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Studies on fatty acid-binding proteins

The diurnal variation shown by rat liver fatty acid-binding protein

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The concentration of fatty acid-binding protein in rat liver was examined by SDS/polyacrylamide-gel electrophoresis, by Western blotting and by quantifying the fluorescence enhancement achieved on the binding of the fluorescent probe 11-(dansylamino)undecanoic acid. A 2–3-fold increase in the concentration of this protein produced by treatment of rats with the peroxisome proliferator tiadenol was readily detected; however, only a small variation in the concentration of the protein due to a diurnal rhythm was observed. This result contradicts the 7–10-fold variation previously reported for this protein [Hargis, Olson, Clarke & Dempsey (1986) J. Biol. Chem. **261**, 1988–1991].

INTRODUCTION

Rat liver FABP is an abundant 14000-M_r cytosolic protein that has a high affinity for long-chain fatty acids and their CoA esters, as well as a number of non-polar organic anions (Glatz & Veerkamp, 1985). Although its precise physiological function remains unknown, studies from several laboratories have suggested that it may be involved in the intracellular transport and compartmentation of long-chain fatty acids, or it may protect specific enzymes against inhibition by long-chain acyl-CoA esters (Grinstead *et al.*, 1983; Glatz & Veerkamp, 1985). Thus the concentration of liver FABP is subject to long-term modulation by a number of factors, which include sex-steroid hormones and peroxisome proliferators, and the changes can be correlated with changes in the rate of uptake and utilization of fatty acids by the liver (Renaud *et al.*, 1978; Ockner *et al.*, 1980; Kawashima *et al.*, 1983). In addition, FABP has been shown to modify the activity of several enzymes *in vitro* (Grinstead *et al.*, 1983).

More recently, Dempsey (1984) and Hargis *et al.* (1986) have reported that sterol-carrier protein, which is considered to be identical with liver FABP (Dempsey *et al.*, 1981; Ockner *et al.*, 1982), undergoes a dramatic 7–10-fold diurnal variation in hepatic content during an alternating 12 h-dark–12 h-light cycle due to changes in the rate of synthesis of the protein mediated at the level of translation of sterol-carrier-protein mRNA (McGuire *et al.*, 1985). Furthermore, the proposed diurnal variation corresponds to a diurnal variation of the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (Dugan & Porter, 1977), and it has been suggested that sterol-carrier protein may be implicated in the short-term regulation of lipid metabolism by dietary and hormonal events (Dempsey, 1984; Hargis *et al.*, 1986). In marked constrast, however, Bass *et al.* (1985) have reported that there is no significant diurnal change in the hepatic concentration of FABP during a 12 h-dark–12 h-light cycle. In view of this marked discrepancy between the findings of the two laboratories, we have examined the FABP concentration in liver cytosols prepared from rats maintained on an alternating 12 h-dark–12 h-light cycle by using SDS/polyacrylamide-gel electrophoresis and Western blotting in conjunction with a newly described method (Wilkinson & Wilton, 1986) for quantifying FABP involving the measurement of the fluorescence enhancement of a bound fluorescent fatty acid probe. The results obtained with these different techniques support the view that there is no marked diurnal variation in heptic FABP concentrations.

MATERIALS AND METHODS

Chemicals

11-(Dansylamino)undecanoic acid was obtained from Molecular Probes, Junction City, OR, U.S.A. Tiadenol [2,2′-(decamethylenedithio)diethanol] was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. 3-Hydroxy-3-methyl[^{14}C]glutaryl-CoA and ^{125}I-labelled Protein A were obtained from Amersham International, Amersham, Bucks., U.K. All other chemicals used in this study were of analytical-grade quality.

Animals

Female Wistar albino rats weighing 200 g were used throughout this study. Rats were fed *ad libitum* on a commercially available pelleted diet [Labsure CRM(X); K. and K. Greeff, Croydon, Surrey, U.K.). This contained (w/w) 56% carbohydrate (mainly starch), 3.1% fat and 18% protein. Rats of drug-treated groups were fed *ad libitum* for 7 days before death on a diet containing 0.5% (w/w) tiadenol. All rats used for this study were maintained for at least 1 week before death on an alternating 12 h-light–12 h-dark cycle. Animals of drug-treated and chow-diet (control) groups were killed at the mid-dark point of the alternating cycle. Rats used for this study of the diurnal variation of FABP were killed at the mid-dark and end-of-dark points of the diurnal period.

Purification of rat liver FABP

Rat liver FABP was purified as described by Wilkinson & Wilton (1986).

Abbreviation used: FABP, fatty acid-binding protein.

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Assay of FABP in rat liver cytosol

The FABP content of rat liver cytosols was assessed by making use of the fluorescence enhancement observed when the fluorescent fatty acid analogue 11-(dansylamino)undecanoic acid binds to FABP fractions prepared by subjecting cytosols to gel-permeation h.p.l.c. on an LKB TSK-G 2000 SW column as described by Wilkinson & Wilton (1986).

Assay of microsomal 3-hydroxy-3-methylglutaryl-CoA reductase activity

3-Hydroxy-3-methylglutaryl-CoA reductase activity was measured by the method of Shapiro *et al.* (1974) with microsomal fraction prepared from animals maintained on an alternating 12 h-light–12 h-dark cycle. Activity was expressed as pmol of mevalonate synthesized/min per mg of microsomal protein. Analyses were performed at the mid-dark and end-of-dark points of the diurnal rhythm, corresponding to the maximum difference in sterol-binding protein concentrations as reported by Dempsey (1984).

Antibody preparation

Antiserum to homogenous rat liver FABP was produced by injecting New Zealand White rabbits with FABP (250 μg) in complete Freund's adjuvant. This primary injection was followed by two booster injections at 1-month intervals with FABP in incomplete Freund's adjuvant. A partially purified IgG fraction was prepared from whole serum by $(NH_4)_2SO_4$ fractionation (Johnstone & Thorpe, 1982), and this preparation was used for Western blotting. Monospecificity of the antiserum was confirmed by Ouchterlony immunodiffusion analysis and by Western blotting.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis of cytosolic proteins was performed in vertical slab gels according to the method of Laemmli (1970). Gels were stained with 0.125% Coomassie Brilliant Blue R-250 in methanol/acetic acid/water (9:1:10, by vol.). After destaining, the gels were dried down on to Whatman 3MM paper for photography. Alternatively, the destained gel was analysed by using a Joyce–Loebl Chromoscan scanning densitometer.

Western blotting

Western blots of FABP and total liver cytosolic proteins were performed as described by Burnette (1981) with modifications. Proteins were separated by electrophoresis on SDS/polyacrylamide gels (15% acrylamide), and then were electrophoretically transferred to a nitrocellulose membrane (pore size 0.2 μm) by using an electrophoretic transfer buffer containing 25 mM-Tris base, 192 mM-glycine and 20% (v/v) methanol, pH 8.3. A 5% (w/v) solution of non-fat dried milk in Tris-buffered saline (150 mM-NaCl/20 mM-Tris/HCl buffer, pH 7.4) was used as the blocking solution and antibody incubation buffer. Filter-bound antibody–antigen complexes were detected by using ^{125}I-labelled Protein A followed by autoradiography.

RESULTS

Peroxisome proliferators, such as the drugs clofibrate and tiadenol, have been shown to produce marked

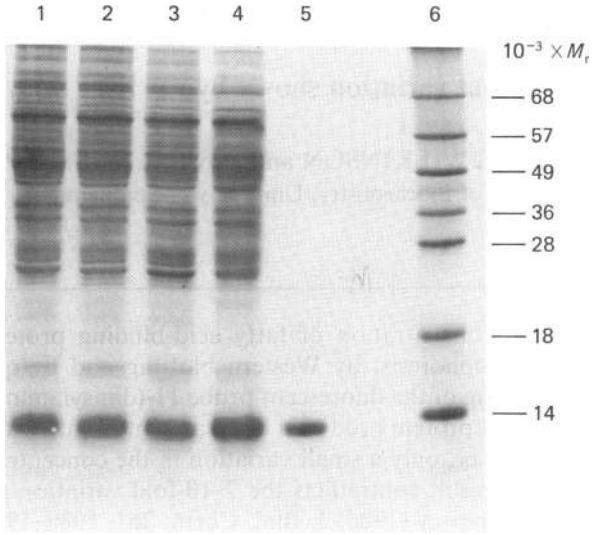

Fig. 1. SDS/polyacrylamide-gel-electrophoresis profiles of total liver cytosolic proteins

Track 1, cytosolic proteins (62.5 μg) obtained from rats at the mid-dark point of diurnal cycle; track 2, cytosolic proteins (62.5 μg) from the end-of-dark point of diurnal cycle; track 3, cytosolic proteins (62.5 μg) obtained from rats fed on normal chow diet; track 4, cytosolic proteins (62.5 μg) obtained from rats fed on a diet containing 0.5% (w/w) tiadenol; track 5, pure FABP (2 μg); track 6, M_r markers.

increases in the liver cytosolic content of FABP (Kawashima *et al.*, 1983; McTigue *et al.*, 1985) in addition to their well-documented ability to induce the peroxisomal β-oxidation enzyme system (Berge & Aarsland, 1985; Watanabe *et al.*, 1985). Thus tiadenol produces a 2–3-fold increase in the cytosolic content of FABP (McTigue *et al.*, 1985). Fig. 1 shows that such a change in FABP concentration can be readily demonstrated as an increase in the intensity of the band corresponding to the FABP polypeptide in Coomassie-Blue-stained SDS/polyacrylamide-gel-electrophoresis profiles of total liver cytosolic proteins obtained from rats fed on a diet containing tiadenol.

On the basis of this observation, it was assumed that the dramatic 7–10-fold diurnal variation in liver FABP content observed by Dempsey (1984) and Hargis *et al.* (1986) should be readily demonstrable by SDS/polyacrylamide-gel-electrophoretic analysis of total liver cytosolic proteins. Therefore liver cytosols prepared from animals killed at the extremes of the diurnal period were subjected to SDS/polyacrylamide-gel-electrophoretic analysis. In the present paper the extremes of the diurnal period refer to the mid-dark and end-of-dark points of the diurnal rhythm, which correspond to the maximum difference in sterol-carrier protein concentrations as reported by Dempsey (1984) and Hargis *et al.* (1986). Fig. 1 demonstrates that no significant change in the concentration of the polypeptide in the FABP region could be detected by this method. These results are presented in a more quantitative manner in Fig. 2, which shows the scanning-densitometer profile of tracks 1–4 of Fig. 1.

The apparent lack of a change in the concentration of FABP at the extremes of the diurnal period was

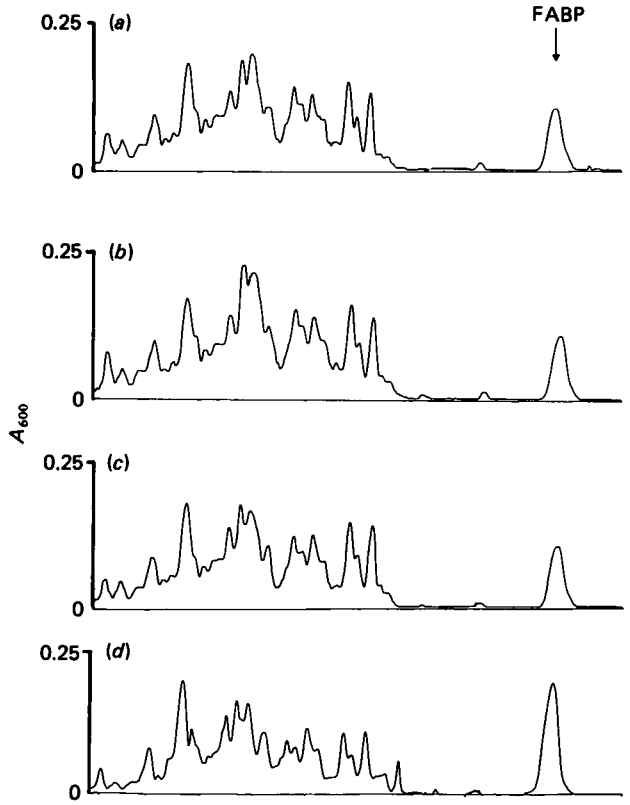

Fig. 2. Scanning-densitometer tracings of SDS/polyacrylamide-gel-electrophoresis profiles of total liver cytosolic proteins

Total liver cytosolic proteins were subjected to SDS/polyacrylamide-gel electrophoresis. After staining with Coomassie Brilliant Blue R-250, densitometer tracings were made with a Joyce–Loebl Chromoscan scanning densitometer. Traces (*a*), (*b*), (*c*) and (*d*) correspond to tracks 1, 2, 3 and 4 respectively of Fig. 1. The position of migration of pure FABP is indicated.

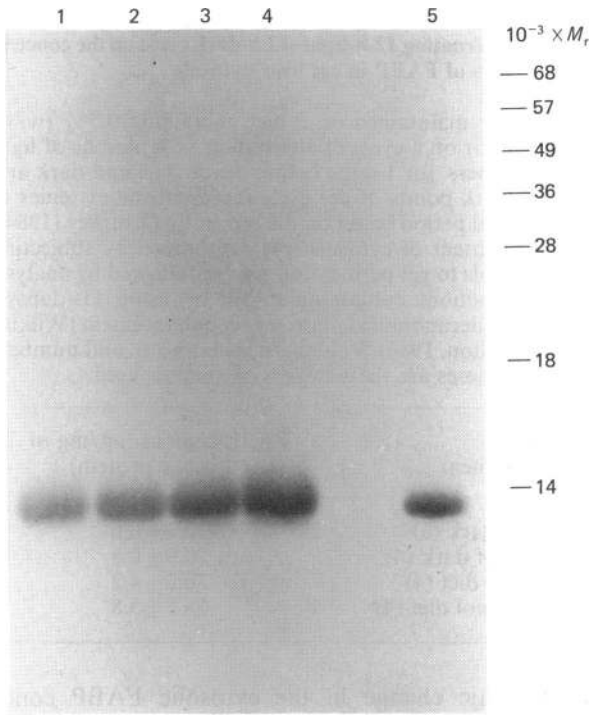

Fig. 3. Western blots (autoradiographs) of total liver cytosolic proteins showing the effects of diurnal cycle and tiadenol treatment

Samples of total liver cytosolic proteins (62.5 μg) were subjected to SDS/polyacrylamide-gel electrophoresis followed by electrophoretic transfer on to nitrocellulose. FABP was detected by using a monospecific polyclonal antiserum raised against FABP in conjunction with the detection of immunocomplexes by using ^{125}I-labelled Protein A. Tracks 1 and 2, total liver cytosolic proteins obtained from rats killed at the mid-dark and end-of-dark points respectively of an alternating 12 h-light–12 h-dark cycle; tracks 3 and 4, total liver cytosolic proteins obtained from rats fed on normal chow and a diet containing 0.5% (w/w) tiadenol respectively; track 5, pure FABP. Marker M_r values are shown.

confirmed by the use of Western blotting. Samples of total liver cytosolic proteins obtained from animals at the extremes of the diurnal period were subjected to SDS/polyacrylamide-gel electrophoresis, and the polypeptides were electrophoretically transferred to nitrocellulose followed by detection of FABP by using a monospecific polyclonal antiserum directed against FABP in conjunction with ^{125}I-labelled Protein A. Fig. 3 demonstrates that no diurnal change in the concentration of FABP could be detected by this method (compare tracks 1 and 2). The increase in the concentration of FABP after tiadenol administration, however, could be clearly demonstrated (Fig. 3, tracks 3 and 4). These observations were confirmed in a semi-quantitative manner by scanning the autoradiograph with a densitometer. Thus lanes 1, 2 and 3 were identical whereas lane 4 showed a 1.8-fold increase in the density of the band.

In order to confirm that the animals used in these experiments had adapted to the imposed 12 h-light–12 h-dark cycle after 1 week, the activity of 3-hydroxy-3-methylglutaryl-CoA reductase was measured in microsomal preparations obtained from rats killed at the extremes of the diurnal cycle as defined in the work of Dempsey (1984). The observed diurnal variation in 3-hydroxy-3-methylglutaryl-CoA reductase activity (261 ± 15 and 71 ± 6 pmol/min per mg of protein for the mid-dark and end-of-dark phases of the light cycle respectively) confirmed that the rats had adapted to the alternating 12 h-light–12 h-dark regime. It is therefore clear that the failure to demonstrate a diurnal variation in FABP concentration is not due to the use of animals that had not adapted to the alternating light–dark regime.

Finally, in order to show that the absence of diurnal change in the amount of FABP is reflected by a lack of alteration of the functional activity of FABP, the FABP content of liver cytosol samples was measured directly from the fluorescence enhancement produced when the fluorescent fatty acid probe 11-(dansylamino)undecanoic acid binds to FABP fractions obtained after gel-permeation h.p.l.c. fractionation of the cytosol samples (Wilkinson & Wilton, 1986). The results obtained with this functional assay in FABP are shown in Table 1. Only a modest diurnal change in cytosolic FABP content was detected by this method. This is in marked contrast with

Table 1. Effect of administration of tiadenol and of an alternating 12 h-light–12 h-dark cycle on the concentration of FABP in rat liver cytosols

Rats were maintained on a diet containing 0.5% (w/w) tiadenol, or on a cycle of alternating 12 h periods of light and darkness, for 1 week before death. The mid-dark and end-of-dark points of the cycle represent the extremes of the diurnal period based on the report by Dempsey (1984). FABP content of cytosols was determined by subjecting the cytosols to gel-permeation h.p.l.c. followed by analysis of the fractions containing FABP by using 11-(dansylamino)undecanoic acid fluorescence enhancement (Wilkinson & Wilton, 1986). Values are means ± S.D. and numbers in parentheses are the numbers of animals used.

Treatment	FABP content (μg/mg of cytosolic protein)
Mid-dark (3)	30.3 ± 0.6
End of dark (4)	20.9 ± 0.8
Chow diet (4)	30.2 ± 4.2
Tiadenol diet (4)	78.5 ± 5.8

the dramatic change in the cytosolic FABP content produced by the administration of tiadenol in the diet (Table 1).

DISCUSSION

Rat liver FABP is an abundant cytosolic protein that may be involved in the intracellular utilization of fatty acids (Glatz & Veerkamp, 1985). The cytosolic concentration of FABP in rat liver is subject to modulation by a number of factors, which include sex-steroid hormones and drugs that produce peroxisome proliferation (Ockner *et al.*, 1980; Kawashima *et al.*, 1983). These changes in hepatic FABP concentration are accompanied by changes in the utilization of fatty acids by the liver, and such observations provide circumstantial evidence for an involvement of FABP in fatty acid metabolism. Such changes in the concentration of FABP seem to be mediated at the pre-translational level, and the changes occur over relatively long periods of time (days), indicating that if FABP is indeed involved in the regulation of fatty acid metabolism the protein is involved in more-long-term aspects of the regulation of lipid metabolism. However, Dempsey (1984) has reported that sterol-carrier protein, which is considered to be identical with liver FABP (Ockner *et al.*, 1982; Dempsey *et al.*, 1981), undergoes a 7–10-fold diurnal variation, which represents the greatest change in FABP concentration reported in the literature. The possibility that FABP is subject to a rapid modulation might indicate that FABP has an important role to play in the short-term (hours) regulation of lipid metabolism. More recently, however, the findings reported by Dempsey (1984) have been challenged by Bass *et al.*, (1985), who reported that there was no significant diurnal variation in the cytosolic concentration of FABP. In the light of this marked discrepancy between the findings reported by Dempsey (1984) and Bass *et al.*, (1985), we decided to investigate the diurnal variation of FABP in an attempt to clarify the issue.

In the present study we have demonstrated that the marked and well-documented (Kawashima *et al.*, 1983; McTigue *et al.*, 1985) increase in the liver cytosolic content of FABP produced by the peroxisome-proliferator tiadenol can be readily demonstrated by SDS/polyacrylamide-gel electrophoretic analysis of rat liver cytosols. Thus the Coomassie-Blue-staining intensity of the FABP polypeptide in cytosol samples subjected to electrophoresis increases after tiadenol treatment when compared with controls. In view of the results obtained after tiadenol treatment (which represents a 2–3-fold change in cytosolic FABP content), the 7–10-fold diurnal variation in liver FABP content reported by Dempsey (1984) should be readily detected by electrophoretic analysis of liver cytosol samples. The results obtained, however, failed to show any change in the cytosolic concentration of FABP as detected by scanning densitometry of stained gels. Furthermore, analysis of the FABP content of liver cytosol samples by the use of Western blotting also indicated that there was no diurnal change in FABP content.

Finally, functional FABP binding activity was assessed by using an assay procedure based on the fluorescence enhancement observed when a fluorescent fatty acid derivative binds to FABP. With this technique only a modest diurnal variation in cytosolic FABP content was observed. Furthermore, this modest diurnal change was not enhanced by adaptation of the rats to the alternating light–dark cycle for a longer (3 weeks) period (results not shown). Thus the results obtained with the three different methods support the view that there is no marked diurnal variation in hepatic FABP concentration.

The reasons for the discrepancy between the results reported by Dempsey (1984) and those presented by Bass *et al.* (1985), which are supported by the present report, are not readily apparent. Dempsey and colleagues (Hargis *et al.*, 1986) have suggested that the radial-immunodiffusion technique used by Bass *et al.* (1985) to quantify FABP concentrations may not be sensitive to free and lipid-associated FABP, and that this might form the basis for their failure to observe a diurnal variation. This explanation ignores, however, the fact that Bass *et al.* (1985) also determined FABP concentrations by a functional assay based on the binding of [^{14}C]oleate to FABP in liver cytosol and this technique failed to show any marked diurnal variation in FABP concentration.

In summary, we have shown that rat liver FABP is not subject to a major diurnal variation in concentration. This result is consistent with the data presented by Bass *et al.* (1985) but contradicts the results reported by Dempsey and colleagues (Dempsey, 1984; Hargis *et al.*, 1986). At present, there is no satisfactory explanation for the difference in results. In view of the possible implications of these results with respect to the role of FABP in lipid metabolism, further studies may be necessary in order to clarify this particular aspect of FABP regulation.

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