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Differential inhibition of human placental monooxygenase activity: evidence for multiple forms of 7-ethoxycoumarin O-deethylase

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Preparations of human placental microsomes or homogenates from maternal smokers have been shown to catalyse the *in vitro* hydroxylation of zoxazolamine [1], benzo(α)pyrene [2] and 2,5-diphenyloxazole [3], the *N*-demethylation of aminoazo dyes [4] and the *O*-deethylation of 7-ethoxycoumarin [5] and 7-ethoxyphenoxazone [6]. Of these activities only ethoxycoumarin *O*-deethylation has been observed to any significant extent in placentas from non-smokers [2, 5], suggesting the possible existence of both a basal (constitutive) and an induced form of deethylase activity in the placenta. Multiple forms of cytochrome P-450 have been found in various animal tissues [7–10] and have been distinguished by their sensitivity to various inhibitors of monooxygenase activity. In this study we investigated qualitative differences between basal and induced human placental ethoxycoumarin *O*-deethylase activities using α-naphthoflavone, metyrapone and dimethylsulphoxide as selective monooxygenase inhibitors. Metyrapone and dimethylsulphoxide are known to inhibit one form of cytochrome P-450, α-naphthoflavone having little or no effect, whilst the reverse situation occurs with a different form of cytochrome P-450. Samples of placental homogenates were obtained from a number of maternal smokers and non-smokers and also from an epileptic receiving phenytoin anticonvulsant therapy throughout her pregnancy.

Materials and Methods

Placentas were obtained from three non-smoking women, three women who smoked between 15 and 30 cigarettes per day during their pregnancy and from a non-smoking epileptic woman who had received phenytoin (350 mg/day) throughout her pregnancy; her plasma concentration of phenytoin at delivery was found to be 14.5 μg/ml. All patients delivered at term after a normal pregnancy and labour. The placentas were immediately stored at −20° after delivery until required for assay when they were thawed out overnight at 4° before being minced using a Bauknecht tissue mincer after first removing the membranes and umbilical cord. A portion of the mince was homogenised (14,500 rpm, 1 min) in ice-cold 50 mM Tris–HCl buffer (pH 7.5) using a Silverson homogeniser to produce a 20% w/v homogenate.

Placental 7-ethoxycoumarin *O*-deethylase activity was measured using a modification of the method of Greenlee and Poland [11]. The enzyme reaction was initiated by adding 1.0 ml of a mixture of NADPH (2.4 mM) and 7-ethoxycoumarin (0.67 mM) in 50 mM Tris–HCl buffer (pH 7.5) to placental homogenate (0.5 ml). After incubation (10 min, 37°) the reaction was stopped by addition of 4.5 ml of chloroform : acetone (9 : 1 v/v). The tubes were vortex-mixed (15 sec), centrifuged (1500 *g*, 5 min) and the lower, solvent layer back-extracted into 1.0 ml of 50 mM Tris buffer (pH 9.8) by vortex mixing (15 sec). After centrifugation (1500 *g*, 5 min) the fluorescence of the upper aqueous layer was determined in an Aminco-Bowman spectrophotofluorometer at uncorrected excitation and emission wavelengths of 368 and 456 nm respectively. The mean recovery of metabolic product (7-hydroxycoumarin) was found to be 50.0 ± 1.9% [12]. The effect of inhibitors upon 7-ethoxycoumarin deethylase activity was determined by adding appropriate amounts of each of the inhibitors to the corresponding assay medium. Dimethylsulphoxide and metyrapone inhibitors were dissolved in 50 mM Tris–HCl buffer (pH 7.5) and α-naphthoflavone was dissolved in methanol. Inhibitor concentrations used were: α-naphthoflavone (0.1–2.0 μm), metyrapone (0.05–1.0 mM) and dimethylsulphoxide (10–200 mM).

Results

The optimum assay conditions required for the determination of 7-ethoxycoumarin deethylase activity in homogenate preparations from induced and non-induced placentas were found to be virtually identical. Incubation of

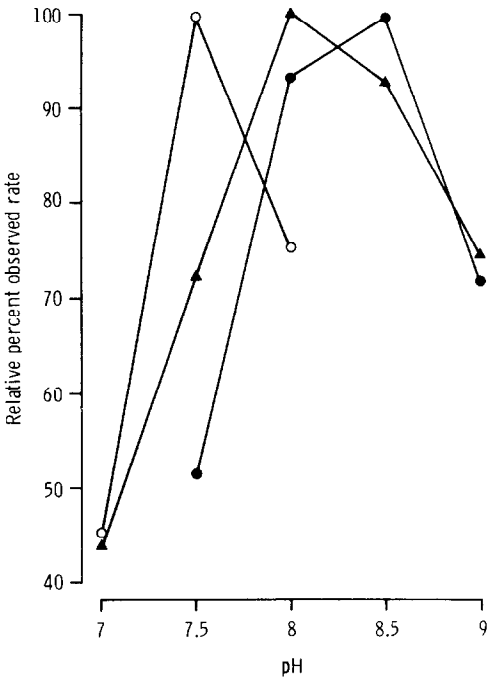

Fig. 1. The effect of pH upon 7-ethoxycoumarin *O*-deethylase activity in human placental homogenates from a smoker (○), a non-smoker (●), and an epileptic patient receiving phenytoin anticonvulsant therapy (▲).

a number of homogenates in a range of different pH environments, however, showed the pH required for maximum monooxygenase activity to be different for induced and non-induced preparations (Fig. 1). Normal assay conditions involved incubating all placental homogenates at pH 7.5.

The effects of α-naphthoflavone, metyrapone and dimethylsulphoxide upon the 7-ethoxycoumarin *O*-deethylase activity present in placental samples from three maternal smokers, three non-smokers and an epileptic patient receiving phenytoin anticonvulsant therapy are shown in Fig. 2 (a–c). The *O*-deethylase activity present in placentas from both the epileptic and the non-smokers was only weakly inhibited by α-naphthoflavone compared with the marked inhibition of deethylase activity in the placentas from maternal smokers. This contrasts with the results obtained using metyrapone and dimethylsulphoxide as inhibitors where *O*-deethylase activity in placentas from both the epileptic and the non-smokers was found to be strongly inhibited in comparison with the activity in placentas from smokers which was relatively weakly inhibited.

Additional data in the form of inhibitor concentrations producing 50% inhibition of maximum *O*-deethylase activity (I_{50} values) are shown in Table 1. The I_{50} value for α-naphthoflavone inhibition of *O*-deethylase activity in placental homogenates from non-smokers is about eight-fold greater than the corresponding value obtained for smokers. The inhibitory effects of metyrapone and dimethylsulphoxide upon placental *O*-deethylase activity are the reverse of those shown by α-naphthoflavone, I_{50} values being about 3- and 11-fold greater respectively for smokers than for non-smokers. The I_{50} values obtained for inhibition of *O*-deethylase activity in placental samples from the epileptic patient receiving phenytoin appear to resemble more closely those found for non-smokers than those found for smokers. There is a suggestion that prolonged administration of phenytoin may cause some enhancement of placental *O*-deethylase activity (0.062 nmoles 7-hydroxycoumarin formed/g/min) compared to that found in placentas from non-smokers (0.036, 0.036 and 0.046 nmoles 7-hydroxycoumarin formed/g/min). Deethylase activities in placentas from smokers were found to be 0.18, 0.47 and 0.68 nmoles 7-hydroxycoumarin formed/g/min.

Discussion

The existence of multiple forms of cytochrome P-450 in various mammalian tissues is now well established, though at the present time only a small number of these have been adequately characterised and shown to be distinctly different [9]. Whilst it has proved extremely difficult to purify cytochrome P-450 from the human placenta indirect evidence points to the existence of at least five distinct P-450 forms [13].

The data presented in this communication provide strong evidence for the existence of at least two different forms of 7-ethoxycoumarin *O*-deethylase activity in the human placenta. One form, induced by maternal smoking, is strongly inhibited by α-naphthoflavone, moderately inhibited by metyrapone and weakly inhibited by dimethylsulphoxide and appears to behave in a similar fashion to the 7-ethoxycoumarin *O*-deethylase activity found in rat liver microsomes following pretreatment with 3-methylcholanthrene [14]. This result complements other findings from differential inhibition studies [15] which show human placental aryl hydrocarbon hydroxylase (AHH) to resemble hepatic AHH from 3-methylcholanthrene-treated rats with regard to inhibition by α-naphthoflavone.

The other (non-induced or basal) form of deethylase activity present in placentas from non-smokers exhibits a reversal of this inhibition pattern as evidenced by its increased sensitivity towards inhibition by both dimethyl-

Table 1. Effect of α-naphthoflavone, metyrapone and dimethylsulphoxide on 7-ethoxycoumarin *O*-deethylase activity in human placental homogenates

	Inhibitor (I_{50})		
Patient	α-Naphthoflavone	Metyrapone	Dimethylsulphoxide
Smokers (3)	$5.5 \pm 3.1 \times 10^{-7}$	$8.1 \pm 2.2 \times 10^{-4}$	$52 \pm 6.0 \times 10^{-3}$
Non-smokers (3)	$44 \pm 10.0 \times 10^{-7}$	$2.4 \pm 0.8 \times 10^{-4}$	$4.8 \pm 0.9 \times 10^{-3}$
Epileptics (1)	50×10^{-7}	1.3×10^{-4}	3.9×10^{-3}

The I_{50} value is defined as the molar concentration of each inhibitor required to inhibit enzyme activity by 50%. I_{50} values (mean ± S.D.) are derived from the intercept at 50% inhibition of deethylase activity when the percentage inhibition of deethylase activity is plotted against the logarithm of the molar concentration of the inhibitor. Each value represents the mean of duplicate results. Figures in parentheses indicate the number of different placental homogenates used in the study.

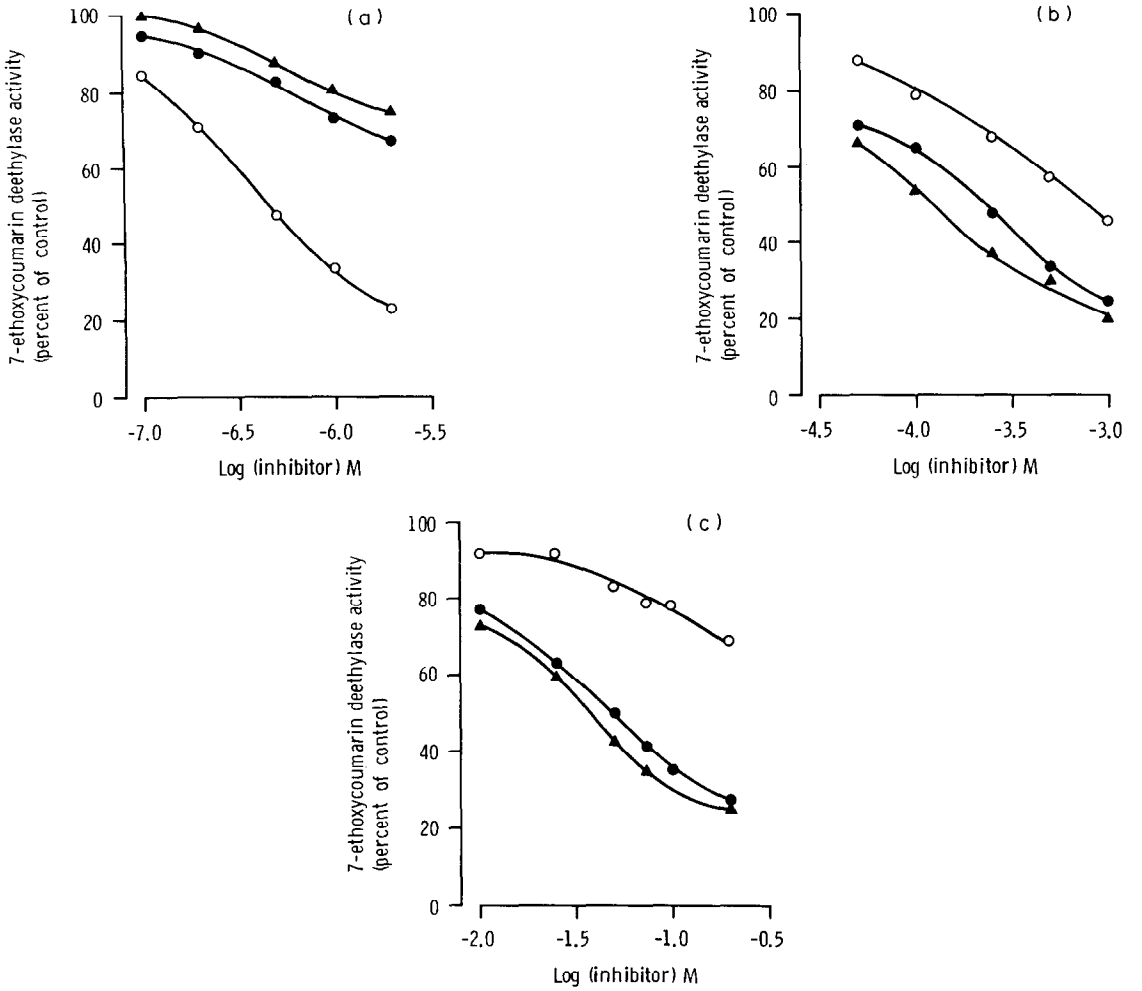

Fig. 2. Inhibition of 7-ethoxycoumarin *O*-deethylase activities by; (a) α-naphthoflavone, (b) metyrapone and (c) dimethylsulphoxide in human placental homogenates from smokers (○), non-smokers (●) and an epileptic patient receiving phenytoin (▲). In the case of smokers and non-smokers values given are the means of duplicate results from assays of three different placental samples.

sulphoxide and metyrapone and its weak inhibitory response towards α-naphthoflavone. The behaviour of this latter form of deethylase activity appears to resemble that found in hepatic microsomes from control (non-induced) and phenobarbitone pretreated rats [16].

The ability of a number of water-miscible solvents, including dimethylsulphoxide to inhibit certain NADPH-dependent enzyme systems, probably by acting as competitive substrates [17] has been reported in the literature [18]. Dimethylsulphoxide has been shown to inhibit *in vitro* microsomal 7-ethoxycoumarin *O*-deethylase activity in various animal tissues [18, 19] and, as both the work described here and elsewhere [20] demonstrate, this reagent possesses the useful property of being able to discriminate effectively between different forms of cytochrome P-450.

It is of interest to note that placental samples from the epileptic patient showed some enhancement of 7-ethoxycoumarin *O*-deethylase activity, presumably as a result of phenytoin administration. The response of this activity towards the inhibitors used here appears to bear more resemblance to the basal activity present in placentas from non-smokers than it does to the activity induced in placentas as a result of maternal smoking. No significant activity towards either hydroxylation of 2,5-diphenyloxazole or deethylation of 7-ethoxyphenoxazone substrates was found to occur, a result in accord with a previous study of monooxygenase activity in the placentas of epileptic patients [21]. It has been reported that phenytoin induces monooxygenase activity in a manner similar to phenobarbitone [22]. It must be stressed, however, that the data obtained from the patient receiving treatment with phenytoin, whilst consistent with this finding, should be viewed with caution until such a time as a larger epileptic population has been analysed.

In conclusion, it would appear that the human placental monooxygenase system parallels closely that present in rat liver with regard to its behavioural response to inhibition of ethoxycoumarin deethylase activity. Our studies show that the deethylase activity found in placental samples from non-smokers is most obviously associated with cytochrome P-450 and is qualitatively different to the activity induced as a result of maternal smoking which appears to be associated with cytochrome P-448.

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