this laboratory on enzyme induction in mice exposed to PAHs suggest that methoxyresorufin may be readily metabolized by CYP isozymes other than CYP1A2 [5]. Therefore, more information is needed to determine the suitability of methoxyresorufin and acetanilide in enzyme induction studies with mice.

The development of a *Cyp1a2*(−/−) KO mouse enables exploration of the metabolism of putative 1A2 substrates in the absence of 1A2 protein. We used hepatic microsomes derived from 1*a*2 KO and from the parental strains of mice originally used to derive the KO mice, C57BL/6N and 129Sv, to determine the specificity of CYP1A2 substrates. In addition, animals from each group were exposed to CYP1-inducing compounds TCDD and 4-PeCDF, or to a CYP2B inducer, PCB153, in order to further examine the substrate specificity in induced microsomes.

MATERIALS AND METHODS

Chemicals

[1,6-^{3}H]TCDD and [^{14}C]4-PeCDF were obtained from Chemsyn Science Laboratories. [^{14}C]PCB153 was obtained from the Sigma Chemical Co. These chemicals had a

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‖ *Abbreviations:* ACOH, acetanilide-4-hydroxylation; EROD, ethoxyresorufin-*O*-deethylase; KO, knockout; MROD, methoxyresorufin-*O*-demethylase; PCB153, 2,2′,4,4′,5,5′-hexachlorobiphenyl; 4-PeCDF, 2,3,4,7,8-pentachlorodibenzofuran; and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin;

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reported purity >98%, and the purity of [1,6-^{3}H]TCDD was confirmed by HPLC [6]. Radiolabeled compounds were used to facilitate disposition studies on these compounds; these data are presented elsewhere [6]. Acetanilide, 2-acetamidophenol, and 3-acetamidophenol were purchased from the Aldrich Chemical Co. 4-Acetamidophenol was purchased from the Sigma Chemical Co. Resorufin and resorufin ethyl and methyl ethers were purchased from Molecular Probes. All remaining chemicals were of the highest purity commercially available.

Animals

Cyp1a2(−/−) mice were produced as described [7], and breeding pairs supplied by Dr. Frank P. Gonzalez at the NCI/NIH Laboratories were used to develop a colony at the animal facility of the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency. Parental mice strains, C57BL/6N and 129/Sv, were obtained from the Charles River Breeding Laboratories and Taconic Farms, respectively. Upon receipt, animals were housed individually in Nalgene metabolism cages with a 12:12 hr light:dark cycle, at an ambient air temperature of 22 ± 1°, 55 ± 5% humidity, and fed and watered *ad lib.* with dustless pellet feed (BioServe). Animals were acclimated under these conditions for 1 week, such that all animals were 19 weeks of age upon initiation of the study.

Treatment/Tissue Preparation

A group of male mice (5 animals/group) from each genetic stock was administered a single oral dose of 25 μg [1,6-^{3}H]TCDD/kg body weight, 300 μg [^{14}C]4-PeCDF/kg body weight, or 35.8 mg [^{14}C]PCB 153/kg body weight in corn oil. Doses were based upon previous studies that suggested that these doses result in maximal CYP induction [5, 8]. The dosing volume was 10 mL/kg body weight. Control animals received corn oil alone. Four days after treatment, animals were euthanized by CO_2 asphyxiation, and livers were removed. An approximately 200-mg portion of liver was placed in a cryovial, snap frozen in liquid nitrogen, and stored at −80° until analysis. Frozen livers were removed from the freezer and allowed to thaw on ice, and microsomes were prepared as previously described [9]. Protein content of diluted microsomes was determined by the method of Bradford [10], using BioRad protein assay reagents and a Beckman DU-65 spectrophotometer (Beckman Instruments, Inc.). Bovine serum albumin was used as the standard.

Western Blot Analyses

1A2 protein was detected using reagents supplied in the Amersham Life Science rat cytochrome P450 1A2 western blotting kit according to the manufacturer's instructions unless specified otherwise. Briefly, 50 μg of microsomal protein per well was loaded onto a 10% polyacrylamide gel and electrophoresed; the proteins were transferred to a nitrocellulose membrane (BioRad), blocked for 2 hr, and stored overnight at 4°. The membrane was incubated with rabbit anti-rat CYP1A2 antibody, followed by incubation with donkey anti-rabbit biotinylated antibody, and finally with streptavidin–horseradish peroxidase conjugate. Following washes, the membrane was reacted with chemiluminescent reagents, and the blot was used to develop x-ray film (Hyperfilm™ ECL™, Amersham Life Science).

Microsomal Metabolism Assays

ACOH was determined by the method of Liu *et al.* [4]. Briefly, 20 μL of microsomes was added to 1 mL of assay buffer (50 mM Tris, 30 mM $MgCl_2$, 1 mg/mL BSA, and 0.6 mmol NADPH/mL, pH 7.5), reactions were initiated by the addition of 25 μL of 20 mM acetanilide, and tubes were incubated at 37° for 30 min. Following incubation, reactions were terminated with ice-cold ethyl acetate, and 0.2 μg internal standard (3-hydroxyacetanilide) was added. Samples were extracted with additional ethyl acetate, the extract was taken to dryness and resuspended in 200 μL mobile phase (77.5% water, 17.5% methanol, and 0.5% acetonitrile), and metabolites were analyzed by reverse-phase HPLC. Recovery of internal standard was greater than 95%.

EROD and MROD were determined by the method of Pohl and Fouts [11] and Chaloupka *et al.* [12] as modified by DeVito *et al.* [9, 13]. Briefly, microsomal protein was diluted and added to 0.1 M KPO_4, 5 mM Mg_2SO_4, and 2 mg bovine serum albumin/mL at pH 7.5 containing ethoxyresorufin or methoxyresorufin (final concentration 1.5 nM). Samples were preincubated at 37° and reactions were initiated by the addition of 100 μL NADPH (5 mg/mL). Resorufin accumulation was monitored spectrofluorometrically with excitation and emission wavelengths of 522 and 586 nm, respectively.

Statistics

Levels of statistical significance were analyzed by ANOVA using StatView 512+ (Abacus Concepts) for the MacIntosh, followed by a Fisher PLSD-test as a *post hoc* test to compare means between different treatment groups. Differences were considered significant if $P < 0.05$.

RESULTS AND DISCUSSION

As expected, microsomes from *Cyp1a2*(−/−) mice did not have immunodetectable CYP1A2 protein (Fig. 1). Despite the absence of CYP1A2 protein, these microsomes had measurable MROD (25.5 ± 6.1 pmol/min/mg protein) and ACOH (0.64 ± 0.04 nmol/min/mg protein) activities (Table 1). While this drop in basal levels of activity compared with those of parental strains of mice indicates that CYP1A2 actively metabolizes both substrates, the

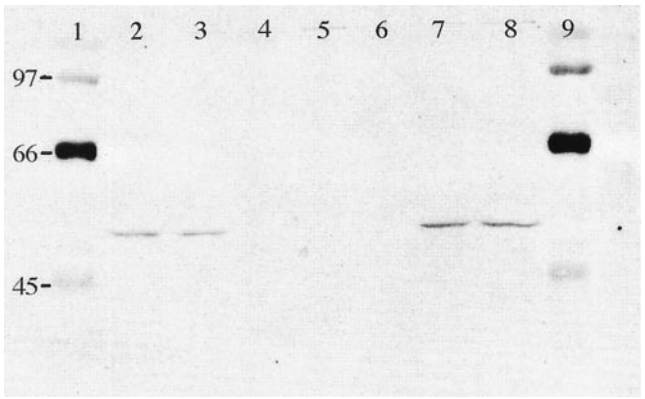

FIG. 1. Western blot analysis of CYP1A2 in hepatic microsomes derived from *Cyp1a2(−/−)* KO, C57BL/6N, and 129Sv mice. Lanes 1 and 9 = molecular weight markers (kDa), lanes 2 and 3 = 50 μg of C57BL/6N hepatic microsomal protein, lanes 4–6 = 50 μg of *1a2* KO hepatic microsomal protein, and lanes 7 and 8 = 50 μg of 129Sv hepatic microsomal protein.

residual enzymatic activity suggests that enzymes other than CYP1A2 metabolize these substrates.

Furthermore, substantial induction of EROD in 1*a*2 KO mice by TCDD was accompanied by a greater than 70-fold increase in MROD activity. In contrast, ACOH was only induced 2-fold (Table 1). As with 1*a*2 KO mice, the parental strains exposed to TCDD or 4-PeCDF showed substantial EROD and MROD induction with much lower induction of acetanilide metabolism. PCB153 resulted in a low level of both EROD and MROD induction, as was noted previously [5]. These results are in agreement with the observations of Sinclair *et al.* [14], who noted that administration of 3-methylcholanthrene to *Cyp*1*a*2(−/−) mice resulted in MROD induction.

While resorufin ethers are useful substrates, there is some overlap in substrate specificity. For example, TCDD induces PROD activity, a marker of CYP2B1, without an increase in immunodetectable CYP2B1 protein [5]. Exposure to PCB 153, a CYP2B inducer, also results in increases in MROD and EROD activity, and these findings were suggested to result from a lack of substrate specificity [5]. In addition, exposure of C57BL/6 mice to the classical CYP1A1 inducer 3-methylcholanthrene induced EROD 54.5-fold but also induced MROD, PROD, and BROD 3- to 5-fold [15]. Furthermore, exposure to a carotenoid, β-apo-8′-carotenal, induced metabolism of the above four marker enzymes, and while CYP1A2 protein was increased, CYP1A1 protein remained unchanged, suggesting that CYP1A2 can metabolize these substrates in C57BL/6 mice [15].

While selective inhibition of EROD with an anti-CYP1A1 antibody has been reported [3], there are indications of incomplete specificity of MROD in mice. In NIH/Swiss mice, a dose–response study with Arochlor 1254 showed no differences in induction of EROD and MROD activity for a given Arochlor concentration [3]. In addition, it was reported that the ability of 7,8-benzoflavone to inhibit MROD and EROD resulted in almost identical K_i values in control mice but nearly an order of magnitude difference with rat tissues [3].

Some overlap in substrate specificity is not uncommon between CYP isoforms, and the residual substrate metabolism in the 1*a*2 KO mouse was not unexpected. However, use of microsomes from 1*a*2 KO mice demonstrated that induction of MROD activity occurs in the absence of CYP1A2 protein to a degree equivalent to that of EROD induction and suggests that MROD is an unsuitable marker for measuring CYP1A2 induction in mouse liver.

TABLE 1. Enzyme activity in microsomes derived from parental and *Cyp1a2(−/−)* KO strains of mice

Treatment	ACOH (nmol/min/mg protein)	EROD (pmol/min/mg protein)	MROD (pmol/min/mg protein)
C57BL/6N			
Control	0.89 ± 0.06	75.91 ± 10.46	129.56 ± 24.87
TCDD	5.11 ± 0.88* (5.7)	5623.77 ± 649.43* (74)	3722.42 ± 73.66* (29)
4-PeCDF	4.68 ± 0.15* (5.3)	4853.20 ± 136.27* (64)	3194.53 ± 88.84* (25)
PCB 153	2.30 ± 0.04* (2.6)	321.79 ± 63.02 (4)	701.77 ± 41.86* (5)
129Sv			
Control	1.07 ± 0.04	105.88 ± 13.65	155.25 ± 19.12
TCDD	3.33 ± 0.22 (3.1)	2979.28 ± 153.95 (28)	2635.15 ± 149.71 (17)
4-PeCDF	3.36 ± 0.17 (3.2)	2036.90 ± 162.28 (19)	2103.96 ± 164.96 (14)
PCB 153	1.45 ± 0.04 (1.8)	144.61 ± 5.29 (1)	278.03 ± 17.76 (2)
1*a*2(−/−)			
Control	0.64 ± 0.04†‡	91.59 ± 10.77	25.46 ± 6.08†‡
TCDD	1.29 ± 0.05†‡ (2.0)	5782.92 ± 453.01‡ (63)	1894.82 ± 193.64†‡ (74)
4-PeCDF	0.99 ± 0.03†‡ (1.6)	5294.50 ± 611.97‡ (58)	1846.71 ± 168.15† (73)
PCB 153	0.80 ± 0.13†‡ (1.3)	879.40 ± 124.38†‡ (10)	314.07 ± 44.51† (12)

Values are means ± SEM (N = 5). Fold induction is indicated by the number of parentheses.

* Statistically significant relative to 129Sv ($P < 0.05$).

† Statistical significance between 1*a*2(−/−) and C57BL/6N ($P < 0.05$).

‡ Statistical significance between 1*a*2(−/−) and 129Sv ($P < 0.05$).

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