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Heterogeneity of carboxylesterases in rat liver cells

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Abstract—Rat liver cells were separated into parenchymal cells (PC), Kupffer cells (KC) and endothelial cells (EC). The distribution of carboxylesterases (EC 3.1.1.1) between these cell types was investigated by PAGE and chromatogenic substrate staining, and compared with the results for total liver preparation and individual isoenzymes isolated by chromatofocusing. All of the liver carboxylesterase isoenzymes could be detected in the PC, whereas in both KC and EC only those with isoelectric point (pI) 6.4/6.2 could be detected. Use of carboxylesterase inhibitors like bis-(4-nitrophenyl)phosphate and paraoxon, and organophosphorus compound hydrolase inhibitors like 4-hydroxymercuribenzoate and EDTA confirmed that these esterases were of the carboxylesterase type.

In rat liver, carboxylesterase (EC 3.1.1.1) activity is localized within both the endoplasmatic reticulum and the cytosol [1], but the microsomal carboxylesterase isoenzymes have been characterized most extensively [2]. Six carboxylesterases have been purified from rat liver microsomes by isoelectric focusing or by chromatofocusing and named by their isoelectric points (pI 6.4, 6.2, 6.0, 5.6, 5.2 and 5.0) [3]. Four of the esterases have different primary structures and differ in their specificity for ester and amide substrates [4]. Liver carboxylesterases are strongly involved in the metabolism of xenobiotics, and natural substrates have also been reported [5].

It is known that 60% of the liver cells are parenchymal cells (PC*), 22% endothelial cells (EC) and 10% Kupffer cells (KC) [6]. By electron microscopic autoradiography of liver slices labeled with [^{3}H]diisopropyl phosphorofluridate it was demonstrated that the bulk of organophosphate-sensitive esterase sites were localized in the PC, the Kupffer cells were not significantly different from the background [7].

The problem concerning the individual localization of enzymes in different types of cells is often neglected, and the present study was undertaken to examine whether each type of liver cell has its own carboxylesterase pattern.

Materials and Methods

Chemicals. Acrylamide, *N,N'*-methylene-bis-acrylamide, ammonium persulphate and TEMED were from Bio-Rad (Richmond, CA, U.S.A.). 4-Nitrophenyl butyrate, α-naphthyl acetate, Fast Blue B Salt, bis-(4-nitrophenyl)-phosphate and 4-hydroxymercuribenzoate were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Paraoxon (diethyl 4-nitrophenyl phosphate) was from Koch-Light Laboratories (Colnbrook, U.K.). Nycodenz was obtained from Hafslund Nycomed A/S (Oslo, Norway). All other chemicals were of analytical-grade quality.

Animals. Male Wistar rats (200–250 g) were used for all experiments. The animals were purchased from the National Institute of Public Health, Oslo, Norway. The animals were given a standard laboratory diet and water *ad lib.*

Preparation of PC, KC and EC. Experimental animals were anesthetized with pentobarbital, and total liver cell suspension was obtained by enzymatic perfusion of the rat liver [8] following a modified two-step procedure [9]. The liver cell suspension was filtered through nylon gauze and sedimented by centrifugation (2 min, 16 *g*). The pellet containing PC was twice resuspended in incubation buffer [10] and centrifuged.

Non-parenchymal liver cells were sedimented from the supernatant by centrifugation (4 min at 310 *g*) according to Magnusson *et al.* [11]. The cells were resuspended in 50 mL of incubation buffer containing 1% BSA and centrifuged (2 min, 16 *g*), and the resulting supernatant was recentrifuged (4 min, 310 *g*). Any PC remaining in the pellet were quantitatively removed by centrifugal elutriation at 1500 rpm. The non-parenchymal cells were separated further by centrifugal elutriation at 2500 rpm, and the EC were collected in 100 mL at a flow rate of 22 mL/min. The sediment (containing the KC) was resuspended in incubation buffer containing 20% (w/w) Nycodenz and carefully overlaid with incubation buffer containing 1% BSA. The KC were collected from the interface after centrifugation (15 min, 1450 *g*), washed and resuspended in incubation buffer containing 1% BSA. The EC were further purified by resuspending the cells in incubation buffer containing Nycodenz as described for the KC preparation.

Cell viability was assessed by the Trypan blue exclusion test, and the purity of PC, KC and EC was found to be at least 99% [12]. The PC, KC and EC were frozen before they were dissolved in 20 mM Tris–glycine, pH 8.5, containing 2.0 M glycerol, and applied to the gels.

Preparation of rat liver fraction for chromatofocusing. Liver from one to two animals was rinsed with ice-cold 50 mM Tris, pH 7.5, containing 0.1 M NaCl and homogenized in 4 vol. of the same buffer with a Polytron instrument for 20 sec. The homogenate was filtered through glass wool to remove fat and heated at 55° for 15 min, followed by centrifugation at 9000 *g* for 20 min at 4°. The supernatant was mixed with saponin to a final concentration of 1% (w/w) for 1 hr at 4° and centrifuged at 100,000 *g* for 60 min. The high-speed supernatant was fractionated by ammonium sulphate precipitation. The 35–70% saturated ammonium sulphate fraction was desalted by eluting through Sephadex G-25M (Pharmacia PD-10 column) and applied to a chromatofocusing column as described previously [13].

PAGE. The gels were run and stained by the method of Øien and Stenersen [14]. The PAGE was run with a Mini Protean II cell from Bio-Rad. Gel slabs 7 × 8 cm^2, 1.00 mm thick were used with 7.5% acrylamide and 0.25% *N,N'*-methylene-bis-acrylamide (stacking gel: 3.0% acrylamide and 0.10% *N,N'*-methylene-bis-acrylamide) in 0.375 M Tris–HCl buffer, pH 8.8 (stacking gel-buffer: 0.125 M Tris–HCl, pH 6.8). Tris–glycine (5 mM, pH 8.5) was used as electrode buffer. Samples of 10–20 μL were applied after addition of Bromophenol blue (0.01%) as marker. A Pharmacia LKB model power supply was used (190 V for approximately 1.5 hr).

The staining solution contained 50 mM Tris–HCl buffer, pH 8.0 and 10 mg of α-naphthyl acetate in 1 mL acetone per 100 mL buffer, and 50 mg Fast Blue B salt per 100 mL

* Abbreviations: PC, parenchymal cells; KC, Kuppfer cells; EC, endothelial cells; BSA, bovine serum albumin.

buffer was added as a powder. The bands developed for 20 min at room temperature.

Inhibitors like paraoxon (1.0×10^{-4} M), bis-(4-nitrophenyl)phosphate 2.0×10^{-4} M), 4-hydroxymercuribenzoate (1.0×10^{-3} M) and EDTA (1.0×10^{-3} M) were used in parallel runs to non-inhibited gels. These inhibitors were added 1 hr before addition of substrate and Fast Blue B Salt.

Other analytical methods. Carboxylesterase activity was measured spectrophotometrically with 4-nitrophenyl butyrate as substrate [15].

Protein was determined as described by Lowry *et al.* [16], with BSA as standard.

Results

Total carboxylesterase activity in different types of rat liver cell is given in Fig. 1. These results illustrate that the main carboxylesterase activity is localized in the PC, but activity is also present in KC and EC. Therefore, it was of interest to examine the distribution of the different isoenzymes of carboxylesterase in each of the liver cell types.

Figure 2 illustrates a non-denaturing polyacrylamide gel stained with α-naphthyl acetate and Fast Blue B salt. The purified fraction of PC migrated as a cascade of distinct bands (lane 2). The slowest migrating band corresponded to the isoenzyme pI 6.0, which occurs as a trimer in a non-denaturing gel (lane 7). The next band has been described as being the monomeric form of the isoenzyme pI 6.0 [17]. The isoenzymes with pI 6.4/6.2 migrated as two bands, whereas the isoenzyme pI 5.6 appeared heterogeneous. A comparison of the PC fraction (lane 2) with the homogenate fraction (lane 1) shows that the bands were nearly identical, with differences in the intensity of staining only. This illustrates that the PC contain all of the main forms of carboxylesterase which can be isolated from rat liver. The fractions of KC (lane 3) and EC (lane 4) migrated identically, but with only two main bands. These two protein bands were similar to the pI 6.4/6.2 (lane 6). In some instances we could see traces of the band corresponding to isoenzyme pI 6.0. The fastest migrating band (a weak band at the bottom of the gel in lanes 3 and 4) did not disappear when the gel was treated with the inhibitors paraoxon and bis-(4-nitrophenyl)phosphate, but did when treated with 4-hydroxymercuribenzoate and EDTA, which confirms that this protein was not of the carboxylesterase type.

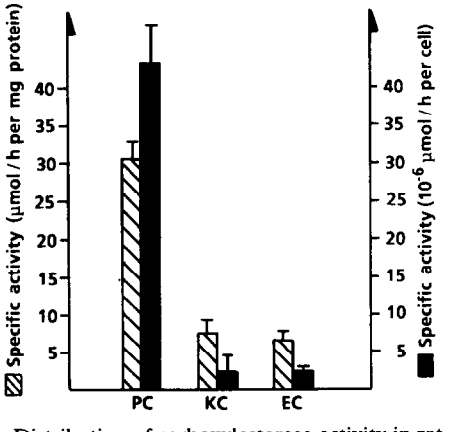

Fig. 1. Distribution of carboxylesterase activity in rat liver cells. Results are means ± SEM (N = 4).

Discussion

Isoenzyme pI 6.0, the predominant esterase in rat liver parenchymal cells, has the highest activity towards short aliphatic esters and also butanilicaine [3]. This isoenzyme is probably involved in the detoxication of xenobiotics, but also in the metabolism of natural substrates. In the EC and KC this isoenzyme appears to be of minimal importance.

In sharp contrast to the heterogeneity of carboxylesterases in PC, the KC and EC contain mainly the isoenzyme pI 6.4/6.2. These two closely related isoenzymes hydrolyse retinyl palmitate, palmitoyl-CoA and monoacylglycerols. The two forms, coded by one gene locus (ES-4B), hardly differ in their catalytic properties [18]. They possess an identical amino acid sequence, but possibly these two forms differ in their glycosylation [2]. Robbi and Beaufay [19] have identified two active enzyme forms of pI 6.4 with slightly different polypeptide chain lengths, but immunoblots revealed a single form with $M_r \approx 62{,}000$ and they explained the heterogeneity as a result of proteolysis, without inactivation of the enzyme, the PAGE results for the isolated pI 6.4/6.2 fraction show two separate bands (lane 6). Surprisingly, the pI 6.4 fraction changed from migrating like the slowest band to migrating like the fastest band of the pI 6.4/6.2 fraction (lane 6), when the solution was kept for some months at 4° (shown in lane 5). This observation

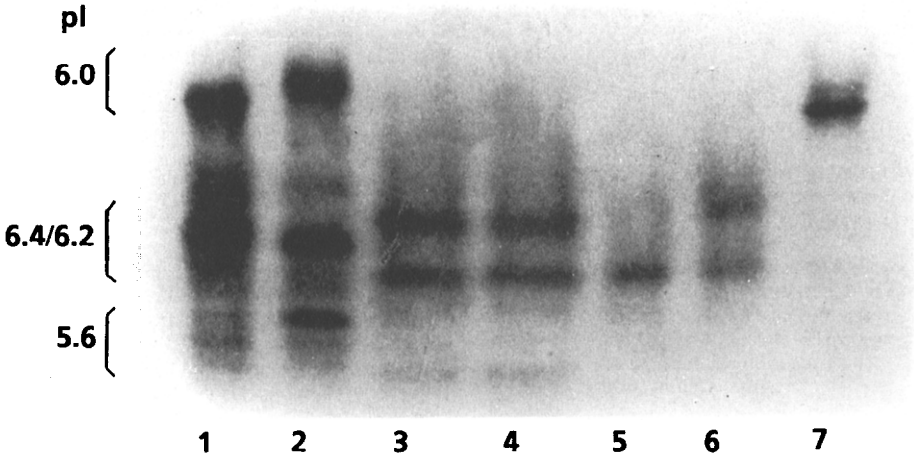

Fig. 2. Esterase banding patterns following PAGE of rat liver. The gel was stained with α-naphthyl acetate. Lane 1, rat liver extract; lane 2, PC; lane 3, KC; lane 4, EC; lane 5, carboxylesterase pI 6.4; lane 6, carboxylesterase pI 6.4/6.2; lane 7, carboxylesterase pI 6.0.

agrees with the findings of Robbi and Beaufay [19] that a peptide chain is cleaved from the rest of the pI 6.4 isoenzyme. A comparison of the isolated pI 6.4/6.2 isoenzymes with correlating bands for PC and liver homogenate illustrate differences in electrophoretic mobility. The mismatching might well be generated by differences in proteolysis of the authentic isoenzymes.

Only the PC show the three bands corresponding to isoenzyme pI 5.6 (lane 2). The differences in relative mobilities are due to the heterogeneity at the N-terminal end [3]. The isoenzymes pI 5.2/5.0 (not shown in Fig. 2) were detected in both PC and homogenate when the staining time was considerably extended (from 40 to 60 min).

The metabolic function of the PC depends on their specific position in the liver, and each individual cell probably has the potential for all hepatic functions, including the detoxication reactions. This illustrates the versatile functions among the PC compared with the more specialized functions of the KC and EC. In relation to these properties a heterogeneous pattern of the carboxylesterases in PC would be expected.

Both KC and EC are able to endocytose a large variety of different particles and molecules, and they contain high specific activities of lysosomal enzymes [20]. KC and EC are in direct contact with the bloodstream and they constitute a coordinated defence system that protects PC against injury [21]. The carboxylesterase pI 6.2/6.4, also termed monoacyglycerol lipase (EC 3.1.1.23) [22], in both EC and KC may serve to protect the liver by hydrolysis of the long chain monoacyglycerols in the degradation of lipopolysaccharides.

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