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Different effects of carbon tetrachloride toxicity and cirrhosis on substrate binding to rat hepatic microsomal cytochrome P-450

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In humans, impaired drug metabolism is often associated with hepatic disease, especially cirrhosis, and this may lead to toxic complications of drug therapy [1]. Levels of hepatic cytochrome P-450, the principal component of the mixed function oxidase system which is active in drug metabolism, are lowered in experimental animals rendered cirrhotic by chronic exposure to carbon tetrachloride (CCl_4) [2]. However, attempts to use this model to study altered drug metabolism in cirrhosis may be complicated by the acute destructive effects of CCl_4 on cytochrome P-450 [3]. In the present study, the binding of representative Type I and Type II substrates [4] to cytochrome P-450 has been examined in cirrhotic and control rat liver microsomes. In addition, the binding changes observed in cirrhotic liver have been compared with those found in microsomes from rats acutely exposed to CCl_4.

Methods

Animals. Hepatic cirrhosis was produced in male Wistar rats (from the Institute of Clinical Pathology and Medical Research, Westmead, N.S.W.) receiving drinking water containing 0.1% sodium phenobarbitone (PB) by exposure to CCl_4. This was administered by inhalation twice weekly for ten weeks, essentially as described elsewhere [5]. Animals were sacrificed ten days after the final exposure to CCl_4 and PB. Control animals received PB-water for the same period of time as cirrhotic rats.

Acute CCl_4 intoxication was produced in rats receiving PB-water by three exposures, for a period of 10 min each, in six days. Unlike the procedure used to generate cirrhosis, acutely poisoned rats were not permitted to recover after the third exposure to CCl_4. Instead, PB was stopped, access to normal drinking water was restored, and forty-eight hours later rats were killed and microsomal fractions were prepared. Controls received PB for the same length of time.

Preparation of microsomal fraction. Washed hepatic microsomal fractions were prepared by differential centrifugation as described previously [6]. Microsomes were stored as pellets at −20° and were used within three weeks of preparation.

Assays. Cytochrome P-450 was determined by the method of Omura and Sato [7] employing an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ for the cytochrome P-450 ferrous carbonyl spectral complex.

Difference spectra. Optical difference spectra were determined in an Aminco Chance DW-2a spectrophotometer operating in the split beam mode. Microsomal pellets were resuspended in 0.1 M potassium phosphate buffer (pH 7.4) to a final protein concentration of 1 mg/ml, divided equally between two cuvettes, and a baseline of equal light absorbance established. Ethylmorphine HCl (McFarlan Smith Ltd., Edinburgh, Scotland) or metyrapone (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) were selected as examples of Type I and Type II substrates, respectively, and were introduced to the sample cuvette in microlitre quantities from stock solutions in 0.1 M phosphate buffer. The difference spectrum was recorded between 380 and 500 nm. Spectral dissociation constants (K_s; μM) and maximal absorbance changes (ΔA_{max}; absorbance units/nmoles cytochrome P-450) were determined from the ordinate and abscissa intercepts, respectively, of double reciprocal plots of ligand concentration vs ΔA (peak to trough; 386–417 nm for ethylmorphine and 426–392 nm for metyrapone; 4–6 ligand concentrations).

As changes in affinity (K_s) and capacity (ΔA_{max}) may occur independently, the ratio of $\Delta A_{max}/K_s$ was calculated to assess the net effect of substrate binding alterations. The significance of this ratio, the efficiency of binding, is analogous to that which the ratio of V_{max}/K_m has in catalytic studies [9].

Statistics. Differences between mean values (K_s, ΔA_{max} and cytochrome P-450 content) were assessed using the unpaired Student's *t*-test (two-tailed).

Results and discussion

Changes in substrate binding to cirrhotic microsomes. Changes in the binding of Type I (EM) and Type II (MET) substrates to cytochrome P-450 varied between microsomes from cirrhotic rat liver and those from rat liver acutely poisoned with CCl_4 (Table 1). In cirrhotic liver microsomes, the K_s values for both EM and MET were identical to those in control liver microsomes. In contrast, there was a significant decrease in the extent of the interaction (ΔA_{max}) of both compounds in cirrhotic microsomes compared with controls. Thus, it is apparent that the affinity of substrate binding to oxidised cytochrome P-450 is unaltered in hepatic cirrhosis but that the proportion of cytochrome P-450 undergoing the spectral interaction is reduced.

The extent of the decrease in substrate binding capacity (ΔA_{max}) was 35% for the Type I substrate (EM) and 20% for the Type II compound (MET) (Table 1). The percentage decrease in binding capacity for the Type I interaction is almost twice that of the decrease in the Type II interaction, a finding that supports the assertion that the microsomal Type I and Type II sites are distinct. It seems apparent that the two binding sites (apoprotein and haem sites) are affected to a different extent by the cirrhotic process.

The values of the ratio $\Delta A_{max}/K_s$ for both Type I and Type II interactions with cytochrome P-450 in cirrhotic rat liver microsomes were lower than those in control microsomes. This reflects the reduced capacity of the cirrhotic

Table 1. Cytochrome P-450 content and spectral binding of ethylmorphine and metyrapone in hepatic microsomal fractions from differently treated rats*

Microsomal type†	Cytochrome P-450 content	Substrate: Ethylmorphine			Substrate: Metyrapone		
		(K_s)	$(\Delta A_{max} \times 10^2)$	$\left(\frac{\Delta A_{max}}{K_s}\right)$	(K_s)	$(\Delta A_{max} \times 10^2)$	$\left(\frac{\Delta A_{max}}{K_s}\right)$
Control	1.21 ± 0.07	21 ± 2	1.69 ± 0.06	805	4.7 ± 0.8	3.49 ± 0.24	7430
Cirrhotic	0.99 ± 0.07‡	21 ± 3¶	1.09 ± 0.14‖	519	4.9 ± 1.0¶	2.76 ± 0.26‖	5630
PB-induced	1.92 ± 0.05	5.2 ± 1.3	0.65 ± 0.19	1250	1.7 ± 0.2	3.32 ± 0.09	19500
PB-induced + acute CCl_4	0.86 ± 0.12‡	3.2 ± 0.9‖	0.40 ± 0.10§	1250	2.6 ± 0.4‖	2.72 ± 0.15‖	10500

* Values are mean ± S.D. from 4 individual microsomal preparations. Each determination was made from spectral change titrations using four to six different substrate concentrations within the range 1–30 μM (ethylmorphine) or 1–32 μM (metyrapone). Units are: cytochrome P-450, nmoles/mg protein; K_s, μM; ΔA_{max}, absorbance units/nmole cytochrome P-450; $\Delta A_{max}/K_s$, absorbance units/nmole cytochrome P-450/μM.
† Difference between means compares "cirrhotic" to "control" microsomes, and "PB-induced plus acute CCl_4" to "PB-induced".
‡ Difference between means significant at the 0.001 level.
§ Difference between means significant at the 0.05 level.
‖ Difference between means significant at least at 0.02 level.
¶ Difference between means not significant at 0.05 level.

rat liver to bind drug substrates and could account for the lowered metabolism in cirrhosis that has been documented by others [5].

Effect of PB on substrate binding to cytochrome P-450. Induction of cytochrome P-450 by PB produced an increase in binding affinity for both EM and MET of around four- and three-fold, respectively (Table 1). In contrast the ΔA_{max} value for EM binding was lower in PB-induced than in control microsomes; the ΔA_{max} for MET binding was similar in both types of microsomes.

Effect of acute CCl_4 exposure in vivo *on substrate binding to cytochrome P-450* in vitro. The acute exposure of rats to CCl_4 had a complex effect on substrate binding to microsomal cytochrome P-450 *in vitro*. In the case of EM, the K_s and ΔA_{max} values were both lower in microsomes from rat liver after acute CCl_4 exposure than in microsomes from PB-induced rat liver; the ratio $\Delta A_{max}/K_s$ had the same value in both types of microsomes (Table 1). In the case of MET, the Type II compound, acute *in vivo* exposure of rats to CCl_4 decreased the extent of the binding interaction (ΔA_{max}) relative to that in PB-induced microsomes and also decreased the binding affinity (Table 1). The ratio $\Delta A_{max}/K_s$ in microsomes isolated from rat liver after acute CCl_4 exposure was approximately half that in PB microsomes. Thus it appears that the proportion of cytochrome P-450 involved in substrate binding is decreased in acute CCl_4 poisoning but changes in the affinity of binding are dependent upon the particular substrate.

Studies using SDS-polyacrylamide gel electrophoresis have shown that a major PB-inducible form of cytochrome P-450 is preferentially lost from rat liver microsomes after acute exposure to CCl_4 [10]. The decreased affinity of Type II binding is consistent with destruction of cytochrome P-450 near the haem moiety, particularly as the PB-inducible form demonstrates a high affinity for MET [11]. The increased affinity of EM for cytochrome P-450 in hepatic microsomes isolated after acute exposure to PB and CCl_4 is not as readily accounted for as the effect observed with MET, but lipid factors appear important in the metabolism of Type I compounds [12] and CCl_4 alters the microsomal lipid content via a peroxidative effect [13–15]. Consequently changes in the K_s and ΔA_{max} for EM binding in microsomes from acutely poisoned rat liver may reflect an altered lipid environment near the Type I binding site.

General discussion

A previous study by Tsyrlov and Lyakhovich documented the effects of the chronic exposure of rats to CCl_4 on the binding of Type I and Type II compounds with ferricytochrome P-450 [16].

These workers reported changes in both K_s and ΔA_{max} values but it is significant that, in the protocol used to generate experimental cirrhosis, the animals were permitted only two to three days to recover after the final CCl_4 treatment. It is evident from the present data that changes observed two days after CCl_4 administration are quite different from those in cirrhotic rats permitted ten days to recover. Clearly, in studies of experimental cirrhosis, the time period over which the animals recover should be carefully chosen so that the residual acute effects of the last CCl_4 and PB treatments are minimised.

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Effect of combinations of deoxyguanosine and 8-aminoguanosine with 2,3-dihydro-1*H*-imidazo[1,2-*b*]pyrazole on L1210 cell crowth in culture

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Since ribonucleotide reductase catalyzes the rate-limiting step in the formation of the 2′-deoxyribonucleoside 5′-triphosphate precursors of DNA, this pathway represents a controlling step in DNA replication. Lowe and Grindley [1] reported that deoxyguanosine inhibits the growth rate of L1210 cells in culture and that this inhibition of cell growth is correlated with decreased intracellular levels of dTTP and dCTP. While deoxyguanosine has been shown to be toxic to cells, high concentrations are required because of the rapid phosphorylysis of deoxyguanosine by purine nucleoside phosphorylase [2]. Kazmers *et al.* [3] showed that, by selective inhibition of purine nucleoside phosphorylase with 8-aminoguanosine in intact cells, the toxicity of deoxyguanosine in T lymphoblasts is greatly enhanced. This is correlated with a marked increase in the dGTP levels in these cells. B lymphoblasts which are not sensitive to the combination of deoxyguanosine plus 8-aminoguanosine do not show elevations in intracellular dGTP. Stoeckler *et al.* [4] showed that 8-aminoguanine and 8-aminoguanosine are very effective inhibitors of purine nucleoside phosphorylase and that in combination with guanosine or deoxyguanosine lead to increased intracellular pools of GTP or dGTP respectively.

We have shown previously that mammalian ribonucleotide reductase consists of two non-identical protein components [5], each of which can be specifically and independently inhibited [6]. Studies with combinations consisting of agents directed at the individual subunits of tumor cell ribonucleotide reductase indicate the feasibility of such an approach to combination chemotherapy [7]. It has been shown that deoxyadenosine (presumably through the accumulation of dATP) in combination with hydroxyurea or 2,3-dihydro-1*H*-imidazo[1,2-*b*]pyrazole (IMPY) result in synergistic inhibition of L1210 cell growth in culture provided that the deamination of deoxyadenosine is decreased markedly with the aid of an adenosine deaminase inhibitor such as erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) [8].

In the current studies, experiments were carried out determine if combinations of drugs which included deoxyguanosine and IMPY could be generated which synergistically inhibited L1210 cell growth in culture. It was anticipated that 8-aminoguanosine would be needed to protect deoxyguanosine from degradation. The rationale for these combinations was that dGTP would inhibit the effector-binding subunit of ribonucleotide reductase while IMPY would inhibit the non-heme iron subunit.

Materials and methods

The L1210 cells were grown in suspension in RPMI 1640 medium supplemented with sodium bicarbonate (2 g/l), gentamicin sulfate (50 mg/l) and 10% horse serum. The cells were grown at 37°. Each group, control or drug-treated, was set up in triplicate. The flasks were seeded at day-zero with 0.15×10^6 cells/ml. At daily intervals, aliquots (1 ml) were removed for cell counts. Each cell sample was counted in duplicate in a model ZBI, Coulter Counter. The L1210 cell cultures were tested for mycoplasma contamination by the method of Schneider *et al.* [9].

8-Aminoguanosine was synthesized from 8-bromoguanosine by the method of Holmes and Robins [10]. The 8-aminoguanosine was characterized by its u.v. spectrum and analyzed for purity by thin-layer chromatography and high performance liquid chromatography (HPLC). Finally, it was tested and found to be a potent inhibitor of purine nucleoside phosphorylase [4]. 8-Bromoguanosine was purchased from the United States Biochemical Corp., Cleveland, OH. IMPY was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, through the assistance of Dr. Ven L. Narayanan. Desferal was a gift of the Ciba-Geigy Corp., Summit, NJ. Deoxyguanosine and gentamicin were purchased from the Sigma Chemical Co., St. Louis, MO. The horse serum, RPMI 1640 culture medium, and the sodium bicarbonate were purchased from the Grand Island Biological Co., Grand Island, NY.

Results and discussion

Deoxyguanosine is toxic to cells due to the buildup of dGTP which, in turn, presumably inhibits ribonucleotide reductase activity [1, 3, 5, 11–14]. Normal cells have high concentrations of purine nucleoside phosphorylase which degrades deoxyguanosine and prevents the accumulation