Biochemical Pharmacology, Vol. 39, No. 5, pp. 973–975, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Microsomal reduction of dimethylaminoazobenzene (DAB). Selective effects of carbon dioxide asphyxiation versus decapitation of animals and variation with age, sex and species

(*Received* 30 *May* 1989; *accepted* 19 *September* 1989)

Dimethylaminoazobenzene (DAB), an azo dye hepatocarcinogen, is readily reduced by rat liver microsomal cytochrome P450 (P450) and slightly by NADPH–cytochrome P450 reductase [1–6]. The reaction is insensitive to both oxygen and carbon monoxide, although catalysis by P450 is well established [5]. Activity is induced *in vivo* or in primary hepatocyte cultures by a hypolipidemic drug, clofibrate, but not by other hypolipidemic drugs [4, 7, 8] or the commonly used inducing agents, phenobarbital, 3-methylcholanthrene, β-naphthoflavone, isosafrole and pregnenolone-16α-carbonitrile [4]. Laurate hydroxylase is also catalyzed by a form of P450, which is selectively induced by clofibrate [9, 10]. However, 10-undecynoic acid suppresses laurate hydroxylase but not DAB azoreductase, suggesting that these two activities are catalyzed by different forms of P450 [5, 11].

In view of the unusual nature of DAB azoreductase, it was of interest to determine how this activity varied with age, sex and species. An unexpected finding was that measurable activity in certain species depended on the method of killing.

For the sex and age study, Sprague–Dawley male and female rats were used. Livers were removed from fetuses taken from female rats whose date of pregnancy was accurately established. Other ages were established from known dates of birth. For the species study, the following young adult male animals were used: Sprague–Dawley and Wistar rats, 6 weeks; New Zealand White rabbits, 5 months; guinea pigs, 12 weeks; Syrian Golden hamsters, 6 weeks; and CD-1 mice, 6 weeks. Some animals were killed by placing them in an atmosphere of carbon dioxide, others by decapitation. Livers were homogenized in 0.1 M KCl in 10 mM Tris–HCl, pH 7.4; microsomes were prepared by differential centrifugation, washed twice, and stored at −70°. Microsomal DAB azoreductase activity was measured by fluorometric determination of reduced products as previously described [4]. Benzphetamine demethylase was measured by the rate of formaldehyde formation [12] and ethoxycoumarin deethylase by the formation of 7-hydroxycoumarin [13]. Microsomal P450 was measured by the method of Omura and Sato [14].

The sex of the fetuses could not be determined, but DAB azoreductase activity was undetectable in microsomes prepared from twelve fetal livers at 18 and 21 days, using groups of three livers for each preparation. Within 2 days after birth, high activity was seen (Fig. 1) which was maximal at 3–4 weeks, after which there was a rapid decline.

Table 1. Hepatic microsomal cytochrome P450 from rat, mouse, guinea pig, hamster and rabbit: variation with method of killing

Species	Cytochrome P450* (nmol/mg protein)	
	Carbon dioxide	Decapitation
Sprague–Dawley rat	0.79 ± 0.34	0.73 ± 0.10
Wistar rat	0.92 ± 0.02	0.93 ± 0.16
Mouse	1.00 ± 0.30	1.15 ± 0.30
Guinea pig	0.72 ± 0.28	0.86 ± 0.32
Hamster	1.29 ± 0.05	1.23 ± 0.30
Rabbit	1.15 ± 0.47	1.60 ± 0.20

* Each value is the mean ± SD from three animals. Each experiment was performed in triplicate.

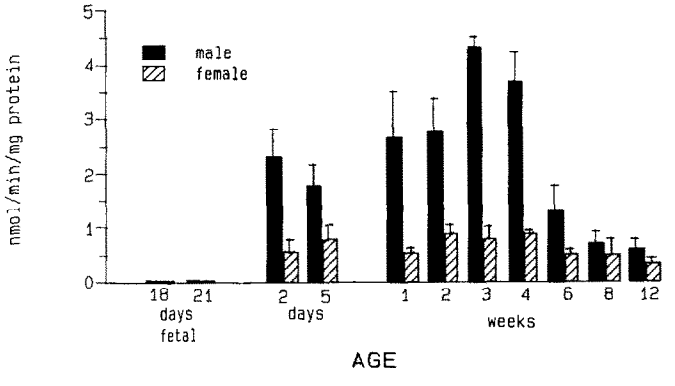

Fig. 1. Variation with age and sex of rat liver microsomal DAB azoreductase activity. Microsomes were prepared from livers of both sexes at various ages, although the sex of fetal animals could not be determined. They were incubated at 37° with DAB, 0.1 mM, and an NADPH-generating system in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) buffer, pH 7.4. Primary amine metabolites were partitioned into hexane, then 0.1 M sodium acetate, pH 4.0, and quantitated fluorometrically [4]. Values are means ± SD; N = 12–14 for fetal, and N = 3–9 for others.

Table 2. Benzphetamine demethylase and 7-ethoxycoumarin deethylase activities in hepatic microsomes from rat, mouse, guinea pig, hamster and rabbit: variation with method of killing

Species	Benzphetamine demethylase* (nmol/min/mg protein)		Ethoxycoumarin deethylase* (nmol/min/mg protein)	
	Carbon dioxide	Decapitation	Carbon dioxide	Decapitation
Sprague–Dawley rat	2.95 ± 0.35	7.09 ± 0.27	0.19 ± 0.02	0.49 ± 0.03
Wistar rat	4.05 ± 0.12	ND†	0.12 ± 0.05	ND†
Mouse	2.05 ± 0.32	2.70 ± 0.15	0.98 ± 0.02	1.30 ± 0.30
Guinea pig	5.00 ± 0.10	8.60 ± 1.30	0.18 ± 0.02	0.51 ± 0.13
Hamster	2.77 ± 0.40	0.21 ± 0.05	0.85 ± 0.15	0.92 ± 0.22
Rabbit	3.95 ± 0.02	4.40 ± 0.45	0.37 ± 0.05	0.58 ± 0.26

* Each value is the mean ± SD of three animals. Each experiment was performed in triplicate.
† Not done.

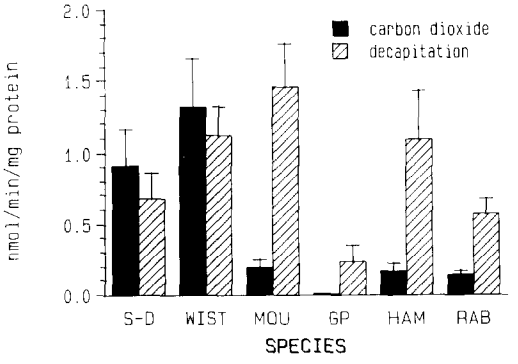

Fig. 2. Species variation of rat liver microsomal DAB azoreductase activity. Hepatic microsomes were prepared from animals killed either by being placed in an atmosphere of carbon dioxide or by decapitation. Assays were performed as described in the legend of Fig. 1. Abbreviations: S–D, Sprague–Dawley rats; WIST, Wistar rats; MOU, CD-1 mice; GP, guinea pigs; HAM, Syrian Golden hamsters; and RAB, New Zealand White rabbits. Values are means ± SD, N = 3.

In other than fetal preparations, rates for females were considerably less than those for males through 6 weeks (Fig. 1), with ratios of male to female activities of better than 2:1.

In the first experiments, animals were killed by placing them in an atmosphere of carbon dioxide. The activities obtained were then compared to those seen after decapitation with a commercial guillotine. The observable degree of stress did not vary between methods. Rabbits and guinea pigs but not other species were quite excitable when handled for either method. When an atmosphere of carbon dioxide was used to kill the animals, microsomal DAB azoreductase activity was a fraction of that seen after decapitation in mouse, guinea pig, hamster and rabbit, whereas no significant differences were seen with rats (Fig. 2). This phenomenon was readily reproducible. Despite these differences, microsomal P450 concentrations were virtually the same in both groups (Table 1). Other P450-catalyzed pathways varied somewhat with method of killing, but did not parallel the differences seen in DAB azoreductase (Table 2). Ethoxycoumarin *O*-deethylase activity was suppressed considerably in carbon-dioxide-killed Sprague–Dawley rats and guinea pigs. Benzphetamine demethylase activity after carbon dioxide was lower only in Sprague–Dawley rats and guinea pigs. Hamsters actually exhibited a reverse relationship; microsomes from decapitated animals had less than 10% of the activity seen in carbon dioxide animals. The reason for these differences is not immediately apparent. However, it is obvious that the method of killing must be considered carefully in studies on xenobiotic metabolism.

The appearance of DAB azoreductase activity soon after birth is consistent with previous reports on xenobiotic metabolism [15–18], although the abrupt diminution of activity after 4 weeks is somewhat more rapid than that observed for other activities [19–24]. Sex differences were pronounced as early as 2 days and continued until 8 weeks, at which time they were no longer significant (Fig. 1). Higher mixed-function oxidase activity in male rats has been attributed in part to specific constitutive forms of P450 [25–28]. These are independently regulated by sex hormones [29]. The sex differences in DAB azoreductase activity diminished with time (Fig. 1), consistent with other P450 pathways [30]. A gradual loss of a male-specific form of P450 [23] appears to account for the age-related loss of sex difference. The exact nature of the form of P450 which catalyzes the reduction of DAB is unknown although, like P452, a laurate hydroxylase [10], it is selectively induced by clofibrate [4].

In summary, there was considerable species variation in hepatic microsomal DAB azoreductase activity. If animals were killed with carbon dioxide, rates were very low compared to decapitation in all species except the rat. This was a rather specific response and was not attributable to loss of P450. Other P450-catalyzed pathways were not similarly affected. Sex differences were prominent immediately after birth but diminished rapidly after 4 weeks.

Acknowledgements—This study was supported, in part, by Grants CA14237 and CA13330 from the National Institutes of Health.

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