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In vitro translation of cytosolic and peroxisomal epoxide hydrolase and catalase on liver polyribosomes from untreated and clofibrate-treated C57B1/6 mice

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Free and membrane-bound polyribosomes were isolated from the livers of untreated and clofibrate-treated male C57B1/6 mice. The in vitro translation products were investigated in a rabbit reticulocyte cell-free system by immunoprecipitation of cytosolic epoxide hydrolase, catalase and albumin. The soluble forms of epoxide hydrolase present in cytosol and in peroxisomes were found to be synthesized on free polyribosomes and could not be distinguished from each other, since only one primary translation product was found with the methods used. Clofibrate treatment was found to increase total protein synthesis, synthesis of soluble epoxide hydrolase and translational efficiency of the isolated polyribosomes.

Clofibrate; Epoxide hydrolase; Catalase; Protein synthesis; Polyribosome; (Mouse liver)

1. INTRODUCTION

Epoxide hydrolases (EC 1.11.1.6) have important functions in the cellular metabolism of xenobiotics and endogenous compounds due to their ability to transform reactive epoxides into the more polar and less reactive products, dihydrodiols [1]. The soluble forms of epoxide hydrolase in the cytosol and in peroxisomes differ in substrate specificity, structure and genetic regulation from the microsomal xenobiotic-metabolizing epoxide hydrolase (review [1]), which furthermore is distinct from the microsomal cholesterol oxide hydrolase [2].

The cytosolic and peroxisomal forms of epoxide hydrolase seem to be closely related, having similar catalytic, immunochemical and structural properties [1]. Our interest in the regulation of these soluble forms of epoxide hydrolase has led us to work with peroxisome proliferators. No investigations concerning the cellular synthesis of soluble forms of epoxide hydrolase have been published so far, however. In an initial attempt to study regulation we therefore decided to carry out an investigation to determine on which type of polyribosomes the synthesis of soluble forms of epoxide hydrolase occurs, to ascertain whether the immediate translation products can differentiate between the cytosolic and peroxisomal forms, and to elucidate the effects of clofibrate on the translation of epoxide hydrolase and total protein, using catalase as a marker protein for effects on peroxisomes.

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2. MATERIALS AND METHODS

Clofibrate (ICI PLC, Macclesfield, England), protein A-Sepharose (Pharmacia-LKB, Uppsala), acrylamide and bis-acrylamide (BDH, Poole, England), phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MO), heparin (Kabi Vitrum, Stockholm), L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride (TLCK) and L-1-chloro-3-(4-tosylamino)-4-phenyl-2-butanone (TPCK) (Merck, Darmstadt), and [^{35}S]-methionine (about 1250 Ci/mmol) and immunopurified [^{125}I]-protein A (30 mCi/mg) (Amersham International, Amersham, England) were all purchased from the sources indicated.

Male C57B1/6 mice (about 20 g, equivalent to 8 weeks of age) (ALAB, Sollentuna, Sweden) were used. Induction was performed by feeding mice for 7 days on the normal diet containing

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0.5% clofibrate. This treatment ensures maximal induction of cytosolic epoxide hydrolase in liver [3]. Male Sprague-Dawley rats (180–200 g) (ALAB) were used to prepare liver cytosol containing an endogenous RNase inhibitor.

Epoxide hydrolase activity towards *trans*-stilbene oxide [4], catalase activity [5], carnitine acetyltransferase activity [6] and protein [7] was determined as described. Total RNA was extracted from liver homogenates and assayed as in [8]. The RNA content of polyribosomes was measured by the absorbance at 260 nm (assuming 1 $A_{260} = 40$ μg RNA). Immunotransfer was performed as described [9] after electrophoretic separation of samples according to Laemmli [10]. Cytosolic epoxide hydrolase was purified from clofibrate-treated mice and antibodies were raised in rabbits as in [11]. IgG was purified from the serum by using protein A-Sepharose.

Free and membrane-bound polyribosomes were isolated essentially as described and stored at -80°C [12–14]. Disruption of livers was routinely performed by homogenizing livers in a Potter-Elvehjem homogenizer using four up-and-down strokes at 440 rpm. This treatment disrupts essentially only hepatocytes while endothelial cells remain unaffected and minimal damage occurs to lysosomes [15]. Rat liver was homogenized in the same way and the cytosol prepared [12] was routinely stored at −80°C. Parts of livers were also used for preparation of different subcellular fractions [16] for immunochemical analysis and other assays.

A rabbit reticulocyte lysate was prepared as in [17]. The reaction mixture contained 0.1 mM unlabelled amino acids and 1 μM [^{35}S]methionine (1 mCi/ml). The reaction was followed by spotting aliquots on filter papers and terminated by addition of SDS to a final concentration of 4% and heating to 100°C for 3 min. A solution containing PMSF, TPCK, TLCK and trasylol was added and the mixture centrifuged whereupon the supernatants were carefully removed. Supernatant fractions were first treated with protein A-Sepharose. After centrifugation, the supernatant was treated with 50 μg preimmune IgG for 1 h at room temperature, protein A-Sepharose added thereafter, and further incubation performed for 30 min before centrifugation. The resulting supernatant was incubated with IgG against cytosolic epoxide hydrolase overnight at 4°C before addition of protein A-Sepharose and centrifugation. This procedure was repeated sequentially with IgG against catalase and albumin. The immunoprecipitates were extensively washed before analysis by SDS-polyacrylamide gel electrophoresis. After electrophoresis [10] gels were stained with Coomassie blue and treated with 1 M salicylate or 20% 2,5-diphenyloxazole and fluorographed at −80°C. Bands corresponding to the different proteins were excised, dissolved and decolorized before addition of acidified scintillation cocktail.

3. RESULTS

Greater amounts of polyribosomes from mouse liver were required to reach maximal incorporation as compared to rat liver polyribosomes (where concentrations over 50 A_{260}/ml do not result in any increase in incorporated radioactivity, unpublished). For free polyribosomes, a slight increase in incorporated radioactivity could be observed up to 80 A_{260}/ml (fig.1A,B), although the radioactivity incorporated, calculated per A_{260} unit, actually decreased 10–20%. A higher translational efficiency was observed for polyribosomes isolated from clofibrate-treated animals vs those from controls. Membrane-bound polyribosomes supported lower incorporation of radioactivity, only slightly above the background of the nuclease-treated lysate. Again, membrane-bound polyribosomes from clofibrate-treated animals had higher translational efficiency (fig. 1A,B). Similar results were obtained when polyribosomes isolated by magnesium precipitation were analyzed.

Immunoblot analysis of hepatic fractions from untreated mice showed a single band of molecular mass 60 kDa for soluble epoxide hydrolase (fig.2A). Analysis of liver fractions with catalase antibodies (fig.2B) revealed proteolysis in the homogenate and light mitochondrial fraction, whereas the cytosol only contained mature catalase, in accordance with other work [18].

The soluble forms of epoxide hydrolase were found to be synthesized on free polyribosomes in mouse liver (fig.2A, lane 5). Only one band was observed in the region of interest on the gels. Upon prolonged exposure, however, some low molecular mass products appeared (fig.2A), which may represent cross-reacting antigens. Membrane-bound polyribosomes were analyzed and showed 4–15-fold lower relative expression of soluble epoxide hydrolase. Synthesis of catalase was also observed to be on free polyribosomes from mouse liver, as expected (fig.2B, lane 4). Only mature catalase was found in this case. The translatability of catalase from membrane-bound polyribosomes was found to be 3–8-fold lower. Synthesis of albumin was found to take place on membrane-bound polyribosomes, as expected (fig.2C). Translation products derived from free polyribosomes also reacted with the albumin antibodies, however the relative expression was 4–13-fold lower vs membrane-bound polyribosomes.

Clofibrate treatment for 7 days was found to affect several hepatic parameters examined (table 1). The hepatomegalic effect is reflected in the pronounced increase in liver weight. The increase in total activity of soluble forms of epoxide hydrolase was found to be 295% of control levels, while that in catalase activity was less pronounced (185% of

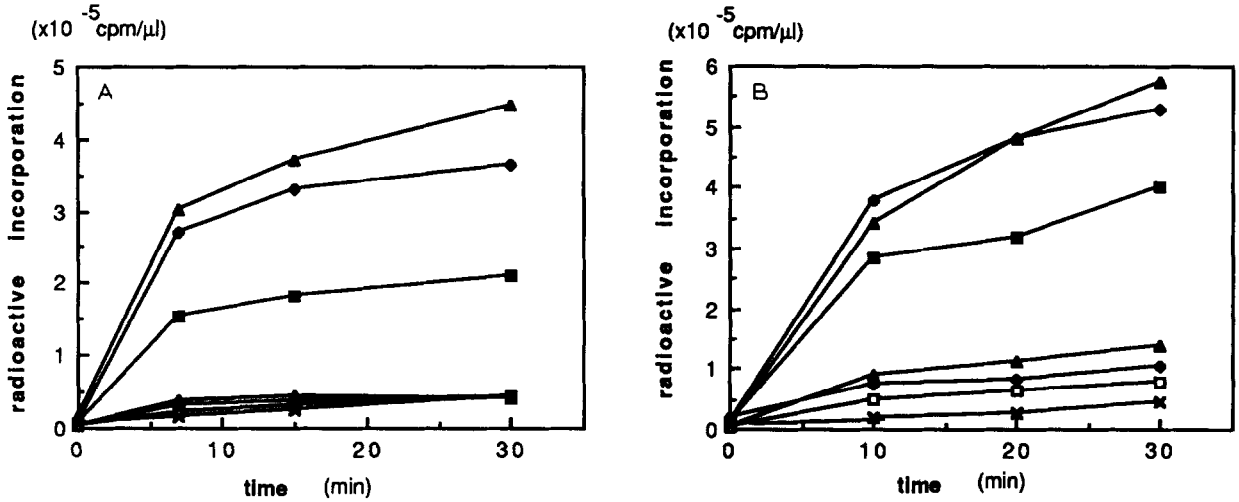

Fig.1. Effects of polyribosomal concentration on incorporation of radioactivity. (A) Free polyribosomes [38 (■), 64 (◆) and 84 (▲) A_{260}/ml] and membrane-bound polyribosomes [24 (□), 32 (◇) and 38 (△) A_{260}/ml] were isolated from livers of untreated mice [12] and assayed for their ability to support methionine incorporation. Nuclease-treated lysate without exogenous RNA added was used as background (x). (B) As A, but with polyribosomes from clofibrate-treated animals.

controls). Carnitine acetyltransferase, often used as a marker for peroxisome proliferation (although this protein is not exclusively localized to peroxisomes), displayed considerably increased activity (780% of control values). The relative levels of translated epoxide hydrolase were also increased

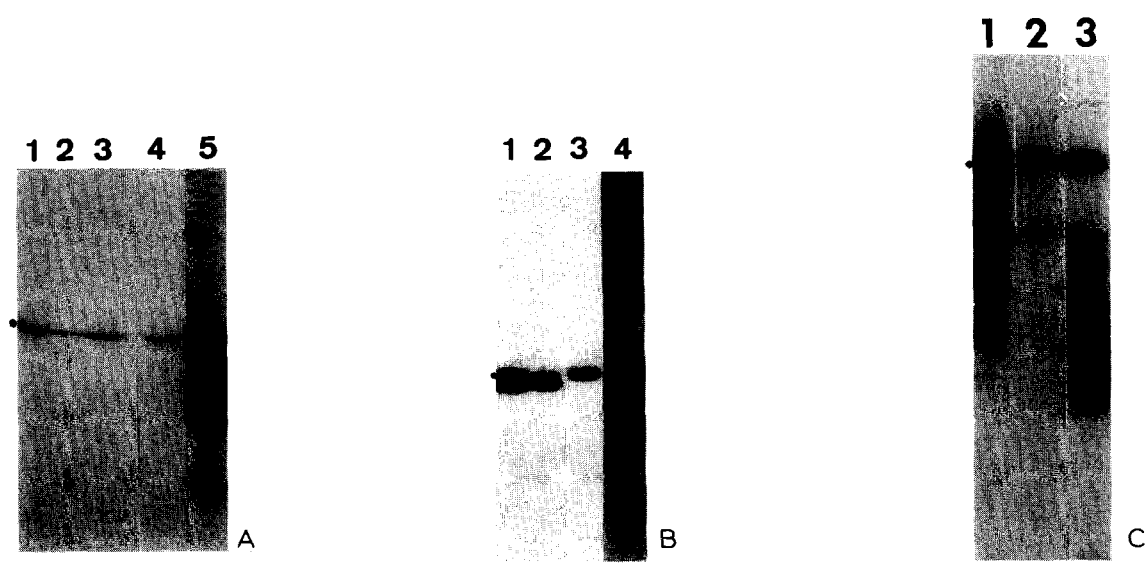

Fig.2. Immunochemical analysis of different fractions. (A) Antibodies against cytosolic epoxide hydrolase were used to probe Western blots of electrophoretically separated liver homogenate (lane 1) (100 µg), light mitochondrial fraction (lane 2) (25 µg), and cytosol (lane 3) (30 µg) from control mice and purified cytosolic epoxide hydrolase (lane 4) (0.5 µg). Lane 5 shows an autoradiograph of an immunoprecipitate, from an in vitro translation assay with free polyribosomes [12]. The dot next to lane 1 indicates the band of interest in each panel. (B) As A but with antibodies against rat liver catalase. Lane 4 contains an immunoprecipitate from an in vitro translation assay with free polyribosomes. (C) Antibodies towards human serum albumin were used to immunoprecipitate translation products from membrane-bound polyribosomes (lane 1), free polyribosomes (lane 3) prepared by magnesium precipitation and free polyribosomes (lane 2) prepared by sucrose gradient centrifugation.

Table 1

Effects of clofibrate treatment on some hepatic parameters

Treatment	Liver somatic index (%)	Content (mg/g liver)		Enzyme activity (mmol/min per g liver)			% of total protein synthesized ($\times 10^{-2}$)[d]	
		RNA	Protein	Epoxide hydrolase	Catalase	Carnitine acetyltransferase	Epoxide hydrolase	Catalase
Untreated	5.44 ± 0.40	8.08 ± 0.48	211 ± 12	0.654 ± 0.031	17.5 ± 3.09	1.38 ± 0.146	2.38 ± 0.64	1.13 ± 0.23
Clofibrate	7.77 ± 0.99[c]	7.56 ± 1.19	261 ± 25[a]	1.93 ± 0.36[c]	32.4 ± 3.38[b]	10.8 ± 0.128[c]	4.29 ± 0.47[b]	1.19 ± 0.20

Values are means ± SD from 3–5 separate experiments performed with control and treated animals (0.5% clofibrate in the diet for 7 days). Statistical significance was determined using the Student's t-test where [a] $P<0.05$, [b] $P<0.01$, [c] $P<0.001$.

[d] Newly synthesized protein was measured as radioactivity precipitable with specific antibodies in % of total trichloroacetic acid-precipitable radioactivity using polyribosomes prepared according to Ramsey and Steele [12]

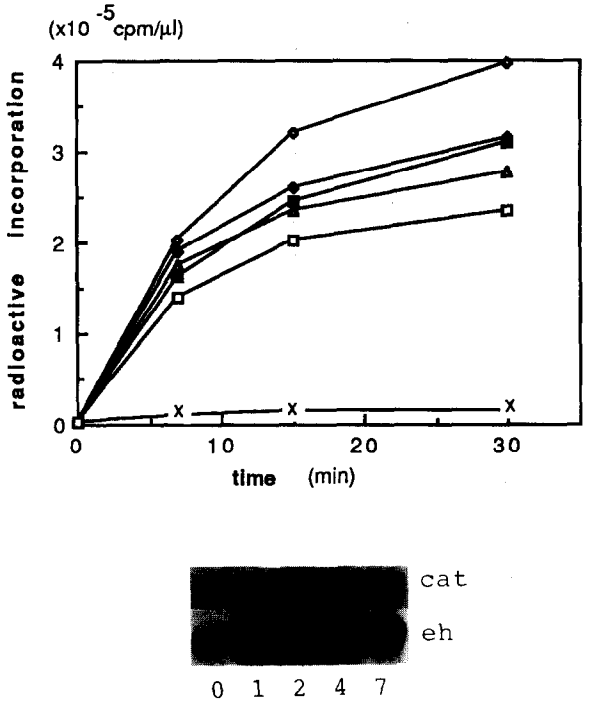

Fig.3. Effects of clofibrate treatment on in vitro translation. Free polyribosomes were isolated [14] from control animals (□) and animals treated with clofibrate for 1 (△), 2 (◇), 4 (◆) and 7 (■) days. Immunoprecipitation of catalase (cat) and epoxide hydrolase (eh) was performed at the 30 min time point for the different preparations. Values shown are means of two experiments.

about 2-fold, whereas those of catalase were not changed significantly.

To examine further the effects of clofibrate on in vitro translation, we analyzed free polyribosomes isolated from animals treated for 1, 2, 4 and 7 days with dietary clofibrate. As shown in fig.3, clofibrate treatment increased the relative translational efficiency at all time points analyzed. Maximum translation was obtained after 2 days and then decreased after 4 and 7 days, but remained above control levels. The relative expression of soluble epoxide hydrolase changed similarly, although the change in relative levels, as calculated from the actual radioactivity (80–100% over control), was not as pronounced as is suggested by the fluorographic image (fig.3).

4. DISCUSSION

Procedures originally established for the isolation of rat liver polyribosomes were effective for isolation of mouse liver polyribosomes with high translational activities as well. Although the method involving sucrose density gradient centrifugation of polyribosomes is time-consuming, it appears to result in preparations with lower contamination of membrane-bound polyribosomes (fig.2C) and in pure polyribosomes (A_{260}/A_{280} = 1.78 ± 0.05, n = 11). The procedure using magnesium precipitation is faster and can be employed with non-starved animals (necessary in the time course experiments), however it results in less pure polyribosomes (A_{260}/A_{280} = 1.60 ± 0.10, n = 25). At high concentrations these polyribosomes also show a tendency to aggregate with time.

Even though cross-contamination of free polyribosomes with membrane-bound polyribosomes occurs (cf. synthesis of albumin) and vice versa, the difference in relative levels of expression makes it possible for one to conclude that the cytosolic and peroxisomal forms of epoxide hydrolase are synthesized on free polyribosomes (fig.2). In contrast, microsomal epoxide hydrolase is synthesized on membrane- bound polyribosomes in rat liver [19]. Approximately the same relative expression of catalase and soluble epoxide hydrolase was found for the different polyribosome classes, which confirms other data suggesting free polyribosomes to be the site of synthesis of catalase in rat liver [20].

The cytosolic and peroxisomal forms of epoxide hydrolase cannot be distinguished using the methods applied thus far (regarding substrate specificity, immunochemical reactivity, native and subunit molecular masses, isoelectric point and sensitivity to inhibitors; summarized in [1]), nor could more than one primary translation product be observed on SDS-polyacrylamide gel electrophoresis (even after short exposure times in fluorography, minimizing blurring, only one band was observed). Thus, these two forms seem to be very similar to one another. Recent data also suggest that a large portion of the cytosolic epoxide hydrolase is in fact derived from peroxisomes, damaged during in vitro fractionation [21]. Of the peroxisomal proteins analyzed to date, only one protein, acyl-CoA thiolase, seems to be processed in mammalian liver after uptake into peroxisomes [20]. The peroxisomal epoxide hydrolase appears to conform to the general rule, judging from comparison of the sizes of the radiolabelled primary

translation product and the stained mature protein. However, import experiments on newly synthesized proteins into peroxisomes have not yet been performed.

Treatment of mice with the peroxisome proliferator clofibrate gives rise to massive proliferation of peroxisomes in hepatocytes, however the peroxisomal proteins are induced to widely differing extents [20]. The relative levels of soluble epoxide hydrolase synthesized were shown to increase after clofibrate treatment (table 1, fig.3), suggesting an increase in transcription or mRNA stability. An interesting observation was the increased translational efficiency of polyribosomes isolated from clofibrate-treated animals (figs.1,3).

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