Detection of acyl-CoA-binding protein in human red blood cells and investigation of its role in membrane phospholipid renewal

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Acyl-CoA-binding protein (ACBP) has been identified in a number of tissues and shown to affect the intracellular distribution and utilization of acyl-CoA. We have detected ACBP in the cytosol but not the membrane of human red blood cells and, using an e.l.i.s.a. with antibodies prepared against human liver ACBP, found that its concentration was 0.5 μ M. To investigate the role of ACBP in human red blood cells, we added purified human liver ACBP and radiolabelled acyl-CoA to isolated membranes from these cells. ACBP prevented high concentrations of acyl-CoA from binding to the membrane but could not keep the acyl-CoA in the aqueous phase at low concentrations. This suggested the presence of a pool in the membrane with a binding affinity for acyl-CoA that was greater

than that of ACBP for acyl-CoA. In the presence of lysophospholipid, this membrane-bound pool of acyl-CoA was rapidly used as a substrate by acyl-CoA:lysophospholipid acyltransferase (LAT) to generate phospholipid from lysophospholipid. We also found that ACBP-bound acyl-CoA was preferred over free acyl-CoA as a substrate by LAT. These results are the first documentation that human red blood cells contain ACBP and that this protein can affect the utilization of acyl-CoA in plasma membranes of these cells. The interactions between acyl-CoA, ACBP and the membrane suggest that there are several pools of acyl-CoA in the human red blood cell and that ACBP may have a role in regulating their distribution and fate.

INTRODUCTION

Although the mature red blood cell lacks the ability to synthesize phospholipids *de novo*, fatty acids of individual phospholipid classes undergo continuous turnover. Plasma fatty acids are taken up by the red blood cell and esterified to CoA in an ATP-dependent reaction catalysed by long-chain acyl-CoA synthetase (lc-ACS) [1,2]. Transesterification of the acyl group from acyl-CoA to lysophospholipid (LPL) is then catalysed by acyl-CoA:LPL acyltransferase (LAT) [3,4].

Acyl-CoA-binding protein (ACBP) is a 10 kDa cytosolic protein that was first identified by its ability to induce medium-chain acyl-CoA synthesis by goat mammary-gland fatty acid synthetase *in vitro* [5]. The amino acid sequence of ACBP has since been determined for a number of different species and all show high homology [6]. ACBP binds acyl-CoA with chain lengths of 14–22 carbon atoms with high affinity [7,8], the binding stoichiometry being 1 mol of acyl-CoA bound/mol of protein [9,10]. The protein shows no affinity for non-esterified fatty acids [10].

Recent studies suggest that ACBP might be involved in acyl-CoA metabolism. Overexpression of a bovine synthetic ACBP gene in yeast showed a positive correlation between the ACBP content and the size of the acyl-CoA pool [11]. Other roles for ACBP in lipid metabolism are supported by the observations that ACBP can (1) donate acyl-CoA for β -oxidation and glycerolipid synthesis [12], (2) interact with specific enzymes involved in lipid metabolism, including lc-ACS and acetyl-CoA carboxylase, and (3) affect the adenine nucleotide translocator [13].

In the present study, we have designed experiments to determine whether human red blood cells contain ACBP and, if they do, whether it affects the distribution and utilization of acyl-

CoA. Our results demonstrate that mature human red blood cells contain a 10 kDa protein that binds acyl-CoA and cross-reacts with antibody directed against ACBP isolated from human liver. ACBP binds acyl-CoA and in so doing affects the distribution of acyl-CoA between the cytosol and membrane. Furthermore, ACBP increases the activity of red blood cell LAT. The results of the experiments included in this report suggest that ACBP has a role in phospholipid remodelling in human red blood cells.

EXPERIMENTAL

Materials

[1-¹⁴C]Arachidonoyl-CoA (specific radioactivity 46.3 mCi/mmol) was purchased from DuPont–New England Nuclear, Boston, MA, U.S.A. [5,6,8,9,11,12,14,15-³H]Arachidonic acid (specific radioactivity 208 Ci/mmol) was obtained from Amersham, Arlington Heights, IL, U.S.A. Econo-Safe scintillation fluid was from Research Products, Mount Prospect, IL, U.S.A. Gelatin, *p*-nitrophenyl phosphate, 5-bromo-4-chloro-1-indolyl phosphate and Nitro Blue Tetrazolium were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. BSA, superoxide dismutase, cytochrome *c*, avidin, biotin, alkaline phosphatase-conjugated affinity-purified swine anti-rabbit IgG, arachidonoyl-CoA, arachidonic acid, CoA, ATP and 1 α -lysophosphatidylcholine (LPC) came from Sigma Chemical Co., St. Louis, MO, U.S.A. All other compounds used were reagent grade.

Purification of ACBP from human liver

Donated human liver was obtained at post mortem from the Institute of Pathology, Hospital of Odense, Odense, Denmark. The liver was homogenized in 2 vol. of 150 mM KCl, pH 7.0,

Abbreviations used: ACBP, acyl-CoA-binding protein; LAT, acyl-CoA:lysophospholipid acyltransferase; lc-ACS, long-chain acyl-CoA synthetase; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; LPL, lysophospholipid; PBS, 10 mM potassium phosphate, 136 mM NaCl, 2.6 mM KCl, 0.02 % Na₂S₂O₅; TBS (for e.l.i.s.a.), 10 mM Tris/HCl, 500 mM NaCl, 2.6 mM KCl, 0.02 % Na₂S₂O₅; TBS (for Western blot), 50 mM Tris/HCl, 150 mM NaCl.

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and centrifuged at 13000 *g* for 30 min. The pellet was discarded and the supernatant precipitated by acid and heat treatment as described by Haunerland et al. [14]. The supernatant was then freeze-dried, redissolved in water and applied to a Sephadex G-100 column (5 cm × 80 cm) (Pharmacia, Uppsala, Sweden) equilibrated in 100 mM ammonium acetate, pH 7.0. Proteins were fractionated at a flow rate of 120 ml/h. The fractions containing ACBP (as determined by cross-reactivity in an e.l.i.s.a. using antibody against bovine ACBP) from the Sephadex G-100 column were pooled, freeze-dried, redissolved in water and applied to a Sephadex G-50 column (5 cm × 80 cm) (Pharmacia) equilibrated in 30 mM Tris/HCl, pH 9.0. Proteins were fractionated at a flow rate of 90 ml/h and fractions containing ACBP were applied to a Q-Sepharose HP ion-exchange column (1.6 cm × 10 cm) (Pharmacia), equilibrated in 30 mM Tris/HCl, pH 9.0. ACBP was eluted with a linear 0–400 mM NaCl gradient in 30 mM Tris/HCl, pH 9.0, at a flow rate of 4 ml/min. The fraction containing ACBP was injected into an h.p.l.c. column (0.8 cm × 10 cm) packed with Dynosphere (PD-102-RE; Dyno Particles, Lillestrøm, Norway). The column was equilibrated in 0.1 % (v/v) trifluoroacetic acid in solution A. ACBP was eluted with a linear gradient of 40–80 % (v/v) solution B [50 % (v/v) propan-2-ol/0.1 % (v/v) trifluoroacetic acid in Raulf Andersen water in solution A] over 40 min at a flow rate of 1 ml/min.

To test the purity of human liver ACBP after each step in the above procedure, 20 % (w/v) polyacrylamide gels in a Pharmacia Phast System were used. Electrophoresis was carried out as recommended by the manufacturer. Gels used for blots were 15 % (w/v) polyacrylamide slab gels, and electrophoresis was carried out as described by Laemmli [15]. The gels were placed in a Bio-Rad Discontinuous Mini-Gel system, run at 50 V until the dye front reached the separation gel and then run at 125 V. The running buffer was 25 mM Tris/HCl, pH 7.9, containing 192 mM glycine and 0.1 % (w/v) SDS. Molecular-mass determination by plasma-desorption time-of-flight mass spectrometry was performed after the final purification step as described previously [9]. To confirm that our purified ACBP bound acyl-CoA, isoelectric focusing was performed as described by Lucas and Granoff [16].

Production and purification of antibody

Polyclonal antibodies against ACBP were produced as described by Knudsen et al. [9]. Antibody titres against ACBP were determined by an e.l.i.s.a., except that 3 % (w/v) gelatin was used instead of 1 % (w/v) BSA, as a blocker to prevent non-specific binding of antiserum. The antisera with the highest titres were pooled. Satd. $(\text{NH}_4)_2\text{SO}_4$ (1 vol.) was added with gentle stirring and the solution was left overnight at 4 °C. After centrifugation at 3000 *g* for 30 min, the pellet was dissolved in PBS, pH 7.3, and dialysed against PBS, pH 7.3, overnight at 4 °C. To eliminate non-specific antibodies, the dialysed antibodies were incubated with a yeast acetone-dried powder for 30 min at 4 °C. After centrifugation at 10000 *g* for 10 min, the supernatant was applied to an affinity column of 2 mg of human liver ACBP coupled to 1 g of CNBr-activated Sepharose 4B (Pharmacia). Coupling was carried out as recommended by the manufacturer. Antibodies were eluted from this column with 1 M propionic acid.

Red blood cell lysate and membrane preparation

Heparinized blood was obtained from normal human donors by venipuncture. Leucocytes and platelets were removed as described by Beutler et al. [17]. The red blood cells were washed three times in a 10-fold volume of 0.9 % NaCl. The lysate was

prepared by haemolysing the cells in 2 vol. of water followed by five cycles of freeze/thawing. The haemolysate was centrifuged for 30 min, at 12000 *g* and the membranes were discarded. Gel filtration of the lysates was performed on a Sephadex G-75 superfine column (5 cm × 120 cm) (Pharmacia), equilibrated with 50 mM Tris/HCl, pH 7.9, containing 0.02 % NaN_3 . The flow rate was adjusted to 16 ml/h. Red blood cell membranes were prepared by the method of Dodge et al. [18], and depleted of LPL by overnight incubation in 10 mM Tris-buffered saline (TBS), pH 7.4, at 37 °C before preparation.

E.l.i.s.a. of ACBP

Red blood cell lysate prepared as described above was diluted 500-fold and 1000-fold in TBS, pH 9.0, containing 0.1 % (w/v) gelatin. Lysate fractions after gel filtration were diluted 2- or 4-fold in a similar manner. The ACBP content was determined by a multiple-layer enzyme-linked immunochemical procedure as described by Pawlak and Smith [19] with the following modifications. Gelatin was used instead of BSA in all steps and the reaction of ACBP with immobilized rabbit anti-ACBP was carried out overnight at 4 °C in TBS, pH 9.0, containing 0.1 % (w/v) gelatin. The layers, attached sequentially to a 96-well micro-titre plate (NUNC, Århus, Denmark), consisted of mono-specific rabbit anti-ACBP IgG, ACBP, biotinylated rabbit anti-ACBP IgG, avidin and biotinylated alkaline phosphatase. The chromogenic substrate was *p*-nitrophenyl phosphate. ACBP content was determined from A_{405} readings of human liver ACBP standards. Biotinylated antibodies against the purified protein were produced as described by Knudsen et al. [9].

Blotting and immunological detection of proteins

Red blood cell cytosolic proteins were separated on 15 % polyacrylamide gels. Before blotting, the gel and the nitrocellulose were equilibrated in buffer² [25 mM Tris/HCl, pH 7.9, 192 mM glycine, 20 % (v/v) methanol] for 20 min. The blotting assembly was prepared in a sandwich configuration, with the membrane placed on the side of the gel facing the anode in cassettes for the Bio-Rad Mini Trans Blot. A transfer time of 1 h at 100 V and a current limit of 0.4 A was used. For blotting of isoelectric focusing gels (5 % polyacrylamide), the blotting buffer was 10 mM 3-(cyclohexylamino)-1-propanesulphonic acid (Caps)/10 % methanol, pH 11, and the transfer time was 1 h at 0.6 A. After transfer, the blots were immersed in TBS, pH 7.9, containing 3 % (w/v) gelatin and incubated at 37 °C for 2 h. The blots were then transferred to TBS, pH 9.0, containing 1 % (w/v) gelatin, 0.05 % (v/v) Tween 20 and affinity-purified anti-ACBP IgG (0.3 µg/ml) and incubated for 2 h at room temperature. After being washed for 3 × 10 min with TBS, pH 7.9, containing 0.05 % (v/v) Tween 20, the blots were transferred to TBS, pH 7.9, containing 1 % (w/v) gelatin, 0.05 % (v/v) Tween 20 and alkaline phosphatase-conjugated affinity-purified swine anti-rabbit IgG (1:1500 dilution) and incubated for 1 h at room temperature. After another wash, the blots were stained in 100 mM Tris/HCl (pH 9.5), containing 100 mM NaCl, 5 mM MgCl_2 , 7 mM 5-bromo-4-chloro-1-indolyl phosphate and 6 mM Nitro Blue Tetrazolium. Reactions were stopped by rinsing with TBS, pH 7.9, containing 20 mM EDTA.

Effect of ACBP on the distribution of arachidonoyl-CoA

Either free [^{14}C]arachidonoyl-CoA or [^{14}C]arachidonoyl-CoA bound to ACBP [ACBP/arachidonoyl-CoA 2:1 (mol/mol)] was added to incubation tubes containing red blood cell membranes (15 µg of membrane protein) in a total volume of 1.2 ml. After

10 min of incubation at 37 °C, the membranes were spun down at 12000 *g*. The supernatant was removed and the total amounts of radioactivity in both the supernatant and the pellet were determined in a liquid-scintillation counter. Amounts of radioactivity in acyl-CoA and phospholipid within the membrane preparations were determined.

Effect of ACBP on lc-ACS

The reaction mixture contained 100 mM Bistris (pH 6.5), 37 μ M [³H]arachidonic acid, 700 μ M CoA, 7 mM ATP, 8 mM MgCl₂, 5 mM dithiothreitol and 40 μ M ACBP in a total volume of 1.2 ml. After 5 min of preincubation at 37 °C, the reaction was initiated by adding LPL-depleted red blood cell membranes (200 μ g of protein, determined by the method of Bradford [20]). After incubation for the indicated time at 37 °C, the reaction was stopped by adding 140 μ l of reaction mixture to a glass tube containing 5 μ l of formic acid. Non-esterified fatty acids and lipids were extracted three times with 1 ml of ethyl acetate. Acyl-CoA was recovered in the water phase [21]. Radioactivity in the water phase and total radioactivity in 100 μ l of incubation mixture were determined in a liquid-scintillation counter.

Effects of ACBP on LAT

To investigate the effects of ACBP on LAT, an incubation mixture consisting of red blood cell membranes, acyl-CoA and ACBP was used. To begin, 20 nmol of LPC was dried down under N₂ and redissolved in 100 mM Bistris, pH 6.5. A suspension of red blood cell membrane protein in buffer (15 μ g) was added and the mixture was preincubated for 2 min at 37 °C. The mixture was then saturated with unlabelled arachidonoyl-CoA (9.8 nmol). Subsequently, either 0.2 nmol of free [¹⁴C]-arachidonoyl-CoA or 0.2 nmol of [¹⁴C]arachidonoyl-CoA preincubated with either 0.4 nmol of ACBP or 0.4 nmol of BSA (30 min at room temperature) was added. BSA was used to determine the specificity of the ACBP reaction. The assay mixtures (total volume 1.0 ml/assay mixture) were incubated at 37 °C for the indicated times and stopped by the addition of 0.5 ml of incubation mixture to 2 ml of chloroform/methanol (2:1, v/v). Lipids were extracted by the method of Bligh and Dyer [22] and the lipid extract was applied to a t.l.c. silica plate (Analtech, Newark, DE, U.S.A.). The plate was developed with the solvent system chloroform/methanol/acetic acid/0.9% NaCl (100:50:16:5, by vol.), and the spot corresponding to phosphatidylcholine (PC) was scraped off and counted for radioactivity. Total radioactivity in 50 μ l of incubation mixture was determined in a liquid-scintillation counter.

RESULTS AND DISCUSSION

The mature human red blood cell is unable to synthesize lipid *de novo* and depends on deacylation and reacylation reactions to remodel its membrane phospholipids. As acyl-CoA is required for reacylation, we undertook a study to determine whether ACBP, a protein that has been shown to affect intracellular acyl-CoA distribution and utilization in other tissues, is present in human red blood cells, whether it affects the cellular localization of acyl-CoA, and whether it influences the utilization of acyl-CoA in phospholipid-renewal pathways requiring LAT and lc-ACS.

Detection and quantification of ACBP in human red blood cells

We recognized that the concentration of ACBP in human erythrocytes might be very low, so, to determine whether ACBP was present in human red blood cells and whether it had a role in phospholipid remodelling, we first purified the protein from

Figure 1 Fractionation of human red blood cell cytosolic proteins

(a) Human red blood cell cytosolic proteins were fractionated on a Sephadex G-75 superfine column. A portion of each fraction collected was analysed by e.l.i.s.a. against human liver ACBP. The absorbance at 280 nm (○) and concentration of ACBP (●) are shown as well as the retention of known molecular-mass standards: BSA (68.0 kDa), superoxide dismutase (32.0 kDa), cytochrome *c* (12.2 kDa), indicated by arrows. (b) Immunostained Western blot. Samples were separated on a 15% polyacrylamide gel, blotted to a nitrocellulose sheet and visualized by immunostaining with antibody against human liver ACBP. Lanes contained the following samples: 1, human liver ACBP (0.5 μ g of protein); 2, red blood cell cytosol (50 μ g of protein); 3, α -globin (5 μ g of protein); 4, fraction 44 from the Sephadex G-75 separation (a); 5, fraction 43; 6, fraction 42; 7, fraction 41; 8, fraction 40.

human liver so that we could use it for *in vitro* incubations and to generate antibodies. After the Dynosphere PD-102-RE h.p.l.c. separation in our ACBP-purification procedure, a single band was found on gel electrophoresis (results not shown). The molecular mass of this protein was determined by plasma-desorption time-of-flight mass spectroscopy to be 9938.2 Da, consistent with it being ACBP. The protein was injected into a rabbit in order to produce antibodies, and an e.l.i.s.a. was developed using these antibodies.

The specificity of the affinity-purified anti-(human liver ACBP) IgG toward human red cell cytosolic proteins was tested in a Western-blot assay. A red blood cell cytosol (50 μ g of protein) preparation, separated by SDS/PAGE and transferred to nitrocellulose, failed to reveal a protein that cross-reacted with our antibody (Figure 1b, lane 2). To load recognizable amounts of

Figure 2 Effect of ACBP on the distribution of arachidonoyl-CoA between different pools in the red blood cell

(a) Red blood cell membranes (15 μ g of protein) were incubated for 10 min with various concentrations of [14 C]arachidonoyl-CoA with (●) or without (○) preincubation with a two times excess (mol/mol) of ACBP. The samples were subsequently centrifuged at 10 000 g. The supernatant was removed and counted for radioactivity in a liquid-scintillation counter. The results are expressed as percentage of total d.p.m. and the curve represents a first-order exponential fit. (b) Relative amount of substrate found in the pellet (■) and supernatant (●) after preincubation with ACBP, or in the pellet (□) and supernatant (○) without preincubation with ACBP. (c) Relative amounts of substrate found in the pellet as arachidonoyl-CoA (●) or PC (■) with preincubation with ACBP. The results are means of three independent experiments.

ACBP on to the gel without overloading with haemoglobin, the cytosol was fractionated on a Sephadex-G-75 superfine gel-filtration column. The individual fractions were analysed by e.l.i.s.a (Figure 1a). The fractions demonstrating the presence of ACBP also yielded positive results in the Western-blot assay (Figure 1b, lanes 4–8). As can be seen in Figure 1(b), a protein that ran in the same position as human liver ACBP cross-reacted with the antibody prepared against human liver ACBP. The multiple bands seen in our human liver preparation represent the monomers and polymers of ACBP [7]. Purified haemoglobin α -

chain (a gift from Dr. M. D. Scott) showed no cross-reactivity with these antibodies (Figure 1b, lane 3).

The concentration of ACBP in the cytosol of mature human red blood cells was found to be $0.45 \pm 0.18 \mu\text{M}$ ($n = 3$). No ACBP could be detected in the red blood cell membrane fraction. The intracellular concentration of ACBP in human red blood cells was lower than the concentration found in human liver ($13.69 \pm 0.21 \mu\text{M}$). Previous reports indicate that the concentration of ACBP in different tissues varies widely and is highest in liver [7]. The variations noted in the amount of ACBP within different tissues and cells is likely to be related to different requirements and functions for acyl-CoA. In the red blood cell, acyl-CoA utilization is limited to plasma-membrane phospholipid remodelling and perhaps protein acylation. We suggest that this limited role explains the relatively low concentrations of ACBP in human red blood cells.

Binding of acyl-CoA by human red blood cell ACBP

The ability of ACBP to bind acyl-CoA was tested by isoelectric focusing on the basis of a report that binding of acyl-CoA by recombinant bovine ACBP shifted the pI of ACBP to a more acidic level [23]. We found that addition of arachidonoyl-CoA to purified human liver ACBP led to a similar acidic shift in pI from 5.9 to 4.5. Addition of arachidonoyl-CoA to fraction 41 from the Sephadex G-75 run of human red blood cell cytosol (Figure 1a), followed by isoelectric focusing, gave similar results: a shift in pI from 5.9 to 4.5. This shift in the isoelectric point confirms that our isolated protein fraction from human red blood cell cytosol, which cross-reacts with antibody prepared against human liver ACBP, contains an active ACBP.

Effect of ACBP on red blood cell acyl-CoA

In the human red blood cell, acyl-CoA is formed from non-esterified fatty acids by the action of lc-ACS. The acyl component of acyl-CoA can be transesterified to LPL by the action of LAT. Both of these steps are involved in phospholipid-acylation pathways of human red blood cells. In our studies, we used arachidonic acid (20:4) as the acyl substrate, as it is rapidly incorporated into the *sn*-2 position of all major phospholipid classes in human red blood cells. Our experimental plan was designed to determine the effects that ACBP might have on the distribution and utilization of acyl-CoA within the human red blood cell.

Effects of ACBP on the cellular distribution of arachidonoyl-CoA

To investigate the effects of ACBP on the cellular distribution of acyl-CoA in the human red blood cell, we first studied partitioning of arachidonoyl-CoA after its addition to a buffered suspension of purified human red blood cell membranes in the absence of ACBP (Figure 2). Various amounts of arachidonoyl-CoA were added to the suspension (15 μ g of protein), and, after 10 min of incubation at 37 °C, the distribution of arachidonoyl-CoA between the membrane and supernatant was determined. At equilibrium, only 30% of the added arachidonoyl-CoA was found in the aqueous phase, and 70% was membrane-bound (Figure 2a). At low levels of arachidonoyl-CoA (0.1–2.0 nmol), this equilibrium was shifted towards the membrane (Figure 2a).

The addition of ACBP to the membrane suspension changed the equilibrium. When arachidonoyl-CoA was preincubated with ACBP for 30 min at 37 °C before addition to the suspension, almost all of the added arachidonoyl-CoA was found in the aqueous phase at higher concentrations of acyl-CoA. In contrast,

Figure 3 Effect of ACBP on the activation of arachidonic acid and subsequent incorporation into PC

(a) Lc-ACS activity. The reaction mixture contained 100 mM Bistris (pH 6.5), 200 μ g of LPL-depleted red blood cell membrane protein, 37 μ M [3 H]arachidonic acid, 700 μ M CoA, 7 mM ATP, 8 mM MgCl_2 and 5 mM dithiothreitol in the presence (●) or absence (○) of 40 μ M ACBP. The reaction was stopped after different times and the amount of arachidonoyl-CoA was measured. The results are means $\pm$ range of duplicates. The curves were generated by computer interpolation of the data. (b) LAT activity. The reaction mixture was the same as that in (a) in the presence (●) or absence (○) of 40 μ M ACBP. In addition, 54 μ M LPC was added. The reaction was stopped after different times and the amount of PC was measured. The results are means $\pm$ range of duplicates.

at low arachidonoyl-CoA concentrations (0.1–2.0 nmol), arachidonoyl-CoA was found in both the aqueous phase and the membrane.

The size of the membrane and supernatant pools of acyl-CoA when incubations were carried out at low concentrations of acyl-CoA (0–5 nmol) is shown in Figures 2(b) and 2(c). In the absence of ACBP, a linear increase is observed in both supernatant and membrane-bound acyl-CoA (open symbols, Figure 2b). In contrast, in the presence of ACBP, the amount of acyl-CoA in the supernatant increases significantly, and the membrane-bound pool is saturated at 0.2–0.3 nmol. As these membranes contained LPC (0.1–0.3 nmol) and LAT, the arachidonic acid in acyl-CoA was used by LAT to generate radioactive PC. Therefore the radioactivity in this membrane-bound pool was found in both acyl-CoA and PC. The arachidonic acid in arachidonoyl-CoA was transferred to PC to a saturation level of 0.12 nmol. Subsequently a pool of acyl-CoA was formed that saturated at approx. 0.12 nmol per 15 μ g of membrane protein, a concentration equal to approx. 8 nmol of acyl-CoA/mg of protein (Figure 2c). When membranes in which LPC was depleted were used in the experiment, a similar-sized pool of acyl-CoA was formed.

In addition to being a substrate for LAT in phospholipid remodelling, acyl-CoA can be converted into acylcarnitine by carnitine acyltransferase. We tested the effect of carnitine acyl-

transferase on the turnover of arachidonic acid in human red blood cells; no radiolabelled arachidonoylcarnitine was detected. Therefore it is unlikely that this enzyme has any effect on the findings we report here for ACBP and arachidonoyl-CoA.

Our results suggest that when arachidonoyl-CoA is added to isolated red blood cell membranes, it rapidly equilibrates between a membrane pool and an aqueous pool. In the presence of ACBP, this equilibrium is shifted to the aqueous phase as most of the acyl-CoA is bound by ACBP. The shape of the curves in Figure 2(a) suggests two membrane-bound acyl-CoA pools with different affinities for ACBP: a large pool (probably membrane-lipid-bound acyl-CoA), which can be competitively bound by ACBP (the difference between the two plateau values in Figure 2a) and a small pool located within a membrane domain that has a higher binding affinity for acyl-CoA than ACBP (low concentrations of acyl-CoA in Figure 2a). In the presence of LPC, LAT can utilize arachidonoyl-CoA within this tightly bound pool to generate PC. We suggest that depletion of acyl-CoA in this pool results in the transfer of acyl-CoA from the cytosolic ACBP-bound pool and that ACBP carries a reserve of acyl-CoA required for acylation of membrane phospholipids.

Effects of ACBP on arachidonic acid incorporation into acyl-CoA

By binding acyl-CoA, ACBP has been shown to diminish the product feedback inhibition that occurs when long-chain acyl-CoA is generated by the action of lc-ACS in rat liver mitochondria [13]. To determine if ACBP has a similar affect on lc-ACS activity in human red blood cells, we prepared LPC-depleted red blood cell membranes so that LAT could not utilize newly synthesized acyl-CoA. Arachidonic acid was added as a substrate for lc-ACS. We found that ACBP did not affect the amount of arachidonoyl-CoA present after different lengths of incubation (Figure 3a). Under the chosen conditions, approx. 1.3 nmol of arachidonoyl-CoA/200 μ g of red blood cell membrane protein (7 nmol of acyl-CoA/mg of protein) was generated in 2 min. As neither CoA nor ATP were limiting factors, our results indicate that, although human red blood cell lc-ACS is feedback-inhibited by arachidonoyl-CoA, feedback inhibition is not affected by ACBP. However, when we performed the same experiment using red blood cell membranes that were either not depleted of LPC or were supplemented with exogenous LPC, ACBP increased the rate of PC formation from arachidonoyl-CoA (Figure 3b). The effect of ACBP on the activity of LAT became clear after 30 min of incubation.

Effects of ACBP binding on the utilization of arachidonoyl-CoA by LAT

To determine whether the binding of acyl-CoA by ACBP influenced the utilization of acyl-CoA by LAT, red blood cell membranes were loaded with LPC. The incubation mixture was then saturated with 9.8 nmol of unlabelled arachidonoyl-CoA. Subsequently, either 0.2 nmol of free [14 C]arachidonoyl-CoA or 0.2 nmol of [14 C]arachidonoyl-CoA preincubated with 0.4 nmol of ACBP was added (Figure 4). The arachidonoyl-CoA bound to ACBP resulted in a twofold higher incorporation of [14 C]arachidonoyl-CoA into PC than free [14 C]arachidonoyl-CoA. In the absence of LPC and ACBP, centrifugation of the assay mixture revealed that the supernatant contained almost 100 % of the added [14 C]arachidonoyl-CoA (results not shown). These findings suggest that ACBP-bound cytosolic acyl-CoA is a better substrate for LAT than free cytosolic acyl-CoA. To determine the specificity of this interaction, and to prove that these results were not due to protection of LAT by ACBP, we used BSA rather than ACBP as the arachidonoyl-CoA-binding

Figure 4 Effect of ACBP on the incorporation of arachidonoyl-CoA into PC

The reaction mixture contained 100 mM Bistris (pH 6.5), 15 μ g of red blood cell membrane protein, 20 μ M LPC and 10 μ M [14 C]arachidonoyl-CoA in the presence of either 0.4 μ M ACBP (●) or 0.4 μ M BSA (×) or in the absence of any binding protein (○). The system was saturated with 9.8 nmol of unlabelled arachidonoyl-CoA. Subsequently either 0.2 nmol of free [14 C]arachidonoyl-CoA or 0.2 nmol of [14 C]arachidonoyl-CoA preincubated with 0.4 nmol of binding protein was added. The reaction was stopped after different times and the amount of radioactivity incorporated into PC was measured. The results are means $\pm$ range of duplicates. The curves were generated by computer interpolation of the data.

Figure 5 Schematic representation of the acyl-CoA pools in the red blood cell

Non-esterified fatty acids (NEFA) are activated by Ic-ACS to acyl-CoA (FACoA). Subsequently acyl-CoA is esterified to LPL by LAT resulting in phospholipid (PL). Acyl-CoA exists in equilibrium in different pools in the membrane and cytosol; as well as being bound to ACBP. A, B, C and D are the acyl-CoA pools.

protein. Although BSA can bind acyl-CoA, the presence of BSA in the incubation mixture did not affect LAT activity (Figure 4).

Hypothetical model of acyl-CoA pools in human erythrocytes

We propose that acyl-CoA exists in an equilibrium between the cytosol and the membrane in the human red blood cell. Our results are consistent with the model shown in Figure 5. Within the cytoplasm, acyl-CoA can be bound to ACBP (pool D) or can be free (pool C). Within the membrane, acyl-CoA can exist in a loosely bound domain (pool B) which is in equilibrium with free acyl-CoA in the cytoplasm (pool C) or in a tightly bound domain (pool A). It is likely that pool A is membrane-protein-bound acyl-CoA and pool B is membrane-lipid-bound acyl-CoA. Pool

A is utilized by LAT to acylate LPL in the phospholipid-renewal pathways. Pool A is the primary site for endogenously synthesized acyl-CoA mediated by Ic-ACS.

The level of acyl-CoA in pool A can increase until a saturation point is reached. In LPL-depleted membranes, the size of this pool is approx. 7 nmol of acyl-CoA/mg of red blood cell membrane protein (Figure 3a). This pool is similar in size to the high-affinity pool (approx. 8 nmol of acyl-CoA/mg of red blood cell membrane protein) found in our studies of the equilibration of acyl-CoA between water and the membrane phase (Figure 2c). At saturation, a significant amount of acyl-CoA can also be detected in pool B. This pool is in equilibrium with pool C in the cytosol (Figure 2a). As the amount of free acyl-CoA in the cytosol (pool C) is very low in the presence of ACBP (Figure 2), the equilibrium between pool C and B is affected by pool D. In other words, pools B and C will be very small in the presence of sufficient amounts of cytosolic ACBP relative to acyl-CoA. If acyl-CoA is needed as a substrate for LAT (signalled by a low level in pool A), the equilibrium will shift from pool D to pool A (Figures 3b and 4).

With regard to the role of ACBP in this model, ACBP provides a source of acyl-CoA when the amount of acyl-CoA in pool A is limited. In addition, ACBP can bind free acyl-CoA in pool C. ACBP can also stimulate LAT by providing a substrate for this enzyme which is preferred to free acyl-CoA.

CONCLUSION

In conclusion, we have demonstrated that the human red blood cell contains 0.45 μ M ACBP and that this protein has a role in the utilization and metabolic fate of acyl-CoA. Using an e.l.i.s.a and a Western-blot assay, ACBP was found in the cytosol but not the membrane. ACBP affected the cellular distribution of acyl-CoA. It has a lower finding affinity for acyl-CoA than a metabolically active membrane-bound pool, used by LAT for the acylation of LPC. ACBP could transfer acyl-CoA into this pool when required. Recent reports suggest that the ACBP gene has many of the characteristics of a housekeeping gene [24,25], and the role of ACBP as a housekeeping protein is in agreement with our results. ACBP could bind most of the cytosolic acyl-CoA and prevent it from being hydrolysed and adversely affecting membrane properties.

Besides its role in determining the cellular distribution of acyl-CoA, our results suggest that ACBP can also affect the acylation of LPL because ACBP-bound acyl-CoA is preferentially utilized over free acyl-CoA by LAT. Recent studies have shown that the complex between retinol and its binding protein is preferred over free retinol as a substrate for lecithin:retinol acyltransferase [26]. It is possible that a similar mechanism exists in the red blood cell and that acyl-CoA bound to ACBP comes into contact with the membrane and is a preferred substrate for LAT.

Taken together, our findings indicate that ACBP exists in human red blood cells and that it has a role in the cellular distribution and utilization of acyl-CoA. As membrane phospholipids are remodelled throughout the life span of the red blood cell, ACBP may assist in the trafficking of substrates required for remodelling. ACBP may also assist by rendering acyl-CoA available for reactions mediated by LAT. Similar pathways requiring acyl-CoA may exist in other haematopoietic cells and it is possible that ACBP may play a role in lipid metabolism in all stem-cell-derived elements of the blood.

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