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Induction of propranolol metabolism by the azo dye sudan III in rats

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Abstract—Effects of the azo dye sudan III, an inducer of cytochrome P450 isozymes belonging to the CYP1A subfamily, on propranolol (PL) *in vitro* and *in vivo* metabolism were investigated in rats. The kinetic parameters of the activity for each metabolic pathway were determined in liver microsomes from control and sudan III-treated rats. Sudan III pretreatment increased extensively PL 4-hydroxylase, 5-hydroxylase and *N*-desisopropylase activities at high but not at low PL concentrations. On the other hand, kinetic parameters of 7-hydroxylase activity were not affected by sudan III pretreatment. Sudan III pretreatment decreased blood concentrations of PL after intraportal infusion of PL at high doses (12.5 and 20 mg/kg), but not at a low dose (5 mg/kg). These observations were consistent with data obtained from the *in intro* studies showing that sudan III pretreatment induced low-affinity but not high-affinity cytochrome P450 isozymes involved in PL metabolism in rat liver microsomes.

Major metabolic pathways of propranolol (PL*) in rat liver microsomes are hydroxylations at the 4-, 5- and 7-positions of the naphthalene ring and *N*-dealkylation of the propanolamine side chain [1, 2]. Of these pathways 4-hydroxylation is common to rats, dogs and humans [3]. Previous studies have shown that PL metabolism in rat liver subcellular fractions is induced by phenobarbital [4, 5], 3-MC [5] or sudan III [5]. The pretreatment of rats with 3-MC or sudan III increased markedly the formation rate of ND-PL in liver microsomes, but phenobarbital treatment caused a less marked induction [5]. None of the inducers, however, affected the PL 4-hydroxylase activity.

In connection with the regioselective induction of PL metabolism, cigarette smoking has been shown to increase the oral clearance of PL in human subjects selectively by induction of side-chain oxidation without affecting the aromatic ring oxidation of PL [6]. The mechanism for this selective effect is assumed to involve differential induction of the P450 monooxygenase system by PAHs, which are components of cigarette smoke.

Sudan III has been shown in rats to induce P450 isozymes which are very similar to those induced by a PAH, 3-MC, with regard to their substrate specificity, electrophoretic pattern and immunochemical inhibition [7–9]. This indicates that sudan III as well as 3-MC is an inducer of P450 isozymes belonging to the CYP1A subfamily [7, 10]. Since sudan III is not a carcinogen, it may be safer than carcinogenic 3-MC as a CYP1A subfamily inducer for use in the laboratory. Therefore, in this study we present *in vitro* and *in vivo* inducing effects of sudan III on PL metabolism in rats.

* Abbreviations: PL, propranolol; 3-MC, 3-methylcholanthrene; X-HO-PL, X-hydroxy propranolol; ND-PL, *N*-desisopropyl propranolol; PAH, polycyclic aromatic hydrocarbon.

Materials and Methods

Chemicals. The sources of reagents were: PL and procaine from the Sigma Chemical Co. (St Louis, MO, U.S.A.); 4-HO-PL from the Sumitomo Chemical Ind. (Osaka, Japan); ND-PL from the ICI Pharmaceutical Co. (Macclesfield, U.K.); *N*-ethyl-PL from Sankyo Co. (Tokyo, Japan) (all were hydrochlorides); sudan III from the Wako Chemical Co. (Osaka, Japan); NADPH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase from the Oriental Yeast Co. (Tokyo, Japan). 5-HO- and 7-HO-PLs were synthesized as hydrochlorides by the published method [11] with minor modifications. Other chemicals were of analytical grade.

Preparation of microsomes. Male Wistar rats (12 weeks old) were given i.p. sudan III (40 mg/kg) dissolved in corn oil or corn oil alone as vehicle control for 4 days. The rats were killed by decapitation 24 hr after the last injection, and liver microsomes were prepared by the method of Omura and Sato [12]. Protein concentrations were determined by the Lowry method [13]. P450 contents and drug-metabolizing enzyme activities for prototypical substrates in liver microsomes (vehicle control rats vs sudan III-treated rats) were: P450 contents, 0.66 ± 0.05 vs 1.32 ± 0.09 nmol/mg protein; 7-ethoxycoumarine *O*-deethylase, 0.24 ± 0.04 vs 2.83 ± 0.04 nmol/min/mg protein; hexobarbital hydroxylase, 0.22 ± 0.03 vs 0.20 ± 0.03 nmol/min/mg protein.

Assay methods of enzymatic activities. The metabolic activities of four PL oxidative pathways in rat liver microsomes were assayed by HPLC as reported previously [14] except for incubation time (1 min) and mobile phase [acetonitrile/methanol/water/acetic acid (35.0:18.8:46.3:0.23, v/v/v/v)].

In vivo *studies.* Male Wistar rats were pretreated by sudan III or control vehicle as described in the liver microsomal study. On day 5, the rats were anesthetized lightly with ether. A polyethylene cannula (PE-50) filled with 0.9% (w/v) NaCl containing heparin (30 IU/mL) was inserted into the femoral artery for blood sampling. Another cannula (PE-10) for PL administration was inserted into the ileocholic vein. PL (5, 12.5 and 20 mg/kg) dissolved in 0.6 mL of 0.9% (w/v) NaCl or control vehicle was infused at constant rates for 50 min into the ileocholic vein. Blood sample (0.1 mL) was drawn from the femoral artery and was hemolysed in 1 mL of the heparin-containing saline. After adding *N*-ethyl-PL (40 ng) as internal standard, 1 M carbonate buffer (1 mL, pH 9.8) and 1.5% (v/v) isoamyl alcohol in heptane (3 mL), the sample was vortexed for 1 min and centrifuged (1500 *g*, 10 min). To the organic phase transferred to another tube, 0.01 N HCl (0.3 mL) was added, and the mixture was vortexed for 1 min. After centrifugation (1500 *g*, 10 min), the organic phase was removed and the aqueous phase was analysed by the following HPLC method.

HPLC conditions. The HPLC equipment for *in vivo* studies was the same as that for *in intro* studies. Other conditions were: column, an Unisil Pack F-3 50A (3 mm × 50 mm, Gasukuro Kogyo, Tokyo, Japan); mobile phase, acetonitrile/methanol/water/acetic acid (30:25:45:0.2, v/v/v/v); flow rate, 1 mL/min; fluorescence detection wavelength, 295 (excitation) and 340 nm (emission).

Analysis of kinetic data. The kinetic parameters were calculated by fitting experimental data to monophasic or biphasic Michaelis–Menten equations using a non-linear least-squares regression based on a simplex method [15]. Statistical significance was calculated by the Student's *t*-test.

Results and Discussion

In metabolic studies using rat liver microsomes, the initial rate of PL 4-hydroxylation measured over 2.5 min loses its linearity with respect to incubation time. This loss of linearity is probably due to further metabolism of 4-HO-PL, because 4-HO-PL disappears rapidly from the incubation mixture [16]. Therefore, in the following *in intro* kinetic studies the reaction was carried out for 1 min.

The kinetic parameters for the activities of main metabolic pathways in liver microsomes of the control and sudan III-treated rats are listed in Table 1. The formation of 4-HO- and 5-HO-PLs was not expressed by a single Michaelis–Menten equation in either control or sudan III-treated rats, and showed biphasic kinetics over the substrate concentration range of 0.1–700 μM. The formation of ND-PL in sudan III-treated rats also showed biphasic kinetics. On the other hand, the formation of 7-HO-PL in both control and sudan III-treated rats, and that of ND-PL in control rats exhibited monophasic kinetics.

Compared with the kinetic parameters from control rats, K_{m_1} and V_{max_1} values of 4- and 5-hydroxylations in sudan

Table 1. Kinetic parameters of PL metabolism in liver microsomes from control and sudan III-pretreated rats

Metabolic pathway	Parameter			
	K_{m_1} (μM)	V_{max_1} (nmol/min/mg)	K_{m_2} (μM)	V_{max_2} (nmol/min/mg)
4-Hydroxylation	0.314 ± 0.069 (0.174 ± 0.029)	0.326 ± 0.058 (0.393 ± 0.034)	52.4 ± 5.5 (194 ± 43)	1.68 ± 0.20† (0.559 ± 0.056)
5-Hydroxylation	0.093 ± 0.023 (0.046 ± 0.012)	0.048 ± 0.004 (0.039 ± 0.002)	35.9 ± 0.7 (67.6 ± 26.2)	0.289 ± 0.040* (0.057 ± 0.009)
N-Desisopropylation	1.14 ± 0.54	0.049 ± 0.012	48.8 ± 2.4* (109 ± 12)	2.71 ± 0.21* (0.651 ± 0.025)
7-Hydroxylation	0.209 ± 0.024 (0.182 ± 0.037)	0.253 ± 0.023 (0.213 ± 0.008)		

Data are shown as means ± SE for three different microsomes obtained from three rats. Values in parentheses are for control rats.

The subscript numbers 1 and 2 denote values for a high- and low-affinity enzyme(s), respectively.

* and †, significantly different from control values ($P < 0.05$ and 0.01, respectively).

Table 2. Effects of sudan III pretreatment on pharmacokinetic parameters of PL after intraportal infusion

Dose (mg/kg)	Pretreatment	AUC (ng min/mL)	C_{max} (ng/mL)
5	Control	43.9 ± 15.0	604 ± 195
	Sudan III	37.0 ± 8.3	535 ± 125
12.5	Control	83.2 ± 13.6	1104 ± 231
	Sudan III	26.7 ± 7.1*	392 ± 72*
20	Control	281.9 ± 26.6	3451 ± 65
	Sudan III	120.3 ± 20.8†	1628 ± 210†

PL was given for 50 min by constant-rate intravenous infusion. Data are shown as means ± SE for three rats.
* and †, significantly different from control values ($P < 0.05$ and 0.01, respectively).

III-treated rats did not alter significantly; nor did the K_m and V_{max} values of 7-hydroxylation (Table 1). On the other hand, the V_{max_2} values of 4- and 5-hydroxylations, and N-desisopropylation increased markedly in liver microsomes from sudan III-treated rats. The K_{m_2} values of 4- and 5-hydroxylations in sudan III-treated rats tended to be lower than those in the control group, but these differences were not significant. However, the K_{m_2} value of N-desisopropylation was decreased in liver microsomes from sudan III-treated rats compared with those from control rats. A high-affinity phase was observed in N-desisopropylation for sudan III-treated rats, but the V_{max_1} value was extremely low. The results obtained in this microsomal study indicate that two groups of P450 isozymes having low and high affinities participate in the metabolism of PL, and that sudan III pretreatment induces the low-affinity enzymes rather than the high-affinity enzymes in control rats. In other words, sudan III pretreatment enhances PL metabolism at high PL concentrations except for 7-hydroxylation. Failure to observe PL 4-hydroxylase induction by sudan III in the previous report [5] probably arose from the rapid metabolism of 4-HO-PL and the non-specific assay for 4-HO-PL.

Blood PL concentrations of control and sudan III-treated rats were determined following intraportal infusion at PL doses of 5, 12.5 and 20 mg/kg. The AUC and C_{max} values of sudan III-treated rats were reduced at doses of 12.5 and 20 mg/kg compared with those of control rats, but not at a dose of 5 mg/kg (Table 2). This observation is consistent with the results obtained from *in vitro* studies described above; PL metabolic formation at high but not low substrate concentrations was enhanced significantly in liver microsomes from rats pretreated with sudan III. It seems from these data that oxidative metabolism of PL was induced by the pretreatment of rats at doses of 12.5 and 20 mg/kg in sudan III-treated rats, but not at a dose of 5 mg/kg.

Since cigarette smoke contains many PAHs, it is thought that the effect of cigarette smoking on blood levels of PL is due to the induction of the CYP1A subfamily by PAHs. Walle *et al.* [6] observed the reduction of plasma PL concentrations of cigarette smoking subjects after a single 80-mg oral dose of PL compared with those of non-smoking subjects. The plasma PL concentrations (<100 ng/mL) of non-smoking subjects were much lower than those after the lower dose (5 mg/kg) in rats (Table 2), where the effect of induction was not observed. These findings suggest that P450 isozyme(s) belonging to the CYP1A subfamily induced in humans by smoking may have a larger V_{max}/K_m value for PL metabolism than those induced in rats by sudan III. In addition, it should be noted that the selective induction of N-desisopropylation in smoking subjects [6] was in contrast with our microsomal induction data in rats, where V_{max_2} values of ring-hydroxylations as well as N-desisopropylation were increased markedly by sudan III.

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