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Sex-related Differences in Rat Liver Microsomal Enzymes and Their Induction by Doxapram

MASAYASU OZAKI, MASAAKI ISHIKAWA, YOSHIO TAKAYANAGI AND KEN-ICHI SASAKI

Department of Pharmacology and Toxicology, Cancer Research Institute, Tohoku College of Pharmacy, Komatsushima Aoba-ku, Sendai 981, Japan

Abstract—The effects of doxapram on the hepatic microsomal mono-oxygenase system of male and female rats were investigated. Male and female rats were administered doxapram (10–120 mg kg^{-1} day^{-1}, i.p.) for 4 days. In female rats, administration of doxapram (20, 40, 60, 80, 100 and 120 mg kg^{-1}) elevated the parameters in a dose-dependent manner while doxapram (100 and 120 mg kg^{-1}) elevated the levels of cytochrome P450 and hexobarbitone hydroxylase in male rats. Doxapram (40 mg kg^{-1}) caused induction of hepatic drug metabolism typified by an increase of hepatic microsomal cytochrome P450 content and activities of hexobarbitone hydroxylase, benzphetamine *N*-demethylase and ethylmorphine *N*-demethylase in female rats, but no change in male rats. These findings were supported by the results of SDS/polyacrylamide-gel electrophoresis. However, 7-ethoxycoumarin *O*-de-ethylase and arylhydrocarbon hydroxylase activities were significantly increased in male rats. NADPH-cytochrome c reductase and NADH-cytochrome c reductase activities, and cytochrome b_5 content were unaffected in rats of both sexes. The sex-dependent cytochrome P450 species may be selectively sensitive to the action of doxapram.

The liver microsomal mono-oxygenase system metabolizes a wide variety of endogenous and exogenous substrates: steroids, fatty acid, prostaglandins, environmental chemicals and drugs, including doxapram (Aranda et al 1988). The activity of the microsomal mono-oxygenase system is inducible by a large number of chemicals and can be broadly categorized into two major groups, the phenobarbitone-type and the 3-methylcholanthrene- or polycyclic hydrocarbon-type on the basis of their ability to induce different pathways of metabolism (Parke 1975). The differential induction has been linked to the ability of the inducers to stimulate the production of one or more isoenzymes of cytochrome P450 (Lu & West 1980; Okey 1990).

Sex differences have been reported in hepatic microsomal metabolism of drugs particularly in the rat in induction experiments (Skett 1988). Recently, we found that doxapram increases cytochrome P450 and related enzyme activities in mice and rats (Ishikawa et al 1991a); in the present study we have examined the sex differences in induction caused by doxapram and compared the differences with the known sex-dependence of the enzymes.

Materials and Methods

Animals

Six-week-old Wistar rats of either sex were purchased from the Japan SLC Co., Hamamatsu, Japan. Rats were housed in groups of six and were allowed free access to food and water. In a preliminary experiment intraperitoneal doxapram (20 mg kg^{-1}) daily for six days was shown to induce hexobarbitone hydroxylase after 3, 4, 5 and 6 days in a time-dependent manner. Therefore, in subsequent studies doxapram was given intraperitoneally daily for four consecutive days and hepatic drug-metabolizing activity was determined 24 h after the last injection of doxapram. The control group of animals received 0·9% NaCl (saline) alone. All drug solutions were prepared immediately before use, so that each animal received 2 mL kg^{-1}. Each experimental group consisted of six animals.

Correspondence: M. Ishikawa, Department of Pharmacology and Toxicology, Cancer Research Institute, Tohoku College of Pharmacy, 4-4-1 Komatsushima, Aoba-ku, Sendai 981, Japan.

Preparation of liver fraction

Animals were killed by cervical dislocation, and liver removed, weighed, perfused with ice-cold 1·15% KCl solution, and homogenized in a Teflon homogenizer with 3 vol cold 0·1 M sodium-potassium phosphate buffer (pH 7·4). The homogenate was centrifuged at 9000 *g* for 20 min, and the resulting supernatant fraction served as the enzyme source for the measurement of the activity of hexobarbitone hydroxylase. To separate microsomes, the 9000 *g* supernatant fraction was centrifuged at 105 000 *g* for 60 min. All steps were carried out at 4°C. Microsomes were stored as frozen pellets at −80°C and were slowly thawed on ice immediately before the assays.

Enzyme and spectral measurement

The standard reaction mixture for the assay of drug metabolism contained substrate (concentration described below), microsomes (0·05 or 3·0 mg), 0·1 M sodium potassium phosphate buffer (pH 7·4) and an NADPH-generating system, in a final volume of 1·0 mL. The NADPH-generating system consisted of 0·33 mM NADP, 8 mM glucose-6-phosphate, 6 mM $MgCl_2$, and 0·2 units glucose-6-phosphate dehydrogenase. The mixture was incubated in a water bath shaker for 15 or 20 min at 37°C under air. The *N*-demethylation of benzphetamine and ethylmorphine were assayed by the method of Nash (1953) using 10 μmol substrate. Aniline hydroxylase was estimated by measurement of *p*-aminophenol according to Imai et al (1966) using

10 μmol aniline. *p*-Nitroanisole *O*-demethylase was estimated by measurement of *p*-nitrophenol according to Netter & Seidel (1964) using 10 μmol *p*-nitroanisole. The activity of 7-ethoxycoumarin *O*-de-ethylase was assayed according to Greenlee & Poland (1978) using 10 μmol 7-ethoxycoumarin.

Arylhydrocarbon hydroxylase activity was determined according to the fluorometric assay of Nebert & Gelboin (1968). The microsomal pellets were resuspended in 50 mM Tris buffer, 3 mM $MgCl_2$, pH 7·5. The incubation was carried out in a final volume of 1 mL containing 80 nmol benzo(a)pyrene added in 40 μL acetone, 0·31 mL H_2O, 0·5 mL 50 mM Tris buffer (pH 7·5), 0·37 μmol NADH, 0·38 μmol NADPH, 0·6 mg bovine serum albumin, 3·45 μmol $MgCl_2$, and 0·15 mL microsomal suspension (about 0·05 mg protein). The incubation was carried out at 37°C for 5 min, and the mixture was shaken with 3·0 mL hexane for 10 min. A 1·0 mL sample of the organic layer was extracted with 2·0 mL 1 M NaOH, and the fluorescence of the extract was measured immediately (396 nm excitation, 522 nm emission). This was compared with the fluorescence of a 3-hydroxy-benzo(a)-pyrene standard solution.

NADPH-cytochrome c reductase and NADH-cytochrome c reductase activities were measured by the method of Phillips & Langdon (1962) and Mihara & Sato (1972), respectively.

Hexobarbitone hydroxylase of the 9000 *g* supernatant was estimated by the method described by Ishikawa et al (1989).

Cytochrome P450 content was determined from the carbon monoxide-induced difference spectrum of dithionite-reduced microsomes, assuming a molar extinction coefficient of 91 $mM^{-1}\ cm^{-1}$ between 450 and 490 nm (Omura & Sato 1964), and cytochrome b_5 content was determined by measuring the dithionite-reduced difference spectrum assuming a value of 185 $mM^{-1}\ cm^{-1}$ for the molar extinction coefficient between 409 and 424 nm (Omura & Takesue 1970), using a Hitachi U-2000 double-beam spectrophotometer. The protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as the standard.

SDS-polyacrylamide gel electrophoresis

The polypeptide composition of the microsomal fraction was analysed by sodium dodecyl sulphate (SDS) polyacrylamide slab gel electrophoresis (Laemmli 1970) and densitometric scanning was performed at 550 nm using a Shimadzu CS-910 chromatoscanner. Molecular weights of microsomal polypeptide were determined using a mixture of standard proteins. Treatment with phenobarbitone and 3-methylcholanthrene for positive controls were at dosages of 80 mg kg^{-1} day^{-1} for two days, and 40 mg kg^{-1} day^{-1} for two days, respectively, and microsomes were prepared 24 h after the last treatment.

Data analyses

Data were analysed for differences between values for the doxapram-treated animals and their respective control values using Student's *t*-test. Differences were considered to be significant at $P < 0.05$.

Results

Intraperitoneal injection of male and female rats with doxapram resulted in a dose-dependent increase in hepatic microsomal cytochrome P450. The increase in cytochrome P450 content was always greater in the female. This result effectively reflected the increases in hexobarbitone hydroxylase of hepatic microsomes caused by doxapram (Fig. 1). Doxapram did not affect the absorption maximum of the carbon monoxide-induced spectral changes of cytochrome P450 in both male and female rats (data not shown).

Table 1 shows the effect of successive administration of doxapram (40 mg kg^{-1}, i.p., daily for 4 days) on the mixed-function oxidase enzyme system. Doxapram pretreatment caused a marked induction in the metabolism of hexobarbitone, benzphetamine and ethylmorphine and cytochrome P450 content in the females but was without any significant effect in the male rats. No significant effects were seen on the induction of aniline hydroxylase, *p*-nitroanisole *O*-demethylase, 7-ethoxycoumarin *O*-de-ethylase and arylhydrocarbon hydroxylase in the females, but the males showed selective induction after doxapram pretreatment. However, liver weight, microsomal protein content, NADPH-cytochrome c reductase, NADH-cytochrome c reductase and cytochrome b_5 content was comparable with the corresponding values for control rats in both sexes.

Fig. 2 shows the SDS-gel electrophoresis of untreated, doxapram-induced, phenobarbitone-induced and 3-methylcholanthrene-induced rat liver microsomes. In phenobarbitone-induced and 3-methylcholanthrene-induced rats, 50 K and 53 K bands are deeply stained, compared with samples from the untreated rat. In doxapram-treated rats, the phenobarbitone-induced 50 K band was more intensely stained than in untreated rats.

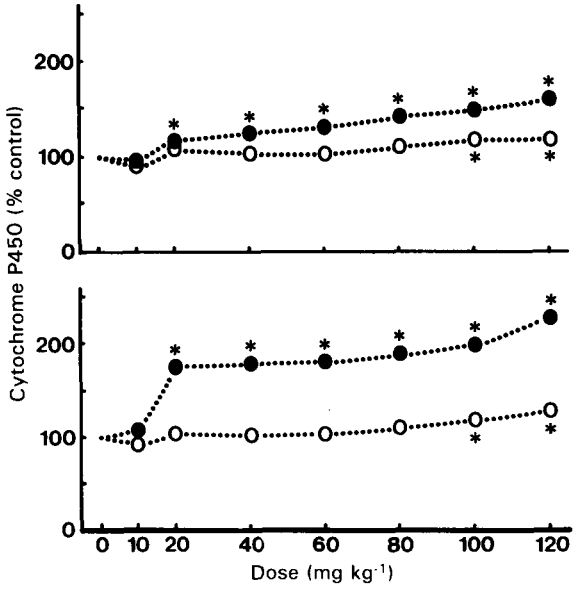

FIG. 1. Dose-dependent increase of cytochrome P450 content and hexobarbitone hydroxylase activity in rats. Male (○) or female (●) rats were injected intraperitoneally for four successive days with doxapram for determination of cytochrome P450 content (upper panel) and hexobarbitone hydroxylase activity (lower panel). Control animals received saline. Each point represents the mean of five values compared with control. *$P < 0.05$ compared with controls.

Table 1. Effect of doxapram on the mixed-function oxidase enzyme system in the rat. Enzyme assays and treatment with doxapram were as described in the text.

Parameters	Control	Doxapram (40 mg kg^{-1})	Phenobarbitone (80 mg kg^{-1})	3-Methylcholanthrene (20 mg kg^{-1})
Cytochrome P450 (nmol (mg protein)$^{-1}$)	0·34±0·020	0·48±0·30*	1·05±0·084*	0·71±0·019*
Hexobarbitone oxidase (units min^{-1} (mg protein)$^{-1}$)	23·2±1·58	39·4±1·18*	61·6±1·88*	20·7±0·83
Aniline *N*-demethylase (nmol/60 min (mg protein)$^{-1}$)	73·3±6·15	120·4±7·86*	259·6±14·88*	57·7±8·57
Ethylmorphine *N*-demethylase (nmol/60 min (mg protein)$^{-1}$)	72·2±8·27	135·0±2·80*	348·9±14·85*	54·8±6·83
7-Ethoxycoumarin *O*-de-ethylase (nmol/20 min (mg protein)$^{-1}$)	1·14±0·015	1·15±0·163	2·57±0·299*	2·20±0·466*
Arylhydrocarbon hydroxylase (nmol/15 min (mg protein)$^{-1}$)	0·58±0·022	0·61±0·011	0·60±0·024	0·79±0·019*

Significant differences from the corresponding control value are indicated as * ($P<0·05$). Each value represents the mean±s.e. of six animals.

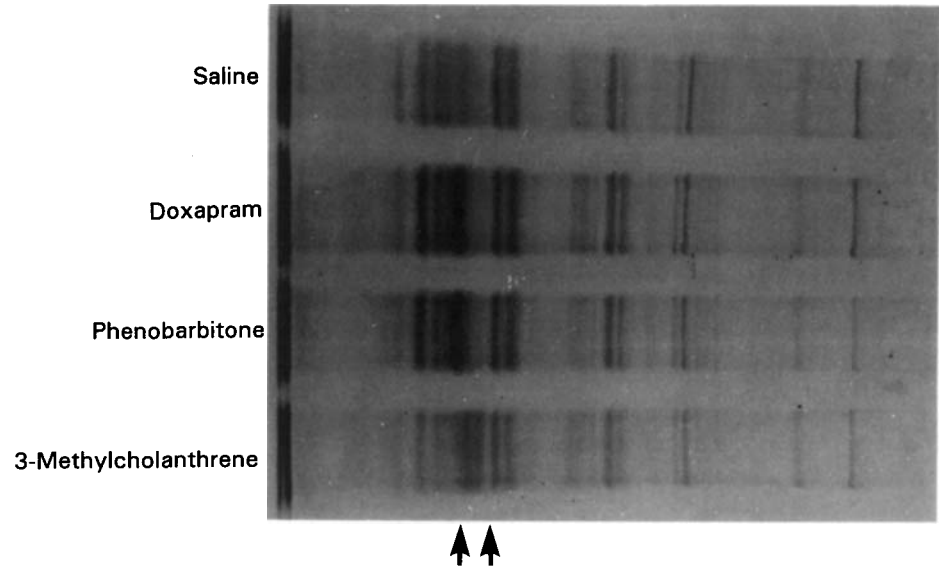

FIG. 2. Results of SDS/polyacrylamide-gel electrophoresis of liver microsomal membrane fraction from doxapram-treated rats. The gel was stained with Coomassie blue. Gel electrophoresis with saline alone, doxapram, phenobarbitone and 3-methylcholanthrene were as described in the text. The arrows indicate the 53 000 (right) and 50 000 (left) molecular weights, respectively.

Discussion

Doxapram dose-dependently induced cytochrome P450 content and hexobarbitone hydroxylase activity and increased cytochrome P450 content, without any change in absorption maximum for the carbon monoxide-induced spectrum of cytochrome P450 in male and female rats. However, induction was always effectively more pronounced in the females, thus indicating a clear sex difference in the effect of doxapram.

Changes in the parameters of drug metabolism as a function of mono-oxygenase system induction can reflect an alteration in the make up of the cytochrome P450 pool. The existence of multiple forms of cytochrome P450 in the rat liver microsomal fraction and changes in the pool composition as a function of induction have been documented (Lu & West 1980). SDS-gel microsomal protein patterns of microsomal protein from doxapram-treated rats more closely resembled those of phenobarbitone-treated than untreated or 3-methylcholanthrene-treated rats.

Studies on spectral binding of doxapram to liver microsomes of female rats have shown that doxapram is a reverse type I compound characterized by a peak at 415 nm and a trough at 390 nm (Ishikawa et al 1991b). Unlike most reverse type I compounds, which usually inhibit the hepatic microsomal drug-metabolizing enzyme activity, doxapram is an inducer of the hepatic drug-metabolizing enzyme system. However, this is not uncommon for reverse type I compounds. For example, macrolide antibiotics cause both induction and inhibition depending on dose and frequency of dosing (Delaforge et al 1983).

Sex differences in phenobarbitone and 3-methylchloranthrene induction in adult rats have been reported (Kato 1974; EL Defrawy EL Masry & Mannering 1974). Phenobarbitone has been found to increase cytochrome P450 more in male rats than in female rats, but spironolactone stimulates more NADPH-cytochrome c reductase in females (Kamataki et al 1983). Several inducers increase the de-alkylation activities of various coumarin derivatives more in females than in males, resulting in a decrease of the sex differences (Kamataki et al 1983). In the present study, doxapram treatment seemed to accentuate the sex difference.

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