Induction of acyl-CoA-binding protein and its mRNA in 3T3-L1 cells by insulin during preadipocyte-to-adipocyte differentiation

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The induction of acyl-CoA-binding protein (ACBP) and ACBP mRNA was investigated in 3T3-L1 cells during growth and insulin-induced differentiation. The level of ACBP relative to both total soluble protein and DNA increased during insulin-stimulated conversion of 3T3-L1 cells from preadipocytes into fully developed adipocytes. So did the total rate of lipogenesis, as measured by incorporation of [$1\text{-}^{14}\text{C}$]acetate. A similar increase in ACBP mRNA relative to total RNA was observed. These results therefore suggest that ACBP plays a specific role in the lipogenic process. However, this role might be indirect, as the increase in lipogenesis preceded the increase in ACBP. The significance of this finding is discussed.

INTRODUCTION

Acyl-CoA-binding protein (ACBP) has previously been identified and purified from bovine and rat liver (Mogensen *et al.*, 1987; Knudsen *et al.*, 1989). The protein from both bovine and rat binds acyl-CoA with high affinity, but shows no binding affinity towards free fatty acids and free CoA (Mikkelsen *et al.*, 1987; Mikkelsen & Knudsen, 1987; Rasmussen *et al.*, 1990). The biological assay for the protein is based on its ability to induce medium-chain fatty acid synthesis by goat mammary-gland fatty acid synthetase (Mogensen *et al.*, 1987). The amino acid composition and primary structure of rat and bovine ACBP have been determined (Mikkelsen *et al.*, 1987; Knudsen *et al.*, 1989). The M_r of bovine and rat ACBP is 9955 and 9938 respectively. The amino acid sequence of rat ACBP showed 83% identity with that of bovine ACBP.

Recently it has been found that ACBP is identical with diazepam-binding inhibitor (DBI) (Knudsen *et al.*, 1989; Knudsen & Nielsen, 1990). DBI, also called endozepine (Shoyab *et al.*, 1986), was first isolated from rat brain (Guidotti *et al.*, 1983), and the biological assay is based on its ability to displace diazepam from the benzodiazepine-binding site on the γ -aminobutyric acid receptor from brain. The primary structures of DBI isolated from human and bovine brain (Webb *et al.*, 1987; Marquardt *et al.*, 1986) and from rat brain (Mochetti *et al.*, 1986; Gray, 1987) have been determined. The sequences of rat and bovine brain DBI are completely identical with those of rat and bovine liver ACBP respectively. The present study was undertaken to obtain information about the regulation of the levels of ACBP and ACBP mRNA during hormone-induced differentiation. Differentiation of cells in culture is accompanied by extensive changes in the cellular content of specific proteins and their respective mRNAs. A suitable model for the study of ACBPs in relation to lipid metabolism is the adipose conversion of 3T3-L1 cells in culture. These cells, which are initially fibroblastic, differentiate upon treatment with insulin and dexamethasone to a cell type possessing the morphological and biochemical characteristics of adipocytes. The differentiation process is accompanied by a dramatic increase in the activities of the enzymes associated with lipogenesis *de novo* and triacylglycerol mobilization. In this report we show that the rate of

triacylglycerol synthesis and the levels of ACBP and ACBP mRNA increased markedly during preadipocyte-to-adipocyte differentiation of 3T3-L1 cells. Interestingly, the rise in triacylglycerol synthesis preceded the rise in ACBP.

MATERIALS AND METHODS

Cell culture

Swiss 3T3-L1 cells were maintained in 80 cm² Nunc culture flasks in Dulbecco's modified Eagle's medium (DMEM), 10% (v/v) calf serum, 100 units of penicillin/ml and 100 μ g of streptomycin/ml in a humidified atmosphere of CO₂/air (1:9) at 37 °C. The cells were subcultured at 600 cells/cm² into 6-well 35 mm-diam. dishes with medium containing 10% (v/v) fetal-bovine serum. After confluence, the cultures were treated with 1 μ M-dexamethasone for 48 h. After removal of dexamethasone, half of the cells received 10 μ g of insulin/ml for the rest of the culture period, whereas the other half did not receive insulin.

Cell extracts

For determination of DNA, protein and lipid synthesis, the cells were sonicated in 25 mM-glycylglycine/150 mM-KCl/5 mM-MgSO₄/5 mM-Na₂EDTA/1 mM-dithiothreitol (pH 7.4): the culture medium was aspirated and 1.2 ml of the sonication medium was added. The cells were disrupted by sonication for 5 s at 40 W with a Branson sonifier. The homogenate was divided into four portions for DNA, protein, ACBP and lipid analysis.

Lipid analysis

Cell monolayers in 35 mm culture wells were incubated for 24 h with 4 μ Ci of [$1\text{-}^{14}\text{C}$]acetate (sp. radioactivity 52 Ci/mol) in the standard culture medium, and cellular lipid was extracted from the cell homogenate by the method of Bligh & Dyer (1959). The lipid extract was applied to Merck HPTLC silica plates with a Camag Linomat IV t.l.c. applicator, and developed in a Camag horizontal development chamber with the solvent system hexane/diethyl ether/acetic acid/methanol (90:20:2:3, by vol.). Incorporation of radioactivity into the lipid fraction was determined by liquid-scintillation counting, and distribution of radioactivity in various lipid classes was determined with a Berthold LB 2821 radioactivity scanner.

Abbreviations used: ACBP, acyl-CoA-binding protein; DBI, diazepam-binding inhibitor.

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Protein and DNA determination

Total soluble protein was determined by using the modified Lowry procedure of Schacterle & Pollack (1973). DNA content was measured by the fluorescence method of Brunk *et al.* (1979).

Determination of ACBP

ACBP was assayed by an e.l.i.s.a. procedure essentially as described by Pawlak & Smith (1986). The layers attached sequentially to a 96-well plastic plate consisted successively of monospecific rabbit anti-ACBP IgG, ACBP antigen, biotinylated rabbit anti-ACBP IgG, avidin and biotinylated alkaline phosphatase; the chromogenic substrate was *p*-nitrophenyl phosphate. The rabbit anti-(rat ACBP) and biotinylated rabbit anti-(rat ACBP) antibodies were produced as described by Knudsen *et al.* (1989).

Isolation of cDNA clones specific for ACBP

Unless otherwise stated, standard procedures (Sambrook *et al.*, 1989) were employed. Clones specific for ACBP were isolated from a Sprague-Dawley rat liver cDNA library prepared in λ gt11 (RL1023b; Clontech, Palo Alto, CA, U.S.A.) by screening with affinity-purified rabbit anti-(rat ACBP) antibodies raised and purified as described by Knudsen *et al.* (1989). To achieve high specificity in the reaction with the anti-ACBP antibodies, the incubation was performed at pH 10.0 (Mandrup *et al.*, 1991). Several clones corresponding to the 3'-region of the ACBP mRNA were isolated. The inserts were subcloned in pBluescript vectors (Stratagene, La Jolla, CA, U.S.A.), and used to re-screen the library by plaque hybridization to obtain a nearly full-length clone.

Isolation of RNA

Total cellular RNA was extracted by the guanidinium thiocyanate/phenol/chloroform procedure (Chomczynski & Sacchi, 1987), and contaminating polysaccharides were removed by washing with 3 M-sodium acetate, pH 6.0 (Palmiter, 1973). The concentration of RNA was estimated by u.v.-absorption spectroscopy ($A_{1\text{ cm}, 260}^{1\%} \approx 250$).

Quantification of mRNA

Serial dilutions of the RNA preparations to be compared were denatured in 10 mM-NaOH/1 mM-EDTA and loaded on to a Zeta-Probe membrane (Bio-Rad, Richmond, CA, U.S.A.) with a Bio-Dot slot format blotting apparatus (Bio-Rad), as described by the manufacturer. The membrane was incubated with a ^{32}P -labelled DNA probe under standard hybridization conditions (Sambrook *et al.*, 1989) and exposed at -70°C with a DuPont Cronex Lightning Plus intensifying screen towards an AGFA Curix RP2 X-ray film. Exposure times varied from 10 min (in which case the screen and cassette were precooled to -70°C) to 3 days. The resulting autoradiograms were scanned with an Ultrosan XL 2222 laser densitometer (Pharmacia LKB, Uppsala, Sweden) and the areas of the peaks in the densitogram were determined by using the GelScan XL software package (Pharmacia LKB). The relative amount of the hybridizing mRNA species in each of the RNA preparations was read from a standard curve for the correlation between peak areas of the densitogram for one dilution series and the corresponding amounts of immobilized mRNA (i.e. the dilution factors). Numerous pilot experiments showed that for any probe and any exposure time a log-log plot of peak area versus membrane-bound RNA is linear from the detection limit of the densitometer and up to at least 25-fold higher amounts of RNA. The mRNA concentrations thus determined are independent of the choice of the reference RNA preparation, and are accurate and reliable

because of the use of internal controls (a specific standard curve is obtained for each membrane, each hybridization reaction, and each autoradiographic exposure). In the present experiments we used 5-fold dilutions of RNA (5.0–0.04 μg of RNA per slot). The hybridization probes were prepared by random primer extension (Sambrook *et al.*, 1989) of DNA fragments isolated from low-temperature-melting agarose gels by using Elutipis as recommended by the manufacturer (Schleicher & Schuell, Keene, NH, U.S.A.). The ACBP-specific probe was the 458 bp cDNA insert of pRL cACBP E2b (see the Results and discussion section). The total amount of RNA in each slot was quantified by rehybridizing the filters to a human rRNA-specific probe corresponding to the most conserved region of 28 S rRNA (nucleotides 3647–4438; Maden *et al.*, 1987).

RESULTS AND DISCUSSION

3T3-L1 cells were cultured for a period of 2 weeks and reached confluence after about 12 days in culture. DNA and protein increased 4–5-fold after insulin treatment relative to control cells (Fig. 1). The ratio of protein relative to DNA therefore remained at a constant value of approx. 40 mg of protein/mg of DNA during the experiment, and likewise the ratio of total RNA to DNA also remained constant (results not shown). The level of ACBP in growing fibroblasts was 1.4 $\mu\text{g}/\text{mg}$ of DNA, and increased after induction of differentiation to 21 μg of ACBP/mg of DNA in fully differentiated adipocytes (Fig. 2). Relative to protein content ACBP was 0.03 $\mu\text{g}/\text{mg}$ of protein in preadipocytes, increasing to above 0.56 $\mu\text{g}/\text{mg}$ in fully differentiated adipocytes. These values may be compared with the content of ACBP in rat tissues (Mikkelsen *et al.*, 1987), which ranged from 0.36 $\mu\text{g}/\text{mg}$ of protein in muscle to 6.35 $\mu\text{g}/\text{mg}$ of

Fig. 1. DNA and protein content in 3T3-L1 cells during differentiation

Cells were grown in standard medium, and at confluence were exposed to dexamethasone for 48 h. Dexamethasone was withdrawn and the cells were cultured in medium with (○, □) or without (●, ■) insulin for the rest of the cultured period. The results shown are means of duplicates from one experiment, but similar results were obtained in two other independent experiments. Bars show the range of measurements.

Fig. 2. Effect of insulin on lipid synthesis and ACBP content in 3T3-L1 cells

Cells were grown in standard medium, and at confluence were exposed to dexamethasone for 48 h. After removal of dexamethasone, cells were cultured with (○, □, △) or without (●, ■, ▲) insulin for the rest of the culture period. The results shown are means of duplicate determinations of (a) ACBP content (○, □, △), triacylglycerol synthesis (○, ●) and (b) phospholipid synthesis (△, ▲) from a single experiment. Similar results were obtained in three independent experiments. Bars show the range of determinations.

protein in liver. The level of ACBP in rat hepatocytes was determined as 250 μg/mg of DNA (results not shown). Thus the level of ACBP in 3T3-L1 cells is relatively low, but the effects of insulin and differentiation are significant. The above results have been repeated in three individual experiments. The level of ACBP in the growing cells (preadipocytes) varied from experiment to experiment, as did that in the fully differentiated adipocytes. However, in all the experiments there was an increase in the ACBP level relative to both DNA and protein content. In some experiments the cells were partly induced to adipocyte differentiation by dexamethasone treatment alone (results not shown), but there was always a pronounced difference between cells that had received insulin and those treated with dexamethasone alone.

Accumulation of triacylglycerol in 3T3-L1 cells increased dramatically after insulin treatment. The increase in triacylglycerol deposition could be seen directly in the light-microscope and could be measured as an increase in lipogenesis *de novo* as measured by [¹⁻¹⁴C]acetate incorporation into cellular triacylglycerol.

The rise in [¹⁻¹⁴C]acetate incorporation could be detected 24 h after initiation of insulin treatment and reached a level about 200 times the preadipocyte value after 72 h of insulin treatment. Relative to control cells, which were not treated with insulin, the

[¹⁻¹⁴C]acetate incorporation increased by a factor of 20. The moderate rise in triacylglycerol synthesis in the control cells may be ascribed to the use of foetal-calf serum, which is known to contain a number of growth factors and hormones. The increase in lipid synthesis of control cells was dependent on the presence of dexamethasone. If this hormone was omitted, no change in lipid synthesis was observed (results not shown).

The increase in the incorporation of labelled acetate into triacylglycerol preceded the increase in the cellular amount of ACBP during differentiation of 3T3-L1 preadipocytes induced by dexamethasone followed by insulin. At 4 days after addition of insulin to the culture medium, the [¹⁻¹⁴C]acetate incorporation had reached 75% of its maximal value, whereas the level of ACBP was unchanged (Fig. 2). By day 5 a dramatic increase in the level of ACBP was observed, and 7 days after start of the insulin treatment a maximal value of the ACBP content was observed. The incorporation rate of [¹⁻¹⁴C]acetate was still maximal 7 days after onset of the insulin treatment. Incorporation of [¹⁻¹⁴C]acetate into phospholipid was also measured, but it was affected by insulin treatment to a lesser extent (Fig. 2a). The above pattern of induction of lipogenesis relative to induction of ACBP was repeated in four individual experiments. Detailed studies on the correlations between induction of lipogenesis and enzymes in the lipogenic pathway show that the increase in rate of lipogenesis precisely correlates with the increase in activities of the enzymes in the lipogenic pathway, e.g. ATP citrate-lyase, acetyl-CoA carboxylase, fatty acid synthetase and glycerol-phosphate acyltransferase (Reed & Lane, 1980; Mackall *et al.*, 1976). The above data therefore strongly indicate that ACBP induction is slightly delayed compared with induction of lipogenic enzymes.

To determine levels of ACBP mRNA during the differentiation of 3T3-L1 cells from preadipocytes to adipocytes, several rat ACBP cDNA clones were isolated from a λgt11 library. The inserts of these clones were subcloned into pBluescript vectors and sequenced. One clone (pRL cACBP E2b) contained an *Eco*RI fragment of 458 bp comprising the entire coding region except for the first two basepairs, and a 3'-trailer of 181 bp. There was no poly(A) tail. The sequence of this ACBP cDNA is identical with that of positions 120–563 in the DBI cDNA sequence reported by Mochetti *et al.* (1986). The insert of pRL cACBP E2b was used as probe for determinations of ACBP mRNA levels.

Incubation with insulin led to a specific increase in the level of ACBP mRNA relative to total RNA (Fig. 3). The increase in ACBP mRNA level preceded the increase in ACBP level.

Whether the increased abundance of ACBP mRNA is caused by an increased transcription of the ACBP gene, an increased stability of the ACBP mRNA or a combination of both phenomena cannot be determined from the present experiments. The observed changes in ACBP and ACBP mRNA levels correspond to the reported insulin-induced changes of the adipocyte lipid-binding protein ('ALBP') and its mRNA in 3T3-L1 cells (Bernlohr *et al.*, 1984). The adipocyte lipid-binding protein, previously referred to as p422 protein, shared 62% sequence similarity with fatty acid-binding protein ('FABP') from rat heart (Sacchettini *et al.*, 1986). These proteins are suggested to be involved in intracellular fatty acid transport.

The observed specific induction of ACBP during differentiation of 3T3-L1 cells from preadipocytes to adipocytes indicates that ACBP plays a specific role in the lipogenic process. However, the fact that the lipogenic rate is increased before the level of ACBP starts to rise and is increased to a greater extent than ACBP indicates that ACBP is not rate-limiting for lipogenesis. A direct involvement of ACBP in transport of acyl-CoA esters from the acyl-CoA ligase to the glycerolipid-synthesizing enzymes is

Fig. 3. Effect of insulin on ACBP and ACBP mRNA content in 3T3-L1 cells

Cells were grown as described in Fig. 2. (○, ●) ACBP mRNA with (○) and without (●) insulin in the culture medium. The level of ACBP mRNA at confluence is set equal to 1. (□, ■) ACBP content of cells grown with (□) and without (■) insulin (same results as shown in Fig. 2). The results shown are means of duplicates from a single experiment, but similar results were obtained in an independent experiment. Bars show the range of determinations.

unlikely, as these enzymes are all located in the endoplasmic reticulum, and acyl-CoA esters diffuse rapidly within the plane of the membrane surface (Sumper & Träuble, 1973). The need for transport of acyl-CoA in soluble form for metabolic purposes is therefore very limited. Furthermore, it is very likely that acyl-CoA is transferred directly from the acyl-CoA ligase to the glycerolipid pathway, because mammalian tissues are known to contain high levels of thioesterases, which would hydrolyse acyl-CoA if it was liberated in free form (Berge & Aarsland, 1985). Acyl-CoA esters are known to be essential for the inter-Golgi protein-transport process (Pfanner *et al.*, 1989), and are reported to regulate the activity of the acetyl-CoA carboxylase (Ogiwara *et al.*, 1978). The partition of acyl-CoA into membranes decreases the content of acyl-CoA in the surrounding aqueous phase to very low levels unless specific binding proteins solubilize the acyl-CoA through complex formation (Rasmussen *et al.*, 1990). It has been shown that ACBP, in contrast with fatty acid-binding protein, is able to extract acyl-CoA esters from membranes and protect them against hydrolysis. A putative function of ACBP could therefore be to form a protected, soluble, pool of acyl-CoA esters for regulatory and synthetic purposes. In order to prove this hypothesis it will be necessary to show that acyl-CoA esters are bound to ACBP *in vivo* and that changed levels of ACBP in cells do affect the amount of acyl-CoA present.

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