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〔一般論文〕

Preliminarily Observation of Sex- and Substrate-Related Induction of Hepatic Drug-Metabolizing Enzyme Activity by Doxapram in Rats

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Abstract

The effects of doxapram on the hepatic microsomal monooxygenase system of male and female rats were investigated. Male and female rats were administered doxapram (10-120 mg/kg/day, i.p.) for 4 days. In female rats, administration of doxapram (20, 40, 60, 80, 100 and 120 mg/kg) elevated the both parameters in a dose-dependent manner while doxapram (100 and 120 mg/kg) elevated the levels of cytochrome P-450 and hexobarbital hydroxylase in male rats. Doxapram (40 mg/kg) caused induction of hepatic drug metabolism typified by increase of hepatic microsomal cytochrome P-450 content and activities of hexobarbital hydroxylase, benzphetamine N-demethylase and ethylmorphine N-demethylase in female rats, but no change in male rats. This data reflected by the results of SDS/polyacrylamide-gel electrophoresis and status of cytochrome P-450 spectra. However, a significant increase 7-ethoxycoumarin O-deethylase and arylhydrocarbon hydroxylase activities in male rats. There were no changes in NADPH-cytochrome c reductase and NADH-cytochrome c reductase activities, and cytochrome b_5 content in both rats. It is possible that the sex-dependent cytochrome P-450 species are selectively sensitive to the action of the doxapram in terms of induction. It is clear that the ability of doxapram to induce the hepatic drug metabolism in rats are sex-related and phenobarbital-like induction.

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Introduction

The liver microsomal monooxygenase system metabolizes a wide variety of endogenous and exogenous substrates: steroids, fatty acids, prostaglandins, environmental chemicals and drugs, including doxapram (1), one of the respiratory stimulant most commonly used in medicine. The activity of the microsomal monooxygenase system is inducible by a large number of chemicals, the best studied being phenobarbital and 3-methylcholanthrene (2), and can be broadly categorized into two major groups, namely phenobarbital-type and 3-methylcholanthrene- or polycyclic hydrocarbon-type on the basis of their ability to preferentially induce different pathways of metabolism. The differential induction has been linked to the ability of the inducers to stimulate the production of one or more isoenzymes of cytochrome P-450 (3, 4).

In most of these studies only one sex of animal has been investigated (usually the male). This is rather unsatisfactory owing to the known sex differences in hepatic microsomal metabolism of drugs seen particularly in the rat. Indeed, when male and female animals have been investigated in induction experiments sex differences have been observed (5). Recently, we found that doxapram increases cytochrome P-450 and related enzyme activities in mice and rats (6-8). For the better understanding of this induction, in the present work was designed to investigate the sex differences in induction caused by doxapram and to attempt to relate the differences to the known sex-dependence of the enzymes under study, which generally has not received much attention in the induction studies.

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 6 and were allowed food and water *ad libitum*. In a preliminary experiment (data not shown), doxapram (20 mg/kg, i.p, daily for 6 days) was shown to induce hexobarbital hydroxylase after 3, 4, 5 and 6 day in a time-dependent manner. Therefore, in subsequent studies doxapram was given intraperitoneally daily for 4 consecutive days and hepatic drug metabolizing activity was determined 24 hr after last injection of doxapram. The control group of animals received equivalent volumes of saline alone. Each experimental group consisted of six animals.

Preparation of liver fraction Doxapram was injected intraperitoneally (i.p.) once daily for 4 consecutive days with a various doses of the drug dissolved in saline (2 ml/100 g). Twently-four hr after the final dose, 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 volumes of cold 0.1 M sodium-potasium phosphate buffer (pH 7.4). The homogenate was centrifugate at 9,000×g for 2 min. and the resulting supernatant fraction served as the enzyme source for the measurement of the activity of hexobarbital hydroxylase. To separate microsomes, the 9,000×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 just prior to the assay of drug metabolism.

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 of 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, aminopyrine, meperidine and ethylmorphine were assayed according to the method of Nash method (9) using 10 μmol of substrate. Aniline hydroxylase was estimated by measuring in p-aminophenol according to Imai *et al.* (10) using 10 μmol of aniline. p-Nitroanisole O-demethylase was estimated by measuring in p-nitrophenol according to Netter and Seidel using μmol of p-nitroanisole (11). The activity of 7-ethoxycoumarin O-deethylase was assayed according to Greenlee and Poland using 10 μmol of 7-ethoxycoumarin (12).

Arylhydrocarbon hydroxylase activity was determined according to the fluorometric assay of Nebert and Gelboin (13). 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 nmoles 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 μmoles NADH, 0.38 μmoles NADPH, 0.6 mg bovine serum albumin, 3.45 μmoles $MgCl_2$, 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 aliquot of the organic layer was extracted with 2.0 ml of 1 N NaOH, and the fluorescence of the extract was measured immediately at 396 nm excitation and 522 nm emission and was compared to the fluorescence of a 3-hydroxybenzo (a) pyrene standard solution.

p-Phenylphenol glucuronyltransferase and p-nitrophenol glucuronyltransferase activities were assayed as described previously (8).

NADPH-cytochrome c reductase and NADH-cytochrome c reductase activities were measured by the method of Philips and Langdon (14) and Mihara and Sato (15), respectively.

Hexobarbital hydroxylase of 9,000×g supernatant was estimated by the method described by Ishikawa *et al.* (16, 17).

Cytochrome P-450 content was determined from the carbon monooxide-induced difference spectrum of dithionite-reduced microsomes assuming a molar extinction coefficient of 91 mM^{-1} cm^{-1} between 450 and 490 nm (18), and cytochrome b_5 content was analyzed by measuring the dithionite-reduced difference spectrum assuming a value of 185 mM^{-1} cm^{-1} for the molar extinction coeffecient between 409 and 424 nm (19), using a Hitachi U-2000 double-beam spectrophotometer. The protein concentration was determined by the method of Bradford (20), using bovine serum albumin as the standard.

SDS-polyacrylamide gel electrophoresis. The polypeptide composition of the microsomal fraction was analysed by sodium dodecyl sulfate (SDS) polyacrylamide slab gel electrophoresis (21) 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 phenobarbital and β-naphthoflavone

for positive control were at dosages of 80 mg/kg/day×2 and 80 mg/kg/day×4, respectively, and microsomes were prepared 24 hr after the last treatment.

Data analyses Data were analyzed 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 the $P<0.05$ level.

Results

Intraperitoneal injection of male and female rats with doxapram resulted in a dose-dependent increased in hepatic microsomal cytochrome P-450 levels, but increase in cytochrome P-450 content was always more preceded and exceeded in the female. This results effectively reflected the increases in hexobarbital hydroxylase of hepatic microsomes caused by doxapram (Fig. 1). Namely, the first significant induction of cytochrome P-450 and hexobarbital hydroxylase by successively administration of doxapram in female and male rats occurred at 20 and 100 mg/kg, respectively. Doxapram did not affect the absorption maximum of the CO-induced spectral changes of cytochrome P-450 in both male and female rats (data not shown).

Data on the effect of successively administration of doxapram (40 mg/kg, i.p., daily for 4 days) on the mixed-function oxidase enzymes system are summarized in Table 1. Doxa-

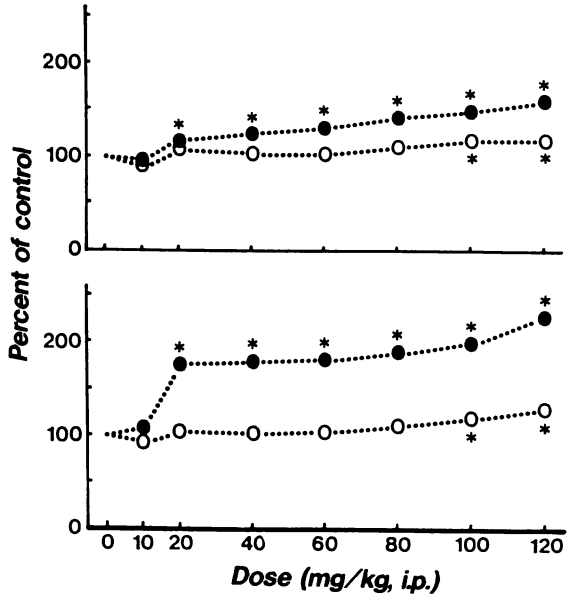

Fig. 1 Dose-dependent increase of cytochrome P-450 content and hexobarbital hydroxylase activity in rats
Male (open symbols) or female (closed symbols) rats were injected i.p. for 4 successive days with doxapram for determination of cytochrome P-450 content (upper panel) and hexobarbital hydroxylase activity (lower panel). Control animals received the saline only. Each point represents the mean of five value compared with control. Significant differences from the control value are indicated as *($P<0.05$).

Table 1 Comparison of Induction by Phenobaribtal, β-naphthoflavone and Doxapram on the Hepatic Drug-metabolizing Enzyme Activities in Female Rats

Parameters	Control	Phenobarbital	β-naphthoflavone	Doxapram
Cytochrome P-450[I]	0.34± 0.020	1.05± 0.084[a]	0.71± 0.019[a]	0.48±0.030[a]
Cytochrome b_5[I]	0.15± 0.005	0.19± 0.015[a]	0.22± 0.016[a]	0.18±0.017
Aminopyrine N-demethylase[II]	73.3 ± 6.15	259.6 ±14.88[a]	57.3 ± 8.571	120.4 ±7.86[a]
Benzphetamine N-demethyase[II]	38.9 ± 5.74	275.8 ±15.18[a]	27.8 ± 7.348	103.2 ±6.04[a]
Ethylmorphine N-demethylase[II]	72.2 ± 8.27	348.9 ±14.88[a]	54.8 ± 6.836	135.0 ±2.80[a]
Meperidine N-demethyalse[II]	165.4 ± 9.08	300.9 ± 9.68[a]	155.7 ± 7.091	202.6 ±8.75[a]
Hexobarbital oxidase[III]	23.2 ± 1.58	61.6 ± 1.88[a]	20.7 ± 0.838	39.4 ±1.18[a]
Aniline hydroxyalse[II]	26.0 ± 1.95	62.6 ± 2.38[a]	24.4 ± 4.373	37.9 ±1.59[a]
Arylhydrocarbon hydroxylase[IV]	0.58± 0.024	0.60± 0.024	0.79± 0.019[a]	0.61±0.011
7-Ethoxycoumarine 0-deethylase[V]	1.14± 0.015	2.20± 0.466	2.57± 0.299[a]	1.15±0.163
p-Phenylphenol glucuronyltransferase[III]	224.6 ±27.86	384.8 ±16.63[a]	236.2 ±32.61	341.8 ±5.72[a]
p-Nitrophenyl glucuronyltransferase[III]	24.4 ± 0.57	23.3 ± 1.87	38.3 ± 0.74[a]	24.0 ±2.05

A group consisting of 6-8 animals was used. Animals were sacrificed 24 hr after the final administration of drugs (phenobarbital 80 mg/kg, *i.p.*, once a day for 2 days; β-naphthoflavone 80 mg/kg and doxapram 40 mg/kg, *i.p.*, once a day for 4 days) and the drug-metabolizing enzyme activities were determined. Control rats were not treated bacause of corn oil (5 ml/kg, *i.p.*) had not effect on the drug-metabolizing enzyme activity. [I] nmol/mg protein, [II] nmol/20 min/mg protein, [III] nmol/60 mg/kg protein, [IV] nmol/10 min/mg protein, [V] nmol/15 min/mg protein. Data represent the mean value±S.E. and were analyzed by Student's *t*-test: [a] $P<0.05$, with respect to the control group.

pram pretreatment caused a marked induction in the metabolism of aminopyrine, benzphetamine, ethylmorphine, meperidine, hexobarbital, aniline and p-phenylphenol glucuronyltransferase and cytochrome P-450 content in the female but was without any significant effect in the male rats. No significant effects are seen on the induction of p-nitroanisole O-

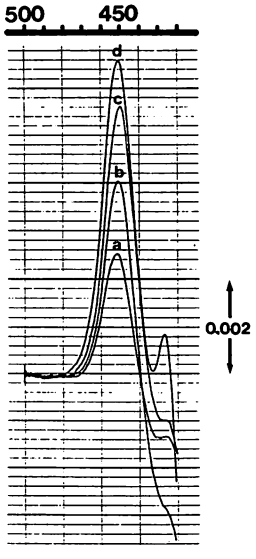

Fig. 2 Status of cytochrome P-450 spectra in doxapram-treated female rats
Animals were sacrificed 24 hr after the final administration of drugs (saline: a, doxpram: b, β-naphthoflavone: c, phenobarbital: d) and total cytochrome P-450 was determined by adding potassium ferricyanide and running the difference spectrum. The experimental conditions were the same as in Table 1.

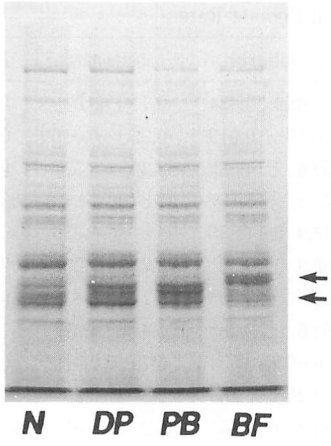

Fig. 3 Results of SDS/polyacrylamide-gel electrophoresis of liver microsomal membrane fraction from doxapram-treated rat
The gel was stained with Coomassie Blue. Gel electrophoresis with saline alone (N), doxapram (DP), phenobarbital (PB) and β-naphthoflavone (BF) were described in the text. The arrow on the right hand side indicate the 53,000 (top) and 50,000 (bottom) molecular weights, respectively. The experimental conditions were the same as in Table 1.

demethylase, 7-ethoxycoumarin O-deethylation, arylhydrocarbon hydroxylase and p-nitrophenol glucuronyltransferase in the female but the male shows selective induction after doxapram pretreatment. However, there were no changes in the liver weight, microsomal protein content, NADPH-cytochrome c reductase, NADH-cytochrome c reductase or cytochrome b_5 content compared with the corresponding values for control rats in both rats.

The data summarized in Fig. 2 confirm that the spectral property of rat pretreated with doxapram are similar to those observed for phenobarbital-induced animals. Cytochrome P-450 levels increased and the reduced cytochrome P-450 : CO binding spectrum peak did not shift from 450 to 448 nm. The ethylisocyanide-difference spectrum 455/428 ratio decreased from 0.59 to 0.5 with the 455 peak and the shift of 428 nm peak is not observed. Like the spectral characteristics of microsmes prepared from phenobarbital-treated rats, the spectral characteristics of microsomes harvested from doxapram-pretreated rats were intermediate to microsomes prepared from the phenobarbital-pretreated rats.

On the other hand, the microsome of rats pretreated with β-naphthoflavone, cytochrome P-450 levels increased and CO binding spectrum peak shifted 2.0 nm downfield from 450 to 448 nm and the ethylisocyanide-difference spectrum 455/428 ratio increased from 0.59 to 1.51 with the 455.0 peak shifted 452.0 and the 428.0 nm peak shifted upfield to 429.4 nm.

SDS-gel electrophoresis of non-treated, doxapram-induced, phenobarbital-induced and β-naphthoflavone-induced rat liver microsomes are shown in Fig. 2. In phenobarbital-induced and β-naphthoflavone-induced rats, 50 K and 53 K bands are deeply stained, respectively, compared with the non-treated rats. In doxapram-treated rats, the bands of phenbarbital induced 50 K band were deeper than those in non-treated rats.

Discussion

In the present investigation, the dose-dependent induction of cytochrome P-450 content and hexobarbital hydroxylase activity by doxapram was observed, and doxapram increases in cytochrome P-450 content without any change in absorption maximum for the CO-induced spectrum of the cytochrome P-450 in male and female rats, but induction was always

effectively more pronounced in the females. There is a clear sex difference in the effect of doxapram.

Furthermore, the sex-dependent monooxygenase (22) (hexobarbital hydroxylase, benzphetamine N-demethylase, ethylmorphine N-demethylase) was effectively induced by doxapram, whereas the sex-independent monooxygenase (23) (p-nitroanisole O-demethylase, 7-ethoxycoumarin O-deethylase, arylhydrocarbon hydroxylase) does not induced in female rats. However, in female rats, the effects of the sucecctive administration of doxapram upon the induction of aniline (sex-independent hydroxylase) were puzzling. Changes in the parameters of drug metabolism as a function of monooxygenase system induction can reflect an alteration in the make up of the cytochrome P-450 pool. The existence of multiple forms of cytochrome P-450 in the rat liver microsomal fraction and changes in the pool composition as a function of induction have been documented (3). SDS-gel microsomal protein patterns and status of cytochrome P-450 spectra from doxapram-treated rats more closely resembled those of phenobarbital-treated than untreated or β-naphthoflavone-treated.

Studies on spectral binding of doxapram to female rats liver microsomes revealed that doxapram is a reverse type I compound characterized by a peak at 415 nm and a trough at 390 nm (17). 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 (24).

Sex differences in the phenobarbital and 3-methylcholanthrene induction in adult rats have been reported (22, 25). Phenobarbital has been found to increase cytochrome P-450 more in male rats than in females, but spironolactone stimulates more female NADPH-cytochrome c reductase (26). Several inducers increase the dealkylation activities of various coumarin derivatives more pronouncedly in females than in males, which consequently results in a decrease of the sex differences (26). In the present study, doxapram treatment seemed to extend the sex difference.

Sex differences exist in the metabolism of the substrates under study (25) and it is interesting to note that the marked sex differences in effects of the inducers are related to the sex-dependent enzymes. Hexobarbital hydroxylase, benzphetamine N-demethylase and ethylmorphnie N-demethylase is higher in the male than in female and it is seen that doxapram increases these activities in the female and dose not induce them in the male. It, thus, seems likely the differences in effects of the inducers in the rats are related to the sex differences in drug metabolism noted previously. It would appear that the sex-dependent cytochrome P-450 species, described by Kamataki *et al.* (26), have different susceptibilities to induction, repression and/or inhibition by the compounds under study, e.g. the N-dealkylation in the male is not inducible by phenobarbital whereas that in the female is inducible.

Because doxapram is an inducer of the hepatic drug-metabolizing enzyme system, it may interact with concomitantly administered drugs by enhancing their metabolism, and thus reduce their therapeutic efficacy or increase their toxicity if their metabolic products are less effective or more toxic than the parent compound.

In conclusion, sex differences exist in the effect of doxapram on aminopyrine N-

demethylase, benzphetamine N-demethylase, ethylmorphine N-demethylase, meperidine N-demethylase, hexobarbital hydroxylase and p-phenylphenol glucuronyltransferase. These differences are related to the sex-dependence of hepatic drug metabolism in the rat. The effects seen may be related to sex specific induction, inhibition or repression of the enzymes involved. Studies on the possible mechanism for these increases are now in progress.

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