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Induction of dihydrolipoamide dehydrogenase in 3T3-L1 cells during differentiation

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The activity and turnover of dihydrolipoamide dehydrogenase (E_3), the common component of the three 2-oxoacid dehydrogenase complexes, were measured during the differentiation of 3T3-L1 preadipocytes into 3T3-L1 adipocytes. The specific activity of E_3 increased approx. 3–4-fold in 3T3-L1 adipocytes differentiated under a regimen of insulin, dexamethasone and 3-isobutyl-1-methylxanthine for 48 h, followed by insulin alone thereafter. A rabbit antibody to pig heart E_3 quantitatively precipitated the enzyme from 3T3-L1 adipocytes. By using immunoprecipitation and gel electrophoresis, a 3.3-fold increase was observed in E_3 protein in 3T3-L1 adipocytes as compared with 3T3-L1 preadipocytes, on a DNA basis. Pulse-labelling experiments with L-[^{35}S]methionine revealed a 3.5-fold increase in the rate of synthesis of E_3 in 3T3-L1 adipocytes compared with that observed in 3T3-L1 preadipocytes. In contrast, the apparent half-lives of the E_3 in 3T3-L1 preadipocytes (43 h) and 3T3-L1 adipocytes (33 h) were not significantly different. Therefore, the 3–4-fold increase in the specific activity of E_3 in 3T3-L1 adipocytes resulted from an increased rate of synthesis of the enzyme.

INTRODUCTION

Dihydrolipoamide dehydrogenase (E_3) (EC 1.8.1.4) is the common component of the three 2-oxoacid dehydrogenase complexes, pyruvate dehydrogenase complex (PDC), 2-oxoglutarate dehydrogenase complex (2-OGDC), and branched-chain oxoacid dehydrogenase complex (BCOADC). Each complex also contains two other catalytic components specific for each complex: 2-oxoacid dehydrogenase (E_1) and dihydrolipoamide acyltransferase (E_2) [1]. PDC and BCOADC are regulated by covalent modification by their specific kinases and phosphatases acting on the α subunit of E_1 [2,3]. These complex activities are also regulated by metabolites [4–8]. All of these modulations alter active PDC without concomitant alteration in total PDC activity.

Changes in total PDC activity have been found in developing rat brain [9], liver [10], lactating mammary gland [11] and differentiating 3T3-L1 cells [12–14]. The differentiation process converting the 3T3-L1 preadipocytes into 3T3-L1 adipocytes is associated with the acquisition of many morphological and biochemical characteristics of adipose tissue [15–17]. Differentiation is spontaneous in preadipocytes, occurring over several weeks; confluent cells treated with a variety of hormones or drugs (insulin, dexamethasone, 3-isobutyl-1-methylxanthine etc.) differentiate more rapidly [14–19]. Differentiation of the 3T3-L1 preadipocytes into 3T3-L1 adipocytes is accompanied by increases in the activities of enzymes responsible for synthesis of triacylglycerol [20–22] and long-chain fatty acids [23–26].

Since PDC plays a central role in the incorporation of glucose carbon into long-chain fatty acids, changes in PDC activity have also been monitored in the differentiating 3T3-L1 cells [12]. The activity of the complex itself, as well as the pyruvate dehydrogenase component E_1, increase severalfold with differentiation. The increased PDC-E_1 activity coincides with increased PDC-E_1 protein, resulting from increased relative rates of synthesis for the two non-identical subunits of PDC-E_1 [12]. BCOADC activity also increases severalfold during differentiation of 3T3-L1 cells, but the 2-OGDC activity is increased only 3-fold [13]. The component enzymes of PDC and BCOADC are each increased in specific activity during this differentiation [13].

In this paper we report that the increased activity of E_3 is correlated with increased E_3 protein in the differentiated 3T3-L1 cells, and that this increase in protein content results from an increase in the rate of synthesis of the enzyme.

EXPERIMENTAL

Materials

Bovine insulin, 3-isobutyl-1-methylxanthine, dexamethasone, DL-6,8-thioctic acid amide (lipoamide), NAD^+, biotin and dihydrolipoamide dehydrogenase were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) L-[^{35}S]Methionine was purchased from New England Nuclear (Boston, MA, U.S.A.). Highly purified bovine kidney dihydrolipoamide dehydrogenase, isolated from PDC, was very generously provided by Dr. Lester J. Reed of the University of Texas, Austin.

Cell culture and differentiation

3T3-L1 preadipocytes obtained from the American Type Culture Collection (A.T.C.C. CC1 92.1) were grown as described previously [12,13]. At confluence (day 0) the cells were treated in one of two ways. In Treatment I, cells were maintained in supplemented Eagle's minimal essential medium (complete medium)

Abbreviations used: E_3, dihydrolipoamide dehydrogenase; PDC, pyruvate dehydrogenase complex; 2-OGDC, 2-oxoglutarate dehydrogenase complex; BCOADC, branched-chain oxoacid dehydrogenase complex; E_1, 2-oxoacid dehydrogenase; E_2, dihydrolipoamide acyltransferase.

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[12]. Cells thus treated remained as preadipocytes. In Treatment II [12], cells were maintained in given complete medium supplemented with insulin (10 μg/ml), dexamethasone (0.25 μM), 3-isobutyl-1-methylxanthine (0.5 mM) and biotin (8 μg/ml) for 48 h, and maintained thereafter in complete medium supplemented with only insulin and biotin. This treatment caused differentiation of 3T3-L1 preadipocytes into adipocytes.

Enzyme assay

Cell extracts for E_3 assay were prepared from cell monolayers scraped into phosphate-buffered saline (Dulbecco's 'A'); cells were washed thrice in phosphate-buffered saline and resuspended in a hypo-osmotic extraction buffer (20 mM-potassium phosphate, pH 7.5, 1% Triton X-100) containing proteinase inhibitors (2 mM-EDTA, 2 mM-EGTA, 0.2 mM-phenylmethanesulphonyl fluoride, 0.5 mg of leupeptin/l, 0.7 mg of pepstatin/l) [27]. Cell suspensions were maintained on ice (30 min), frozen and thawed thrice, and kept again on ice 30 min before E_3 was assayed. DL-Lipoamide was converted into the reduced form (dihydrolipoamide) chemically [28]. E_3 activity was assayed spectrophotometrically in the direction of dihydrolipoamide oxidation (modified from [29]); the reaction mixture contained 50 mM-potassium phosphate, pH 8, 1.5 mM-EDTA, pH 7, 1.5 mM-NAD^+, 2 mM-dihydrolipoamide and 0.5 mg of Triton X-100/ml. A milliunit (munit) of activity is defined as 1 nmol of substrate oxidized/min at 37 °C. Protein was determined by the method of Lowry *et al.* [30], with bovine serum albumin as standard.

Immunological procedures

Cell extracts, prepared as described above, were centrifuged at 20000 *g* for 30 min at 4 °C. E_3 (approx. 300 munits) was immunoprecipitated from the supernatant fraction with 50 μl of rabbit anti-(pig heart E_3) antiserum [31]. The immunoprecipitates were collected by centrifugation of the pellet through a 'sucrose cushion' (1 M-sucrose/10 mM-L-methionine/0.5% Triton X-100/0.5% Triton X-405) and washed as described previously [12]. The washed precipitates were dissociated by 10 min incubation in 2% (w/v) SDS and 20 mM-dithiothreitol at 100 °C and subjected to electrophoresis [32]. The gels were stained with 0.25% Coomassie Blue, destained [32], and densitometry was performed to determine staining intensity [12].

Immuno-cross-reactivity of the anti-E_3 serum with E_3 in 3T3-L1 adipocytes was measured by immunoprecipitating L-[^{35}S]methionine-labelled E_3 from cells grown in a medium containing L-[^{35}S]methionine for 48 h. The immunoprecipitates were subjected to SDS/polyacrylamide-gel electrophoresis, stained for protein, destained, and cut into 2 mm-thick slices. Gel slices were dissolved in 30% (v/v) H_2O_2 containing 1% (v/v) NH_3, and the radioactivity was determined by liquid-scintillation counting. One major peak of radioactivity was detected, co-migrating with Coomassie-Blue-stained E_3. Furthermore, to demonstrate that all the radiolabelled E_3 had been immunoprecipitated, a sample of cell extract from unlabelled 3T3-L1 adipocytes, containing a known amount of E_3 activity, was added to the supernatant fraction of the first immunoprecipitate. More antiserum was added, and the second immunoprecipitate was analysed as described above. The E_3 band was visible on the Coomassie-Blue-stained gel, but no significant amount of radioactivity co-migrated in this position, indicating that all the labelled E_3 had been precipitated with the first addition of antiserum.

Relative rate of synthesis of E_3

Cell monolayers, at confluence in 60 mm-diam. dishes, were incubated in 2 ml of medium containing 20 μM L-[^{35}S]methionine (100 μCi) [12]. In a preliminary experiment, the incorporation of label into total protein and into E_3 protein in both preadipocytes and adipocytes was observed to be linear over a 2 h period (results not shown). At the end of the labelling period, the medium containing L-[^{35}S]methionine was removed and the cells were washed twice in phosphate-buffered saline containing 2 mM unlabelled L-methionine. The cells were harvested, solubilized, and E_3 was immunoprecipitated. Cellular extract from unlabelled 3T3-L1 adipocytes was added as carrier, if necessary, to give a total E_3 activity of 300 munits. The immnoprecipitates were subjected to SDS/polyacrylamide-gel electrophoresis, and the radioactivity in E_3 was determined. Relative rates of synthesis were calculated as the ratio of the radioactivity in E_3 to the radioactivity in total trichloroacetate-precipitable protein [33].

Degradation of E_3

Cell monolayers of both 3T3-L1 preadipocytes and adipocytes maintained for 6 days after initiation of Treatment I or II were incubated in 2 ml of L-methionine-modified medium containing 100 μM-L-[^{35}S]methionine (100 μCi for preadipocytes, 50 μCi for adipocytes), and the appropriate supplements to maintain Treatment I or II. After 24 h of incubation the cells were washed with sterile phosphate-buffered saline containing 2 mM unlabelled L-methionine, and then incubated in complete medium (400 μM-L-methionine) [12]. At the end of the 5 h chase period, and every 12 h thereafter, cells were harvested, solubilized, and processed for the determination of radioactivity in E_3 and total protein.

RESULTS

Changes in the specific activity of the E_3 in 3T3-L1 cells

We examined the effects of insulin, dexamethasone and 3-isobutyl-1-methylxanthine on the increase in specific activity of 3T3-L1 cells during differentiation (Fig. 1). Confluent 3T3-L1 preadipocytes (Treatment I) showed no increase in E_3 specific activity over the 17-day span of the experiment. Morphologically, the cells remained fibroblast-like for this period. The specific activity of E_3 in the 3T3-L1 adipocyte (Treatment II) increased markedly, reaching a 3.5-fold increase on day 8 of the treatment, and then declined over the next 4 days, to a value twice that seen in the preadipocytes. In a separate experiment, a 9.9-fold increase in E_3 activity per dish and a 2.6-fold increase in the DNA content per dish were observed on day 8 of the treatment. A 3.8-fold induction of the E_3 activity per cell, based on DNA content, was similar to a 3.5-fold increase in the specific activity of E_3 reported in Fig. 1, suggesting no change in the protein/DNA ratio during differentiation of 3T3-L1 cells. This finding is consistent with the observed 4-fold increase in the DNA content and no significant change in the protein/DNA ratio of 3T3-L1 cells during differentiation, found previously by an identical regimen [34].

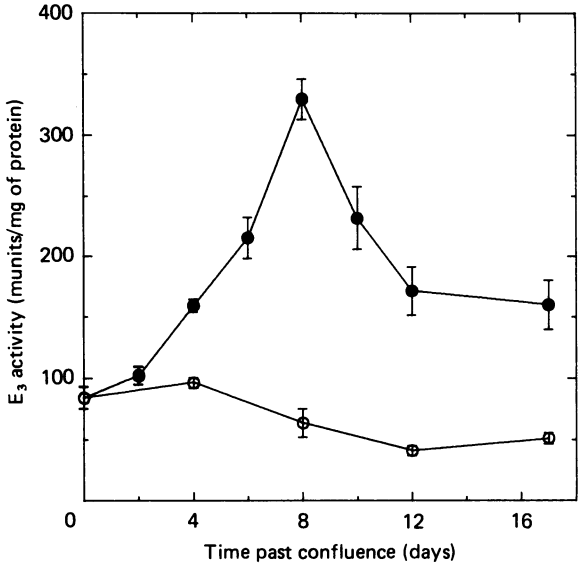

Fig. 1. Differentiation-induced changes in the specific activity of E_3 in 3T3-L1 adipocytes

Monolayer cultures of 3T3-L1 preadipocytes were grown to confluence (day 0) and subjected to either Treatment I (○) or Treatment II (●) as described in the Experimental section. E_3 activity was measured in cell extracts at the times indicated. The results are expressed as means ± S.D. for six separate dishes.

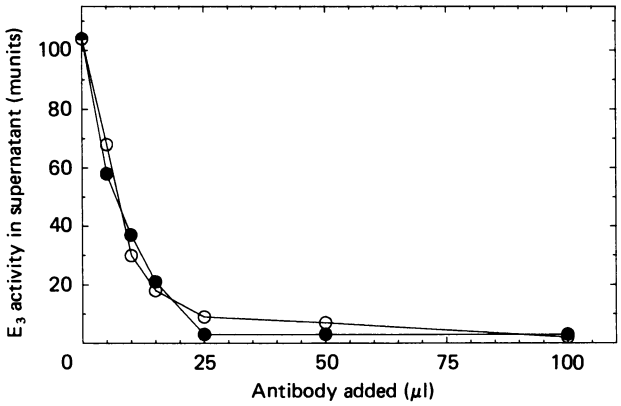

Fig. 2. Immunotitration of E_3 activity in 3T3-L1 preadipocytes and adipocytes

Cell extracts were prepared from 3T3-L1 preadipocytes (○) or adipocytes (●) differentiated for 6 days, and a constant amount of E_3 activity was precipitated with the indicated volumes of anti-E_3 serum as described in the Experimental section. Residual E_3 activity was measured in the supernatant fractions.

To distinguish between an increase in E_3 specific activity being due to increased catalytic activity or to increased E_3 protein, we compared immunotitrations of a constant amount of E_3 activity from both 3T3-L1 adipocytes and preadipocytes with increasing amounts of anti-E_3 serum. Fig. 2 shows that the immunotitration curves were similar, indicating that the specific activity of the enzyme was unchanged during differentiation of the adipocytes. To determine the amount of E_3 in the 3T3-L1 preadipocytes and adipocytes, E_3 was immunoprecipitated from equal numbers of cells (based on DNA) from both cell types (Fig. 3). In this experiment the specific activity of E_3 in the adipocytes was increased 3.9-fold compared with preadipocytes, whereas a 3.3-fold increase in the amount of precipitable E_3 protein was quantified by densitometry of the stained gel. The heavily stained band migrating below the E_3 band is IgG heavy chain.

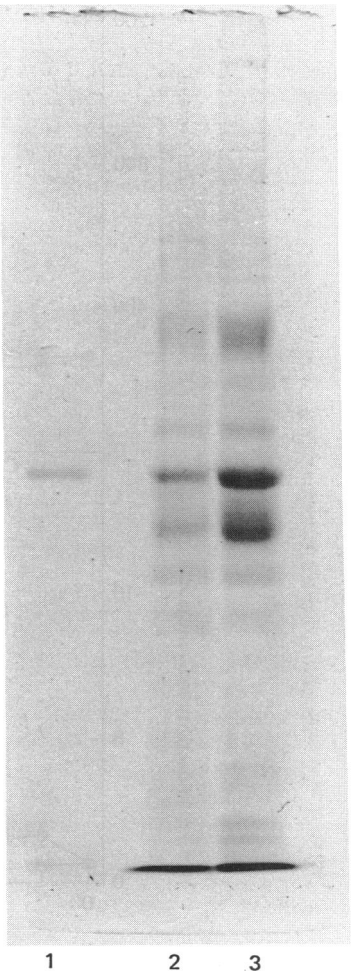

Fig. 3. Determination of immunoprecipitable E_3 in 3T3-L1 preadipocytes and adipocytes

Extracts of 3T3-L1 preadipocytes (Treatment I) or adipocytes (Treatment II for 8 days) were prepared, and E_3 was immunoprecipitated. Immunoprecipitates were subjected to SDS/polyacrylamide-gel electrophoresis, and the gel was stained for protein. Lane 1: purified bovine kidney E_3. Lane 2: 3T3-L1 preadipocyte extract containing 72 μg of DNA. Lane 3: 3T3-L1 adipocyte extract containing 72 μg of DNA.

Turnover rate of E_3

To determine the mechanisms responsible for the increase in E_3 protein, the relative rate of synthesis and apparent rate of degradation of E_3 were determined in differentiating 3T3-L1 cells. As found in other experiments, incubation with insulin, dexamethasone and 3-isobutyl-1-methylxanthine caused a marked increase in the E_3 specific activity in adipocytes; preadipocyte E_3 activity was unchanged over this period (Fig. 4*a*). The

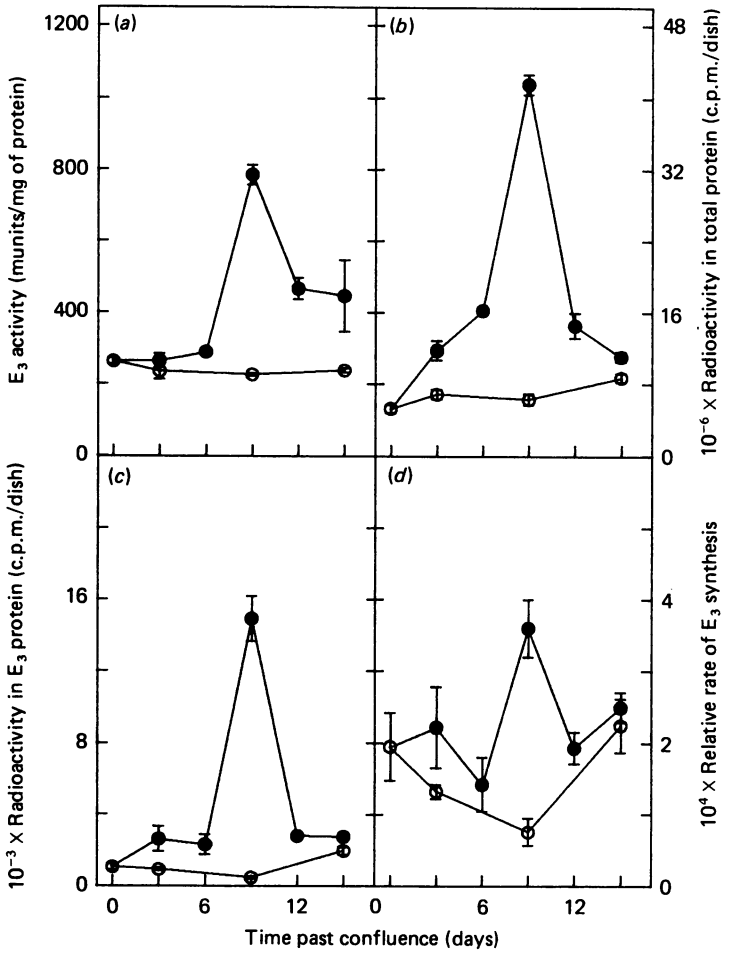

Fig. 4. Relative rate of synthesis of E_3 in 3T3-L1 preadipocytes (○) and adipocytes (●)

(*a*) Changes in the specific activity of E_3. (*b*) Incorporation of L-[^{35}S]methionine into trichloroacetic acid-precipitable cellular protein. (*c*) Incorporation of L-[^{35}S]methionine into E_3. (*d*) Relative rate of synthesis of E_3. Preadipocytes (Treatment I) or adipocytes (Treatment II) were labelled with medium containing L-[^{35}S]methionine, and E_3 was precipitated from the cell extracts as detailed in the Experimental section. The relative rate of synthesis for E_3 is expressed as the ratio of radioactivity in E_3 protein to radioactivity in total cellular protein. Results are shown as means ± S.D. for three separate dishes.

rate of incorporation of L-[^{35}S]methionine into total protein increased approx. 5-fold in differentiating cells on day 9, and declined thereafter (Fig. 4*b*). The radioactivity in total protein in preadipocytes increased only marginally during the 15-day period. Incorporation of L-[^{35}S]methionine into E_3 increased about 16-fold in adipocytes as compared with preadipocytes on day 9 (Fig. 4*c*), and declined thereafter. Incorporation of L-[^{35}S]methionine into E_3 in preadipocytes was essentially unchanged. Incorporation of L-[^{35}S]methionine into E_3 relative to incorporation into total protein (the relative rate of synthesis of E_3) increased approx. 3.5-fold in differentiated 3T3-L1 cells by day 9. The relative rate of synthesis then declined to a value approaching that of the preadipocytes on day 15 (Fig. 4*d*).

To determine the apparent rate of degradation of E_3, cells were labelled with L-[^{35}S]methionine, and the decrease in radioactivity in total cellular protein and in E_3 was monitored over a 60 h period (Fig. 5). The half-life of labelled total protein in adipocytes (68 h) was somewhat longer than that in preadipocytes (48 h) (Fig. 5*a*), in keeping with previous studies [12]. The half-life of E_3 in 3T3-L1 adipocytes (33 h) was not markedly altered compared with that in preadipocytes (43 h) (Fig. 5*b*).

DISCUSSION

Previous work in this laboratory [12–14] has been directed towards an understanding of the mechanisms underlying the induction of the PDC (and the two other 2-oxoacid dehydrogenase complexes) in differentiating 3T3-L1 cells. The activities of PDC, BCOADC and 2-OGDC are all increased during the conversion of 3T3-L1 preadipocytes into 3T3-L1 adipocytes, although the magnitudes of the increases differ greatly: approx. 10-fold for BCOADC, 7-fold for PDC and 3-fold for 2-OGDC [13]. In another experiment, assays of the component enzyme activities for PDC and BCOADC showed a 4–5-fold increase in specific activity for each of the three catalytic components [13]. Studies of the E_1 component of PDC showed that the increase in the specific activity of PDC in differentiated 3T3-L1 cells was associated with increased synthesis of both subunits of E_1 [12].

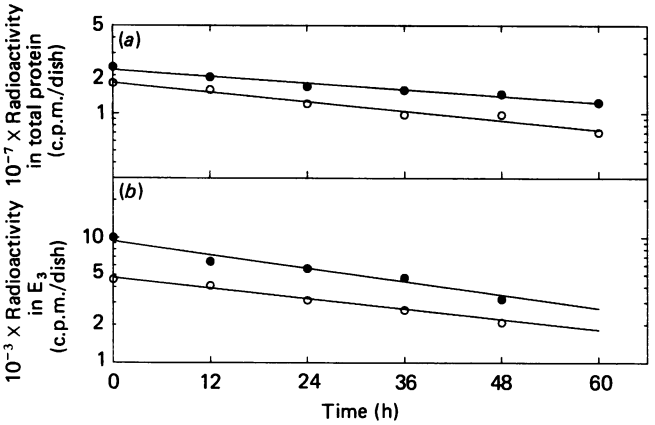

Fig. 5. Apparent rate of degradation of E_3 in 3T3-L1 preadipocytes (○) and adipocytes (●)

(*a*) Total cellular protein. (*b*) E_3. Monolayer cultures of 3T3-L1 preadipocytes or adipocytes were maintained in culture for 6 days, pulse-labelled for 24 h with L-[^{35}S]-methionine-containing medium, and chased for 5 h with unlabelled L-methionine (2 mM) as detailed in the Experimental section. Zero time is designated at the end of the 5 h chase period. At that time, and for every 12 h thereafter, E_3 was immunoprecipitated and the radioactivity in E_3 was measured. Each point is the mean of three separate dishes. Linear-regression analysis was used to fit the line to the data points.

The E_3 component is common to all three 2-oxoacid dehydrogenase complexes. Previous work [13] has demonstrated that E_3 activity increases concordantly with the increased activities of the three complexes. The present study demonstrates that the increase in E_3 activity is due to an increased synthesis of enzyme protein. This is consistent with the induction process observed for the E_1 component of PDC [12]. The apparent variations of increase in the separate components of the multienzyme 2-oxoacid dehydrogenase complexes requires some explanation. In experiments where PDC activity increased approx. 7-fold with differentiation, a 6-fold increase in the E_1 component was measured [12]. However, in a separate set of experiments, when increases in all three component activities were measured during differentiation of 3T3-L1 adipocytes [13], a 4–5-fold increase was observed for all three components of PDC. In contrast, BCOADC-E_1 increased in activity 17-fold and BCOADC-E_2 and -E_3 activities were increased by 4–5-fold, whereas whole-complex activity increased 10-fold [13]. Assuming that the three complexes have similar catalytic efficiencies towards their respective substrates and contain similar amounts of E_3 per molecule of complex, it can be calculated that E_3 in PDC, 2-OGDC and BCOADC represents approx. 90, 8 and 2% respectively of E_3 activity in differentiated 3T3-L1 cells [13]. For this reason, the overall increase in E_3 activity that we observed is consistent with the magnitude of increase in 'total' PDC activity [13]. The measured relative rate of E_1 synthesis (0.02–0.025%) [12] is in good agreement with that reported here for E_3 (0.04%).

The parallel kinetics of induction of PDC and BCOADC suggest that the signals which regulate production of these complexes during differentiation of the 3T3-L1 cells might be similar. In *Escherichia coli* the E_1 and E_2 genes (*aceE* and *aceF* respectively) for PDC and the E_1 and E_2 genes for 2-OGDC are regulated by their respective operons. E_3 is encoded by a gene (*lpd*) linked to the *ace* operon, but it also has its own promoter, which may be regulated by uncomplexed E_3 acting as a repressor [35]. Although eukaryotic genes do not have an operon structure, it is possible that the E_3 gene may be co-ordinately regulated with the genes coding for the other components of PDC. The differentiating 3T3-L1 adipocyte may provide a system in which modulation of component enzymes may be studied at the genetic level.

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