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# An alteration in the liver microsomal membrane of the rat following paracetamol overdose

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Fluorescence polarization of dansyl chloride covalently labelled microsomal membranes revealed an alteration in rat liver microsomal membranes following as a consequence of a paracetamol overdose. This suggests that dansyl chloride would be a useful probe in the study of the effects of paracetamol on microsomal membranes.

Large doses of paracetamol induce hepatic necrosis in man (Davidson & Eastham 1966; Rose 1969) and animals (Boyd & Bereczky 1966; Dixon et al 1971). Chronic liver disease due to smaller amounts occurs when the drug is ingested over a long period (Bonkowsky 1978; Olsson 1978). The hepatotoxicity has been reported as being related to the covalent binding of an electrophilic metabolite of the drug, mediated by cytochrome P450, to tissue macromolecules (Mitchell et al 1973; Dahlin et al 1984). Recently, lipid peroxidation induced by a decrease in glutathione in the liver has been pointed out as a cause of the drug's hepatotoxicity (Fairhurst et al 1982; Albano et al 1983). Such factors may possibly influence the microsomal membrane structure, but the drug's effect on the physical structure of these membranes has not been reported.

We have therefore examined the in-vivo effects of an overdose of paracetamol on the membranes of rat liver microsomes using fluorescence polarization.

*Materials and methods*

Paracetamol and glutamic oxalacetic acid transaminase (GOT) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). NADPH-4$H_2O$, dansyl chloride (DNSCl), and fluorescein isothiocyanate (FITC) were purchased from Sigma Chemical Co. (St Louis, MO, USA). 2-(9-Anthroyloxy)stearic acid (2-AS), 12-(9-anthroyloxy)stearic acid (12-AS) and eosin-5-maleimide (EM) were purchased from Molecular Probes Inc. (Junction city, OR, USA). 8-Anilino-1-naphthalene sulphonate(ANS) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Tokyo Kasei Co. Ltd (Tokyo, Japan). Ethoxybenzamide was supplied from Yoshitomi Co. Ltd. (Osaka, Japan). All other reagents were of analytical grade. The Centriflo ultrafiltration membrane cone was purchased from Amicon Co. (Danvers, MA, USA).

Paracetamol (1·8 g) and carboxymethylcellulose (0·12 g) were mixed in 4·5 mL of saline (150 mmol $L^{-1}$), sonicated and the suspension given i.p. at a dose of 750 mg $kg^{-1}$ to male Wistar rats, 170–250 g. Saline, 0·5 mL, was given i.p. to the controls. 36 h later, just before the rats were killed, serum GOT was determined according to Karmen et al (1955) to measure the extent of the drug-induced hepatotoxicity.

The rats were fasted for about 12 h before the preparation of the microsomes, anaesthetized with ether, injected with heparin sodium (200 units in 0·2 mL), and then the liver was perfused through the portal vein with ice-cold 0·9% NaCl (saline) and excised. The isolated livers were minced with scissors and homogenized in isotonic KCl solution. The homogenate was centrifuged at 9 000 g for 20 min at 4 °C, then the supernatant was centrifuged at 105 000 g at 4 °C. The microsomal pellet obtained was washed and resuspended in 0·1 M sodium phosphate buffer, pH 7·4. The protein concentration of microsomes was determined by the method of Lowry et al (1951) using bovine serum albumin as the standard.

Labelling with the fluorescent probes, ANS, 2-AS, 12-AS, DPH, DNSCl, EM and FITC was by adding a small amount of each probe in solution to the microsomal suspension and incubating for 20–30 min at 37 °C. The unbound fluorescent probes, except ANS, were removed by centrifugation using a Centriflo ultrafiltration membrane cone. The unbound ANS was not removed, since it did not fluoresce in water.

Fluorescence polarization measurements were made as described previously (Kajii et al 1986), using a Hitachi fluorescence spectrophotometer 650-60.

The effects of paracetamol overdose on cytochrome P450 activity were also studied. The microsomal suspension (6 mg protein $mL^{-1}$) to which various concentrations of ethoxybenzamide as a substrate and 5 mM $MgCl_2$ were added, was incubated for 3 min at 37 °C, then 1 mM NADPH was added, followed by incubation for 5 min at 37 °C. The reaction was then stopped by addition of 6 M HCl. The salicylamide produced was determined and the oxidation activity was expressed as the de-ethylation rate of ethoxybenzamide to salicylamide in nmol mg $protein^{-1}$ $min^{-1}$, as previously reported (Lin et al 1980). The data were fitted to the Michaelis-Menten equation by a non-linear least squares computer program (MULTI program, Yamaoka et al 1981).

*Results and discussion*

Serum GOT activity was 324·4 ± 31·6 iu$L^{-1}$ (mean ± s.d.) in the treated rats (n = 4) and 52·8 ± 2·4 iu$L^{-1}$ in

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the control rats (n = 5). The increase in serum GOT activity by a factor of 6 is a clear indication of the hepatotoxicity of a paracetamol overdose.

The effects of the overdose on the rat liver microsomal membranes were studied by measuring the fluorescence polarization of fluorescent probe-labelled microsomes. The DNSCl-labelled microsomes showed a significant increase in fluorescence polarization compared with the controls (Fig. 1). This increase suggests

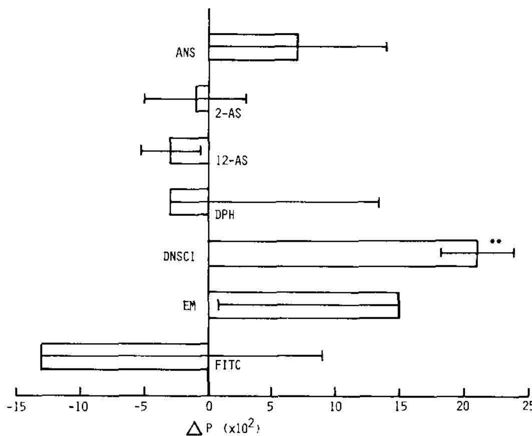


FIG. 1. Effect of paracetamol overdose on fluorescence polarization of liver microsomal membranes labelled by fluorescent probes. ΔP = (fluorescence polarization of fluorescent probe-labelled liver microsomes of treated rats) minus (fluorescence polarization of fluorescent probe-labelled liver microsomes of control rats). Results were obtained from 10 to 13 rats and expressed as means ± s.d. Excitation (Ex) and emission (Em) wavelengths for fluorescent probe, (Ex, Em in nm): ANS (380, 480); 2-AS (395, 455); 12-AS (395, 455); DPH (360, 430); DNSCl (420, 525); EM (527, 553); FITC (492, 527). The light emitted was passed through a 430 nm cut-off filter for ANS, 2-AS and 12-AS, a 390 nm filter for DPH, a 450 nm filter for DNSCl, a 530 nm filter for EM and a 510 nm filter for FITC. ** Fluorescence polarization of treated rat microsomes differed significantly from that of untreated ones ($P < 0{\cdot}01$).

that the mobility of the microsomal membrane is reduced by paracetamol-induced hepatotoxicity. The oxidation activity of the cytochrome P450 in liver microsomes, determined by the de-ethylation rate of ethoxybenzamide to salicylamide, also decreased following the overdose (Table 1) as was also found by Willson & Hart (1977). The maximum velocity, $V_{max}$, for the oxidation activity of control microsomes was 18 nmol mg protein$^{-1}$ min$^{-1}$ and that of DNSCl-labelled microsomes was 1·2 nmol mg protein$^{-1}$ min$^{-1}$. Thus, DNSCl labelling decreased the maximum velocity of oxidation. The microsomal cytochrome P450-dependent mixed function oxidases may be related to a moiety of the membrane which undergoes such perturbation and thus, the information which DNSCl provides may be related to the microsomal mixed function oxidase

Table 1. Effect of an overdose of paracetamol on the oxidation activity of liver microsomal cytochrome P450.

| | $K_m$ (mM) | $V_{max}$ (nmol (mg$^{-1}$ protein)$^{-1}$ min$^{-1}$) |
|---|---|---|
| Control rats (n = 4) | 0·67 ± 0·12 | 17·09 ± 2·22 |
| Paracetamol-treated rats (n = 4) | 0·92 ± 0·38 | 7·96 ± 1·28** |

The de-ethylation rate of ethoxybenzamide to salicylamide was determined in rat liver microsomes 36 h after i.p. injection of paracetamol (750 mg kg$^{-1}$). Controls were given 0·5 mL saline i.p. The values represent the mean ± s.d. of four rats. ** The value differs significantly ($P < 0{\cdot}01$) from that of the untreated rats.

system. The lipid-soluble probes 2-AS, 12-AS and DPH failed to monitor any alteration in membrane lipid domains following the overdose. This may be due to a protection system in-vivo, such as glutathione, glutathione peroxidase and catalase.

In conclusion, the liver microsomal membranes of rats given an overdose of paracetamol underwent an alteration in their structure. This was demonstrated by the fluorescence polarization of DNSCl-labelled microsomes, suggesting DNSCl to be a useful probe for studying the effect of paracetamol on the liver microsomal membrane.

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# High capacity for pulmonary first-pass elimination of propranolol in rats

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Plasma propranolol concentrations after i.v. and i.a. 1, 2·5, 5 and 10 mg $kg^{-1}$ doses of the drug given intravenously or intra-arterially have been compared in 7-week-old male Wistar rats. The areas under the curves after i.a. dosing were almost twice those after i.v. dosing at any dose, despite the elimination half-lives being the same. The difference in total body clearance after i.a. dosing from that after i.v. dosing indicated a significant contribution by pulmonary clearance, which ranged from about 20 to 30 mL $min^{-1}$ $kg^{-1}$, to the overall first-pass elimination after the i.v. administration. The pulmonary extraction ratio was approximately 0·4 to 0·5 at the i.v. doses used. Mean pulmonary transit time was estimated to be about 1 to 2 min. There was no dose-dependence in the pulmonary first-pass elimination kinetics of propranolol.

Propranolol has been thought to be eliminated predominantly by the liver in both man and animals (Shand et al 1971; Shand & Rangno 1972; Shand et al 1972). However, the recent reports have demonstrated that it is also extensively taken up by dog (Pang et al 1982) and rat (Schneck et al 1977; Rikihisa et al 1981) lung after the direct injection into the pulmonary artery or i.v. injection via the tail vein. Furthermore, the hepatic clearance of propranolol after in-vitro perfusion has been found to be substantially lower than the in-vivo total body clearance in rats (Iwamoto et al 1986). However, there have been no reports showing direct evidence of pulmonary first-pass elimination after its i.v. administration. We have compared plasma propranolol levels after i.v. and i.a. administration at 1, 2·5, 5 and 10 mg $kg^{-1}$ in 7-week-old rats to estimate pulmonary clearance, extraction ratio and mean transit time during the first-passage through the lung.

*Methods*

Male Wistar rats (7-week-old, 210–225 g) were chronically cannulated into both jugular vein and artery with silicone polymer tubing (i.d. 1·0 mm; o.d. 1·5 mm, Dow Corning) and fasted overnight. The intravascular end of the venous cannula was inserted into the right atrium as reported by Iwamoto et al (1982) and that (with 1·2 cm tip of bevelled PE-50) of the arterial cannula was inserted into the right pulmonary vein.

Unanaesthetized, chronically cannulated rats (n = 4) were given propranolol (ICI) at 1, 2·5, 5 or 10 mg $kg^{-1}$ either intravenously (via jugular vein) or intra-arterially (via pulmonary vein). Each rat was sham-injected with the same volume (1 mL $kg^{-1}$) of 0·9% NaCl (saline) into an alternative cannula. Periodic blood samples (approximately 0·12 mL at nine time points) were withdrawn from the venous cannula into heparinized micro tubes over 2 h. Plasma (0·05 mL) propranolol concentration was determined according to Iwamoto & Watanabe (1984).

Plasma propranolol concentration (C)-time curves were analysed according to the least-squares regression analysis program MULTI (Yamaoka et al 1981) for a bi-exponential decline expressed as $C = Ae^{-\alpha t} + Be^{-\beta t}$, where A, B, $\alpha$ and $\beta$ are hybrid parameters. Area under the concentration-time curve (AUC) was estimated by the equation, $AUC = A/\alpha + B/\beta$. Total body clearance ($CL_{tot}$) was estimated by the equation,

$$CL_{tot} = dose/AUC.$$

Pulmonary clearance ($CL_p$) and extraction ratio ($E_p$) were calculated by the equations,

$$CL_p = (CL_{tot})_{i.v.} - (CL_{tot})_{i.a.}$$

and

$$E_p = 1 - (AUC)_{i.v.}/(AUC)_{i.a.},$$

respectively, where the subscripts i.v. and i.a. stand for the intravenous and intra-arterial administration, respectively. Mean residence time (MRT) after i.v. or i.a. dosing was estimated by the equation,

$$MRT \int_0^\infty t\,C\,dt/AUC.$$

Mean transit time in the lung ($MTT_p$) was calculated by the equation,

$$MTT_p = (MRT)_{i.v.} - (MRT)_{i.a.}.$$

*Results and discussion*

Fig. 1 represents plasma propranolol concentration-time profiles after i.v. and i.a. administration at

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