J. Pharm. Pharmacol. 1984, 36: 743–747
Received October 31, 1983

Renal aminopyrine demethylation in several species determined by a sensitive radiometric method

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Renal microsomal aminopyrine demethylation activities of several species were measured by a sensitive radiometric method using [dimethylamino-^{14}C-]aminopyrine as a substrate and 2,4-dinitrophenylhydrazine as a trapping agent for the formaldehyde formed. The activity was highest in hamsters (0·75 nmol min^{-1} mg^{-1} protein) and that in rabbits, rats, mice, and guinea-pigs was 19·7, 7·0, 4·5 and 3·7%, respectively, of the hamster values. These species differences did not correlate with species differences in cytochrome P-450 content or in NADPH cytochrome c reductase activity. Aminopyrine demethylation activities in sliced renal tissues of several species were also compared. This activity was also found highest in hamsters (0·54 nmol min^{-1} g^{-1} wet tissue) and the activities in rabbits, rats, and guinea-pigs were 9·2, 1·8 and 2·5%, respectively, of the hamster values.

Species and organ specificity of chemically-induced toxicity is one of the major problems in toxicology. The difference in the metabolic activation by the microsomal mixed function oxidase has been considered to be one of the causes of the specificity. However, the activities in extrahepatic organs were low in general (Jones et al 1980) and it has been relatively difficult to measure the activity of extrahepatic mixed function oxidase. This has been especially so in the case of renal metabolism of aminopyrine, which has been most frequently used as a substrate for the estimation of hepatic and extrahepatic activities of mixed function oxidase, where large discrepancies exist among the reported values (Litterst et al 1975: Pawar et al 1978: Zenser et al 1978; Poland et al 1973).

Previously, we developed the method of determination of renal microsomal cytochrome P-450 without the influence of the contaminating mitochondrial cytochromes. We reported the indication of species differences in the amount of renal microsomal cytochrome P-450 and in the wavelength of the absorption peaks of its CO difference spectra (Ohno et al 1982).

We now report a sensitive radiometric assay method of microsomal aminopyrine demethylation using 2,4-dinitrophenylhydrazine. We have applied this method to the estimation of renal microsomal aminopyrine demethylation of several species, and compared the results with values from sliced tissues of the kidneys.

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MATERIALS AND METHODS

Materials

Metyrapone (Sigma), a-naphthoflavone (Aldrich); [dimethylamino-^{14}C-]aminopyrine and [^{14}C]-formaldehyde [New England Nuclear). Radioactive aminopyrine was partially purified before use by the method of Poland et al (1973). SKF 525-A was donated by Smith Kline and French Labs (Philadelphia). Other chemicals were of reagent grade and obtained from local commercial sources.

Animals

Male rats (Wistar strain, 9–14 weeks), Syrian golden hamsters (9–14 weeks), guinea-pigs (Hartley strain, 9–14 weeks), and mice (ddY strain, 9–14 weeks) were decapitated. Rabbits (Japanese White, ca 3 kg) were anaesthetized lightly with sodium pentobarbitone and exsanguinated.

Microsomal aminopyrine metabolism

Microsomal fractions of the rabbit kidneys were prepared from renal cortex and those of other animals were prepared from whole kidney minus papilla by a method described by Ohno et al (1982). The incubation mixture for the assay of aminopyrine demethylation consisted of 40 mM potassium phosphate buffer (0·5 ml, pH 7·4) containing (μmol) $NADP^+$ 0·5, glucose 6-phosphate 15, $MgCl_2$ 2·5,

semicarbazide 0·5, glucose 6-phosphate dehydrogenase 0·5 unit, aminopyrine 1 μmol (ca 1·5 × 10^5 d min^{-1}) and microsome equivalent to 50–100 mg of wet tissue. For the control $NADP^+$ was removed from the incubation mixture. After incubation at 37 °C, 0·5 ml of the incubation mixture was mixed with 0·1 M NaOH (1·0 ml) and extracted twice with ice cold chloroform (8·0 ml) to remove aminopyrine from the aqueous phase. The aqueous phase (1·0 ml) thus obtained was mixed with 2,4-dinitrophenylhydrazine-saturated 2 M HCl (1·0 ml). After 30 min in the dark, the hydrazone formed was extracted with cyclohexane (9 ml), this was evaporated under reduced pressure, and the residue was extracted twice with toluene (0·5 ml). The radioactivity in the combined toluene extract was measured in the cocktail of a toluene scintillator (DPO 4 g and POPOP 0·1 g l^{-1} toluene) by a liquid scintillation spectrophotometer (Aloka-LSC-637). The values found were corrected by an external standard method.

Aminopyrine metabolism in sliced tissue

Tissue slices (ca 0·6 mm in thickness) were prepared from renal cortex by a tissue slicer (Natsume Co. Tokyo), and were put into ice cold Warburg flasks containing 2·8 ml of Krebs-Henseleit buffer solution. 10% NaOH (0·2 ml) and 2 M HCl (0·5 ml) were put in the centre well and side arm, respectively. After the addition of aminopyrine (280 nmol, ca 2 × 10^5 d min^{-1}) into the tissue suspension, the air in the flask was exchanged with oxygen and the incubation at 37 °C was started. The reaction was stopped by the addition of HCl from the side arm and the flasks were placed upright in an ice bath for 30 min. Radioactivity absorbed in the NaOH solution was measured in a mixture of toluene scintillator and Triton X-100 (2:1) after the decrease of chemiluminescence to the nonsignificant level.

Others

Cytochrome P-450, NADPH cytochrome c reductase, and protein were measured by the methods of Ohno et al (1982), Masters et al (1971), and Lowry et al (1951), respectively.

RESULTS

Aminopyrine metabolism in microsomal fraction

When renal microsomal aminopyrine demethylation activity was measured in rats by the method of Nash (1953), a large amount of formaldehyde (ca 9·3 nmol min^{-1} g^{-1} wet tissue) was formed in the presence of NADPH without the addition of substrate. Further increase of formaldehyde formation by the addition of aminopyrine was not detected. It seemed that the renal microsomal aminopyrine demethylation activity was very low in rats and that it was necessary to use radioactive aminopyrine as a substrate in order to distinguish the formaldehyde formed by the demethylation of the substrate from background formaldehyde.

Poland et al (1973) used [^{14}C]aminopyrine as a substrate and extracted it from the alkaline incubation mixture with chloroform. They measured the radioactivity remaining in the aqueous phase and used this for the estimation of the polar metabolites formed by the demethylation. Aminopyrine demethylation activity in hamster kidney could be measured by this method (Fig. 1). With rat, mouse and guinea-pig kidney, however, the radioactivity in the aqueous phase did not increase over incubation time and it sometimes became lower than in the blank preparation. It seemed that the variation of radioactivity in the blank preparation was too large (780 ± 105 d min^{-1}/tube, which was equivalent to 10·4 ± 1·4 nmol of HCHO/tube; mean ± s.d. of 8 determinations) to allow the measurement of low activity such as in rat kidney.

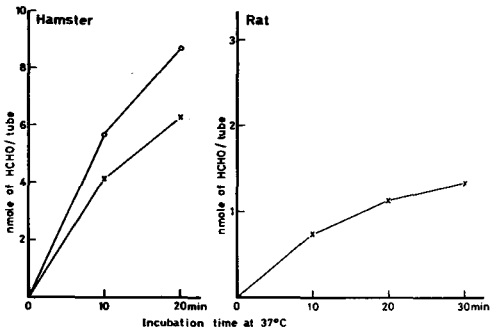

FIG. 1. Renal microsomal aminopyrine demethylation determined by the method of Poland et al (1973) (○) and the method described herein (●). The amount of aminopyrine added to the incubation mixture (0·5 ml) was 1 μmol (ca 15 × 10^4 d min^{-1}). Microsomes equivalent to 50 mg (hamster) or 100 mg (rat) of wet tissue was added to each incubation tube. Each point represents the mean of two determinations.

We therefore used 2,4-dinitrophenylhydrazine to trap the formaldehyde in the aqueous phase as a hydrazone after chloroform extraction of aminopyrine. This hydrazone was extracted with cyclohexane and the radioactivity was measured by a liquid scintillation spectrophotometer. Recoveries of formaldehyde added to the aqueous phase before and after the extraction by chloroform were 82 and 98%, respectively.

The radioactivity in the blank preparation was 77·0 ± 6·3 d min^{-1}/tube (equivalent to 0·98 ± 0·08 nmol of HCHO/tube; mean ± s.d. of 8 determinations) in our method and this value was about 10 times lower than that obtained by the method of Poland et al (1973). The radioactivity was increased significantly up to 30 min of incubation, but this increase was not linear as a function of time (Fig. 1). We obtained similar results in mice, rabbits, and guinea-pigs. Therefore, we estimated the demethylation activity after 10 min of incubation (Table 1).

Renal microsomal aminopyrine demethylation activity obtained by our method was highest in hamsters (8·14 ± 0·42 nmol min^{-1} g^{-1} tissue, 0·76 ± 0·07 nmol min^{-1} mg^{-1} protein), and the activities in rabbits, mice, rats, and guinea-pigs were 53, 9, 8 and 5%, respectively, of hamster values by wet tissue weight base and 19·7, 4·5, 7·0 and 3·7%, respectively, of hamster values by microsomal protein base (Table 1). On the other hand, cytochrome P-450 content and NADPH cytochrome c reductase activity were highest in mice. Cytochrome P-450 contents of rabbits, hamsters, rats, and guinea-pigs were 59, 43, 37 and 36% of mice, respectively, and NADPH cytochrome c reductase activities were 23, 36, 30 and 27% of mice, respectively. Accordingly, species differences in aminopyrine demethylation did not correlate with those of cytochrome P-450 content or cytochrome c reductase. Aminopyrine demethylation activity in kidney was supported by NADPH. Metyrapone (0·1 mM) inhibited the activity to 5% of control in hamsters and to 44% in rats. SKF 525-A (0·25 mM) inhibited the activity to 12% of control in hamsters and to 69% in rats. Inhibition by a-naphthoflavone (0·1 mM) was weak in both hamsters and rats.

Species differences in hepatic microsomal aminopyrine demethylation activity were not so large as those in kidney activity (hamster : guinea-pig : rabbit : rat : mouse = 1 : 0·94 : 0·88 : 0·57 : 0·56 by tissue wet weight base, and = 1 : 1·19 : 1·47 : 0·49 : 0·57 by microsomal protein base) (Table 2).

Aminopyrine demethylation in sliced tissues

To confirm the data obtained by microsomal fraction, we made the experiment using sliced tissues. Radioactive carbon dioxide trapped in the alkaline solution was increased linearly up to 120 min (data not shown in the Fig.). As was the case for microsomal fraction, the activity was highest in hamsters (0·54 ± 0·06 nmol min^{-1} g^{-1} tissue). The activities in rabbits, guinea-pigs and rats were 9·2, 2·5 and 1·8% of that in hamsters, respectively (Table 1).

DISCUSSION

Aminopyrine demethylation activity in the renal microsomal fraction could be measured satisfactorily in various species by the present method. Among the animals examined, the activity was the highest in hamsters. The activity in rabbits was about 20% of that of hamsters by microsomal protein base and those in rats, mice, and guinea-pigs were 3–7% of hamster values.

It seems pertinent to stress that the activities reported herein were lower than 20% of the reported values for rats (Litterst et al 1975; Pyykkö 1980) and about 0·3% of results from guinea-pigs (Pawar et al 1978) obtained by the method of Nash (1953). Our rabbit values were 140 times higher than those for rabbits (Zenser et al 1978) obtained by the method of Poland et al (1973).

Renal microsomal styrene oxidation activity in rabbits was several times higher than that in guinea-pigs, mice and rats (Cantoni et al 1978). The activity of renal aryl hydrocarbon hydroxylase in male hamsters was also several times higher than that in rats, gerbils and guinea-pigs (Bilimoria & Ecobichon 1980). These results were well in line with our present results. On the other hand, pulmonary aryl

Table 1. Species differences in renal aminopyrine demethylation.

Animals	P-450 content nmol g^{-1} tissue	Cyt. c red. nmol min^{-1} g^{-1} tissue	Aminopyrine demethylation* in microsomal fraction nmol min^{-1} g^{-1} tissue	in microsomal fraction pmol min^{-1} mg^{-1} protein	in sliced tissue pmol min^{-1} g^{-1} tissue
Mouse	4·56 ± 0·88† (4)‡	1014 ± 34 (4)	0·72 ± 0·08 (4)	34·0 ± 4·0 (4)	not determined
Hamster	1·99 ± 0·12 (4)	367 ± 15 (4)	8·14 ± 0·42 (4)	756·0 ± 76·0 (4)	540·0 ± 68·0 (4)
Rat	1·67 ± 0·16 (17)	309 ± 39 (17)	0·66 ± 0·12 (17)	52·8 ± 9·4 (17)	9·6 ± 2·2 (4)
Guinea-pig	1·65 ± 0·14 (7)	278 ± 9 (7)	0·46 ± 0·04 (7)	27·8 ± 5·6 (7)	13·4 ± 0·6 (4)
Rabbit	2·72 ± 0·32 (7)	230 ± 6 (7)	4·36 ± 0·74 (7)	149·0 ± 27·0 (7)	49·6 ± 16·8 (4)

* Time of incubation was 10 min for microsome and 120 min for sliced tissues.
† These figures represent the mean ± s.e.m.
‡ Number of animals.

Table 2. Species differences in hepatic microsomal aminopyrine demethylation.

Animals	P-450 content nmol g^{-1} tissue	Cyt. c red. μmol min^{-1} g^{-1} tissue	Aminopyrine demethylation* nmol min^{-1} g^{-1} tissue	nmol min^{-1} mg^{-1} protein
Mouse	12·8 ± 1·1† (5)‡	1·53 ± 0·10 (5)	94·4 ± 5·2 (5)	4·32 ± 0·02 (5)
Hamster	21·0 ± 0·9 (4)	2·98 ± 0·05 (4)	167·0 ± 3·0 (4)	7·50 ± 0·22 (4)
Rat	18·9 ± 1·3 (16)	1·94 ± 0·13 (16)	95·6 ± 13·2 (16)	3·70 ± 0·32 (16)
Guinea-pig	30·0 ± 0·2 (4)	3·07 ± 0·15 (4)	158·0 ± 5·0 (4)	9·94 ± 0·26 (4)
Rabbit	38·2 ± 5·1 (4)	2·48 ± 0·23 (4)	148·0 ± 1·0 (4)	11·00 ± 0·90 (4)

* Time of incubation was 10 min and the amount of formaldehyde formed was estimated by the method of Poland et al (1973).
† These figures represent the mean ± s.e.m.
‡ Number of animals.

hydrocarbon hydroxylation activity in hamsters was lower than that in gerbils, rats and guinea-pigs (Bilimoria & Ecobichon 1980). In the case of intestinal ethylmorphine demethylation, aryl hydrocarbon hydroxylation and 7-ethoxycoumarine deethylation, the activities in guinea-pigs were higher than those in rats, mice and rabbits (Miranda & Chhabra 1980).

A number of different isozymes of cytochrome P-450 were isolated from the livers of rabbits, rats and mice, substrate specificities of which vary greatly among the species (Reviewed by Ichikawa 1981). Characterization of microsomal cytochrome P-450s in kidneys was also attempted and it was found that rabbit renal cortex contained two cytochrome P-450 species (mol. wt 54 500 and 58 900) which were identical to hepatic species on SDS-gel electrophoresis (Ambrecht et al 1979). The molecular weight of cytochrome P-450 purified from phenobarbitone-treated porcine kidney was 56 000 (Masters et al 1980). This enzyme had a high affinity to benzphetamine *N*-demethylation and lauric acid hydroxylation.

Microsomal aminopyrine demethylation was supported by NADPH and inhibited by metyrapone and SKF 525-A in our experiments. Therefore, it seemed that the activity in the kidneys was also dependent on the cytochrome P-450. However, the species differences observed did not correlate with those of cytochrome P-450 and of NADPH cytochrome c reductase. Gray et al (1972) indicated the preence of mitochondrial cytochrome P-450 in chick kidney which had a high affinity to 25-hydroxylcholecalciferol-1-hydroxylation and Yoon et al (1980) purified this enzyme (mol wt 11 900). Renal microsomal fraction contained significant amount of mitochondrial cytochromes. Therefore, we measured the microsomal cytochrome P-450 without the influences of those contaminating mitochondrial cytochromes and found that the absorption peaks of CO difference spectra of renal cytochrome P-450 differed among species (rats: 451·9 nm, hamsters: 450·3, guinea-pigs: 450·7, mice 449·7, and rabbit 450·0) (Ohno et al 1982) and the degree of inhibition by metyrapone and SKF 525-A was different between hamsters and rats. Therefore, there might be differences in the renal microsomal cytochrome P-450 species among animals.

In the case of experiments using sliced renal tissues similar species differences were observed, but the degree was different between sliced tissues and microsomal fractions. We used renal cortex for the preparation of sliced tissues and whole kidney, except for papilla, for the preparation of the microsomal fraction. (Rabbit microsomal fraction was prepared from renal cortex.) This might be one of the causes of these discrepancies. However, the main reason seems to remain a subject for further investigation.

Species differences of aminopyrine metabolism in the kidneys were quite large compared with those in the liver. It has been thought that many of the chemical carcinogens and toxic substances require hepatic or extrahepatic metabolic activation (IARC, 1978, 1979). Therefore, our present data for the kidneys are interesting from the standpoint of the selection of animals for the toxicity evaluation of chemicals.

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