Effect of Clofibric Acid on the Turnover of the Fatty Acid-Binding Protein Identified in Cultured Endothelial Cells from Bovine Aorta

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Several types of fatty acid-binding proteins are found in mammalian cells. Cultured endothelial cells from bovine aorta were shown to contain exclusively the cardiac-type fatty acid-binding protein (cFABP) with a mean concentration of 90 ng cFABP/mg extract protein. Only small variations were observed from passage to passage. In pulse-chase labeling experiments with L-[\textsuperscript{35}S]methionine, a half-life of 4.0 d was measured for cFABP which is about two times longer than the average half-life of the extracted proteins. These data imply that in aortic endothelial cells cFABP is not subject to short-term regulation. However, addition of clofibrate acid to the culture medium led to a shortening of the half-life of cFABP, which was compensated for by an increase in its biosynthesis. The turnover of the bulk of extract proteins remained unchanged when the cells were challenged with clofibrate acid.

Materials and Methods

Materials. L-[\textsuperscript{35}S]Methionine (1100 Ci/mmol) was purchased from Amersham-Buchler (Braunschweig, Germany); Eagle’s minimum essential medium and Hanks’ salt solution were from Seromed (München, Germany). Penicillin and streptomycin were from Boehringer (Mannheim, Germany), protein A-Sepharose and clofibrate acid (2-p-chlorophenyl)-2-oxo-propionic acid) from Sigma (Deisenhofen, Germany) and nitrocellulose paper (0.1 \(\mu \text{m}\)) from Schleicher and Schuell (Dassel, Germany).

Cell cultures, conditions of preincubation and protein labeling. Endothelial cells were derived from bovine aorta, cultured and passaged in Eagle’s minimum essential medium supplemented with 10% of fetal calf serum (Conco, Wiesbaden, Germany) in 25-cm\(^2\) Nun plastic flasks as outlined earlier (10). After cultures reached confluency, the medium was removed and cells were washed two times with 2 mL Hanks’ salt solution. Preincubation with methionine-free Waymouth’s MAB 87/3 medium (Gibco, Eggenstein, Germany) and labeling with L-[\textsuperscript{35}S]methionine were carried out in a manner analogous to that described (11). Per 25-cm\(^2\) flask, 100–150 \(\mu\)Ci were applied in a total volume of 1.9 mL. The pulse was terminated after 5 h either by harvesting or by feeding the cultures with complete tissue culture medium. Clofibrate acid was solubilized in Eagle’s minimum essential medium, and preincubation was carried out as described in the legends to Tables 1 and 2.

Immunoprecipitation. At the end of the incubation time, the cell layer was washed two times with 2 mL Hanks’ salt solution and extracted with 0.4 mL detergent buffer [0.5% (wt/vol) sodium deoxycholate, 0.5% (vol/vol) Triton X-100, 1 M NaCl and proteinase inhibitors in 0.1 M Tris-HCl (pH 7.4)] for 90 min at room temperature and centrifuged (10 min, 10,000 \(\times\) g). An aliquot of the resulting supernatant was treated with 12.5% (wt/vol) trichloroacetic acid (TCA) overnight at 4\(^\circ\)C, the precipitate was washed twice with 10% (wt/vol) TCA and finally dissolved in 0.2 mL of 2.5 M NaOH (“extract proteins”). After neutralization, the radioactivity incorporated into extract proteins was measured by liquid scintillation counting. The remaining supernatant was shaken end over end with preimmune IgG-coated protein A-Sepharose (3 mg/25-cm\(^2\) flask) at 4\(^\circ\)C for 12–24 h. This step was repeated five times to remove unspecifically bound proteins. Sepharose was spun down, and the supernatant was transferred to a tube containing the same amount of protein A-Sepharose coated with affinity purified anti-cFABP-IgG or anti-Cathepsin D-IgG, respectively. After shaking for 20–24 h, the mixture was washed three times with 1 mL detergent buffer (see above) and two times with 1 mL of 10 mM sodium phosphate (pH 7.4) containing 0.15 M NaCl. The pellet was solubilized in sodium dodecyl sulfate...
(SDS) sample buffer as described (11). Sepharose was removed by centrifugation and the immunoprecipitate was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Other methods. SDS-PAGE was performed in slab gels (12.5 cm long and 1.5 mm thick, separating gel 15% and stacking gel 5.85% acrylamide) (12), and the gels were then prepared for fluorography (13). For quantitative determinations, appropriate gel areas were cut out, swollen in water and treated with dimethyl sulfoxide to remove 2,5-diphenyloxazole. There was no loss in radioactivity during these procedures. Before determination of radioactivity, samples were dissolved in 2.2 M ortho-periodic acid for 5–10 min at 80–90°C, and after cooling the radioactivity was counted in 10 mL Instagel (Packard, Frankfurt, Germany). Antibodies against various FABP types were raised, purified and used in Western blots in a manner outlined earlier (2). cFABP was quantitated with the aid of a specific enzyme-linked immunosorbent assay (ELISA) (2) and protein by the bicinchoninic acid assay (14) with ovalbumin as standard.

Statistical analysis. Data are presented as means ± SD of n determinations. The significance of differences was evaluated by Student’s t-test.

RESULTS

Characterization and quantitation of FABP. With all types of FABP known to occur in bovine tissue as well as respective antibodies on hand, SDS-PAGE and Western immunoblotting defined the FABP present in the extract of cultured bovine aortic cells as a cardiac-type protein (Fig. 1). The sensitivity of the sandwich-ELISA employed for quantitation was in the range of 0.05 to 1.0 ng cFABP per mL and allowed the determination of the minute amounts present in the extract. The content was ~90 ng cFABP/mg extract protein with variations of up to ±20% being observed from passage to passage.

Determination of cFABP half-life. Endothelial cells were pulse-labeled for 5 h with [35S]methionine and chased for up to 12 d. [35S]Methionine could be used as radioactive precursor, because bovine cFABP contains three methionine residues (15,16). The immunoprecipitation of [35S]cFABP was of sufficient quality (Fig. 2) and was quantitative, as less than 5% of the total cFABP were found by the ELISA technique in the supernatant after immunoprecipitation. The band appearing around 30 kDa on gels after SDS-PAGE could only be detected after immunoprecipitation, but not in Western blots and it could be quenched by unlabeled cFABP. The radioactivity found in this band was about 68% of that in cFABP. Small molecular mass bands indicate the gel front. For the determination of cFABP half-life a log cpm vs. time plot was constructed which yielded straight lines with correlation coefficients from 0.97 to 0.99 (Fig. 3). From the decay curve, a half-life of 4.0 d was calculated for cFABP (15 kDa protein) and of 1.8 d for total extract proteins. Interestingly, the ~30 kDa cross-reactive protein also exhibited a half-life of 4.0 d. The sequence of the bovine cDNA for cFABP (15) excludes the possibility of the expression of a higher molecular mass precursor of cFABP and hence, the nature of this cross-reacting protein remains to be determined.

Influence of clofibric acid on cFABP metabolism. Clofibric acid was admixed to the culture medium in the concentrations shown in Tables 1 and 2. After administration for 3 to 5 d (order of cFABP half-life), the concentrations of cFABP and of extract proteins were determined. Compared with untreated controls, the values remained constant within the margin of error (Table 1). At a concentration of 5 mM clofibric acid, toxic effects were observed as some of the endothelial cells became detached from the dishes and, therefore, only concentrations up to 1 mM were used in subsequent experiments. Interestingly, clofibric acid did influence the de novo synthesis of cFABP. After preincubation of the cells for 2 d with this drug and a subsequent 5-h pulse with [35S]methionine, a dose-dependent increase of up to 50% of the radioactivity