Biochem. J. (1983) **214**, 177–181
Printed in Great Britain

Induction of the branched-chain 2-oxo acid dehydrogenase complex in 3T3-L1 adipocytes during differentiation

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(*Received 8 February 1983/Accepted 11 April 1983*)

The activities of 2-oxo acid dehydrogenase complexes were measured during hormone-mediated differentiation of 3T3-L1 preadipocytes into adipocytes. Specific activity of leucine-activated branched-chain 2-oxo acid dehydrogenase complex increased approx. 10-fold in 3T3-L1 adipocytes compared with 3T3-L1 preadipocytes. In contrast, specific activity of the 2-oxoglutarate dehydrogenase complex increased by only 3-fold in 3T3-L1 adipocytes. The three catalytic component enzymes of the branched-chain 2-oxo acid dehydrogenase complex and the pyruvate dehydrogenase complex showed concomitant increases in their specific activities. A close similarity in kinetics of induction of the branched-chain 2-oxo acid dehydrogenase complex and the pyruvate dehydrogenase complex in 3T3-L1 adipocytes suggests that a common mechanism may be involved in hormone-dependent increases in the activities of the catalytic components of these two complexes in 3T3-L1 adipocytes during differentiation.

The three 2-oxo acid dehydrogenase complexes are pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes. Each of these complexes is composed of three catalytic components: 2-oxo acid dehydrogenase (E_1), lipoate acyltransferase (E_2) and lipoamide dehydrogenase (E_3) (EC 1.6.4.3) (Reed *et al.*, 1980). The E_1 and E_2 components of each of the three complexes are unique and specific for the substrate decarboxylated, but E_3 is common to all three complexes (Reed *et al.*, 1980). It is known that the pyruvate dehydrogenase complex is regulated by a phosphorylation/dephosphorylation mechanism involving a specific kinase and phosphatase (Linn *et al.*, 1969). Other evidence indicates that the branched-chain 2-oxo acid dehydrogenase complex also is regulated by a phosphorylation/dephosphorylation mechanism similar to that described for the pyruvate dehydrogenase complex (Parker & Randle, 1978; Lau *et al.*, 1981; Hughes & Halestrap, 1981; Odessey, 1982; Harris *et al.*, 1982; Buxton & Olson, 1982). In contrast, 2-oxoglutarate dehydrogenase complex apparently is not regulated by a phosphorylation/dephosphorylation mechanism (Reed *et al.*, 1980).

In a previous study from our laboratory, an approx. 6-fold increase in specific activity of 'total' pyruvate dehdyrogenase complex was observed during hormone-induced differentiation of 3T3-L1 preadipocytes into 3T3-L1 adipocytes (Hu *et al.*, 1983). In that study the dephosphorylation of the complex ('total' activity) was achieved by treating the cells with dichloroacetate, an inhibitor of pyruvate dehydrogenase kinase (Whitehouse *et al.*, 1974). We also showed that the increase in 'total' pyruvate dehydrogenase complex activity in differentiated 3T3-L1 adipocytes resulted from an increase in the content as well as in the apparent rate of synthesis of the E_1 component. Since the three catalytic components of the pyruvate dehydrogenase complex are present in fixed proportion to each other, we inferred that the activities of the other two catalytic components of this complex were also increased in 3T3-L1 adipocytes. In the present paper we provide data to support the previous suggestion. Furthermore, we show that the overall as well as the component activities of the branched-chain 2-oxo acid dehydrogenase complex are also induced in 3T3-L1 preadipocytes during hormone-induced differentiation into 3T3-L1 adipocytes.

Abbreviations used: E_1, 2-oxo acid dehydrogenase; E_2, lipoate acyltransferase; E_3, lipoamide dehydrogenase.

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Materials and methods

Materials

L-[1-^{14}C]Valine, [1-^{14}C]pyruvate and 2-oxo[1-^{14}C]glutarate were obtained from New England

Nuclear, Boston, MA, U.S.A. 3-Methyl-2-oxo[1-^{14}C]butyrate was prepared from L-[1-^{14}C]valine by oxidation with L-amino acid oxidase (Meister, 1953). [1-^{14}C]Acetyl-CoA was obtained from P-L Biochemicals, Milwaukee, WI, U.S.A. 2-Methyl[1-^{14}C]-propionyl-CoA was synthesized enzymically by using medium-chain fatty acid:CoA ligase (EC 6.2.1.2) (Myers & Utter, 1981). Bovine insulin, (+)-biotin, penicillin G, calcium pantothenate, streptomycin sulphate, 3-isobutyl-1-methylxanthine and dexamethasone were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Eagle's minimal essential medium and foetal-calf serum were obtained from Gibco Laboratories, Grand Island, NY, U.S.A.

Cell culture

3T3-L1 preadipocytes obtained from the American Type Culture Collection were grown in Eagle's minimal essential medium as described previously (Freytag & Utter, 1980). When the culture reached confluence (designated as day 0), differentiation was induced by incubating 3T3-L1 preadipocytes in complete medium containing insulin (10μg/ml), dexamethasone (0.25μM), 3-isobutyl-1-methylxanthine (0.5mM) and biotin (8μg/ml) for 48h. The culture was maintained thereafter in complete medium containing only insulin (10μg/ml) and biotin (8μg/ml), and the medium was changed every 2 days (Student *et al.*, 1980). As a control, confluent 3T3-L1 preadipocytes were maintained in complete medium without any addition for the same length of time.

Assays for 2-oxo acid dehydrogenase complexes

To assay the activity of pyruvate dehydrogenase complex, cell monolayers were washed twice with phosphate-buffered saline devoid of $CaCl_2$ and $MgCl_2$ (Dulbecco & Vogt, 1954) containing 100mM, NaF, 10mM-dichloroacetate and 10mM-EDTA to inhibit any change in phosphorylation/dephosphorylation of the complex (Hu *et al.*, 1983). The cells were then scraped in the same buffer and assayed as described previously (Sheu *et al.*, 1981). To measure dichloroacetate-activated pyruvate dehydrogenase complex activity, these cells were further washed twice with phosphate-buffered saline containing 5mM-dichloroacetate, 10mM-$MgCl_2$ and 1mM-$CaCl_2$ and incubated in the same buffer for 15min at 37°C to activate the complex completely before assay of the activity (Hu *et al.*, 1983). The activity of 2-oxoglutarate dehydrogenase complex was assayed as outlined for the pyruvate dehydrogenase complex, except that 0.5mM-2-oxo[1-^{14}C]glutarate (sp. radioactivity 2000c.p.m./nmol) replaced [1-^{14}C]pyruvate (sp. radioactivity 2000c.p.m./nmol) as substrate (Hu *et al.*, 1983). To activate the branched-chain 2-oxo acid dehydrogenase complex, 3T3-L1 cells were first incubated in Krebs–Ringer buffer (Krebs, 1933) for 15min at 37°C, and then in Krebs–Ringer buffer containing 2.5mM-L-leucine for 15min (Frick *et al.*, 1981). The concentration of leucine and the length of time used for activation in the present study gave the maximal stimulation of this complex in 3T3-L1 cells. The overall reaction catalysed by the branched-chain 2-oxo acid dehydrogenase complex was assayed by using disrupted unactivated or leucine-activated 3T3-L1 cells (Chuang *et al.*, 1981). In the present study 'leucine activation' of the branched-chain 2-oxo acid dehydrogenase complex is referred to the experimental protocol. The 'activator' is shown to be 4-methyl-2-oxopentanoate formed on transamination of leucine (Parker & Randle, 1978; Frick *et al.*, 1981; Hughes & Halestrap, 1981).

Assays for components of 2-oxo acid dehydrogenase complexes

The E_1 component was assayed radiochemically with 1-^{14}C-labelled 2-oxo acid as substrate and ferricyanide as an electron acceptor. The E_1 component of the branched-chain 2-oxo acid dehydrogenase complex was assayed by measuring the decarboxylation of 3-methyl-2-oxo[1-^{14}C]butyrate (sp. radioactivity 172c.p.m./nmol) to $^{14}CO_2$ in the presence of ferricyanide at 37°C for 80min (Chuang *et al.*, 1981). Similarly, the E_1 component of the pyruvate dehydrogenase complex was assayed by measuring the oxidation of [1-^{14}C]pyruvate to $^{14}CO_2$. The reaction mixture contained 50mM-potassium phosphate, pH6.3, 2mM-$MgCl_2$, 1mM-dithiothreitol, 0.2mM-thiamin pyrophosphate, 75mM-$K_3Fe(CN)_6$, 1.5mM-[1-^{14}C]pyruvate (sp. radioactivity 2000c.p.m./nmol) and frozen-and-thawed cell suspension (0.1mg of protein) in a final volume of 80μl, and was incubated at 37°C for 10min. The reaction mixture without thiamin pyrophosphate served as a blank. Activity of the E_2 component was measured by using a model reaction (Butterworth *et al.*, 1975) as described previously (Chuang *et al.*, 1981). [1-^{14}C]Acetyl-CoA (sp. radioactivity 400c.p.m./nmol) and 2-methyl[1-^{14}C]propionyl-CoA (sp. radioactivity 400c.p.m./nmol) were used for determination of activity of the E_2 component of the pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes respectively. The E_3 component was assayed in the direction of lipoamide reduction (Stumpf & Parks, 1978) as described previously (Chuang *et al.*, 1981). All assays were linear with time and the amount of protein added. One munit of activity is defined as 1nmol of substrate oxidized or product formed/min at 37°C. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results and discussion

Activities of 2-oxo acid dehydrogenase complexes in 3T3-L1 preadipocytes and adipocytes

To study the effect of differentiation on activity of 2-oxo acid dehydrogenase complexes, confluent 3T3-L1 preadipocytes were treated with insulin, dexamethasone and 3-isobutyl-1-methylxanthine as described above. This treatment mediated a conversion of confluent 3T3-L1 preadipocytes into adipocytes within 8–10 days, whereas untreated 3T3-L1 preadipocytes remained undifferentiated. Table 1 compares specific activities of 2-oxo acid dehydrogenase complexes in 3T3-L1 preadipocytes and adipocytes after 8 days of hormonal treatment. Specific activity of the unactivated branched-chain 2-oxo acid dehydrogenase complex increased by approx. 16-fold in 3T3-L1 adipocytes compared with 3T3-L1 preadipocytes. When the branched-chain 2-oxo acid dehydrogenase complex was activated by incubating cells with 2.5 mM-leucine before assay, specific activity of this complex was approx. 10-fold higher in 3T3-L1 adipocytes than in 3T3-L1 preadipocytes. The same hormonal treatment increased specific activity of the dichloroacetate-activated pyruvate dehydrogenase complex activity by approx. 7-fold in 3T3-L1 adipocytes compared with 3T3-L1 preadipocytes. In contrast, specific activity of the 2-oxoglutarate dehydrogenase complex showed only an approx. 3-fold increase in 3T3-L1 adipocytes. Since total cellular protein content of 3T3-L1 adipocytes per dish increases by about 4-fold over the preadipocyte stage (Student *et al.*, 1980), the actual increases in activities of these three complexes are even higher in 3T3-L1 adipocytes compared with 3T3-L1 preadipocytes.

The induction of the branched-chain 2-oxo acid dehydrogenase and 2-oxoglutarate dehydrogenase in differentiating 3T3-L1 adipocytes was further investigated by measuring the time-course of increased activity under the hormonal treatment over a 16-day period. In confluent 3T3-L1 preadipocytes, the specific activity of leucine-activated branched-chain 2-oxo acid dehydrogenase complex remained essentially unchanged over a 12-day period (Fig. 1). However, treatment with insulin, dexamethasone and 3-isobutyl-1-methylxanthine resulted in an immediate and marked increase in

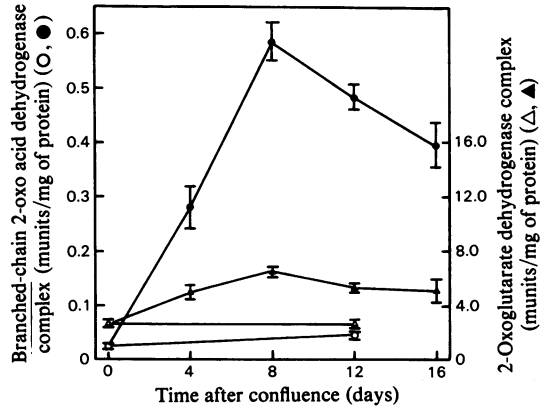

Fig. 1. *Hormone-induced changes in specific activities of branched-chain 2-oxo acid and 2-oxoglutarate dehydrogenase complexes in differentiating 3T3-L1 cells*

Monolayer cultures of 3T3-L1 preadipocytes at confluence (day 0) were incubated either in complete medium containing hormones (●, ▲) or in complete medium only (○, △) as described in the Materials and methods section. Activities of 2-oxo acid dehydrogenase complexes were measured at various times as indicated. Leucine-activated branched-chain 2-oxo acid dehydrogenase complex activity: ○, 3T3-L1 preadipocytes; ●, 3T3-L1 adipocytes. 2-Oxoglutarate dehydrogenase complex activity: △, 3T3-L1 preadipocytes; ▲, 3T3-L1 adipocytes. Values are means ± S.E.M. for four dishes.

Table 1. *Specific activities of 2-oxo acid dehydrogenase complexes in 3T3-L1 preadipocytes and adipocytes*

Confluent monolayer cultures of 3T3-L1 preadipocytes were maintained in complete medium with or without hormones for 8 days. Activities of the three 2-oxo acid dehydrogenase complexes before and after activation were measured as described in the Materials and methods section. Values are means ± S.E.M. for three dishes.

	Activity (munits/mg of protein)	
Enzyme	3T3-L1 preadipocytes	3T3-L1 adipocytes
Branched-chain 2-oxo acid dehydrogenase complex		
Unactivated	0.046 ± 0.002	0.758 ± 0.005
Leucine (2.5 mM)-activated	0.101 ± 0.012	1.034 ± 0.011
Pyruvate dehydrogenase complex		
Unactivated	0.95 ± 0.07	8.96 ± 1.24
Dichloroacetate (5 mM)-activated	6.26 ± 1.07	42.11 ± 3.03
2-Oxoglutarate dehydrogenase complex	1.32 ± 0.08	3.48 ± 0.18

specific activity of the leucine-activated branched-chain 2-oxo acid dehydrogenase complex in differentiating 3T3-L1 adipocytes, with maximal increase occurring at day 8 of the treatment. A decline in specific activity of this complex was observed beyond day 8 in 3T3-L1 adipocytes. This profile is similar in time span to that observed previously for the pyruvate dehydrogenase complex activity (Hu *et al.*, 1983). By comparison, the specific activity of the 2-oxoglutarate dehydrogenase complex increased by about 2-fold in 3T3-L1 adipocytes on day 8 of the treatment (Fig. 1).

Activities of components of the complexes

We have previously shown that the increase in specific activity of the pyruvate dehydrogenase complex in differentiated 3T3-L1 adipocytes was the result of increased content of two subunits of the pyruvate dehydrogenase (E_1) component (Hu *et al.*, 1983). To determine if other components of the complexes were also affected, we measured specific activities of E_2 and E_3 components in 3T3-L1 preadipocytes as well as in adipocytes. Table 2 shows that the activities of the three components of the pyruvate dehydrogenase complex increased approx. 5-fold in 3T3-L1 adipocytes compared with 3T3-L1 preadipocytes. Similarly, the E_1 component of the branched-chain 2-oxo acid dehydrogenase complex assayed with leucine activation showed a 17-fold increase in 3T3-L1 adipocytes compared with 3T3-L1 preadipocytes (Table 2). However, the activities of E_2 (transacylase) and E_3 (dehydrogenase) components of this complex increased only 4–5-fold in 3T3-L1 adipocytes compared with corresponding activities in 3T3-L1 preadipocytes. E_3 is a component common to all three complexes, and hence its combined activity is far greater than that of either the E_1 or E_2 component in any complex (Table 2). In this study we did not measure the activities of the components of the 2-oxoglutarate dehydrogenase complex, because its activity was induced marginally in 3T3-L1 adipocytes during differentiation.

General comments

Previous studies on the branched-chain 2-oxo acid dehydrogenase complex provide convincing evidence that this multienzyme complex is regulated by phosphorylation/dephosphorylation (Hughes & Halestrap, 1981; Odessey, 1982; Harris *et al.*, 1982; Buxton & Olson, 1982), in similar fashion to the pyruvate dehydrogenase complex (Reed *et al.*, 1980). We have used leucine- and dichloroacetate-treated cells to provide an opportunity to measure 'total' branched-chain 2-oxo acid dehydrogenase complex and pyruvate dehydrogenase complex activities respectively. A possible mechanism for the leucine (or 4-methyl-2-oxopentanoate)-dependent activation of the branched-chain 2-oxo acid dehydrogenase complex (Parker & Randle, 1978; Frick *et al.*, 1981) is that 4-methyl-2-oxopentanoate, the 2-oxo acid derived from leucine, inhibits phosphorylation of the α-subunit of the E_1 component (Hughes & Halestrap, 1981). It has been shown that 4-methyl-2-chloropentanoate, an analogue of 4-methyl-2-oxopentanoate, inhibits incorporation of ^{32}P from [γ-^{32}P]ATP into the complex, rendering the enzyme fully active (Harris *et al.*, 1982). Thus the assay with leucine-treated 3T3-L1 cells provided an estimate of total branched-chain 2-oxo acid dehydrogenase complex (Table 1). A similar approach has been adopted to measure total pyruvate dehydrogenase complex activity in dichloroacetate-treated fibroblasts (Sheu *et al.*, 1981).

The studies presented here show that specific activity of the leucine-activated branched-chain 2-oxo acid dehydrogenase complex, like that of the

Table 2. *Activities of components of branched-chain 2-oxo acid and pyruvate dehydrogenase complexes in 3T3-L1 preadipocytes and adipocytes*

Confluent monolayer cultures of 3T3-L1 preadipocytes were maintained in complete medium with or without hormones for 8 days. Activities of E_1, E_2 and E_3 components of both enzyme complexes were measured as described in the Materials and methods section. Values are means ± S.E.M. for three dishes.

	Activity (munits/mg of protein)			
	E_1			
	Unactivated	Activated	E_2	E_3
Branched-chain 2-oxo acid dehydrogenase complex				
Preadipocytes	0.005 ± 0.002	0.008 ± 0.004	0.120 ± 0.034	69.5 ± 2.2
Adipocytes	0.098 ± 0.004	0.138 ± 0.002	0.580 ± 0.094	268.6 ± 10.3
Pyruvate dehydrogenase complex				
Preadipocytes	–	0.18 ± 0.05	2.78 ± 0.06	69.5 ± 2.2
Adipocytes	–	0.83 ± 0.20	12.26 ± 0.46	268.6 ± 10.3

pyruvate dehydrogenase complex (Hu *et al.*, 1983), is increased approx. 10-fold in 3T3-L1 adipocytes during hormone-induced differentiation. This increase involves not only the E_1 component but also the E_2 and E_3 components. Since activities of E_2 and E_3 components are not known to be modulated by post-translational covalent modification, the activity measurements probably represent a realistic assessment of the relative amounts of these components in 3T3-L1 cells. The three catalytic components are present, in the pyruvate dehydrogenase complex, in a fixed proportion relative to each other (Reed *et al.*, 1980). Therefore increases of similar magnitude in the three components activities of the pyruvate dehydrogenase complex support our earlier suggestion that the activities of all three components of this complex are increased in 3T3-L1 adipocytes (Hu *et al.*, 1983). Similarly, the increase in leucine-activated branched-chain 2-oxo acid dehydrogenase complex activity is concordant with increases in its component activities in 3T3-L1 adipocytes.

The increase in the activity of branched-chain 2-oxo acid dehydrogenase complex is consistent with the lipogenic role of this complex, similar to that of the pyruvate dehydrogenase complex, in providing acetyl-CoA for the synthesis of lipids during adipocyte differentiation (Hu *et al.*, 1983). Leucine and isoleucine are shown to be precursors for the synthesis of triacylglycerols and cholesterol in adipose tissue and muscle (Rosenthal *et al.*, 1974). Since the branched-chain 2-oxo acid dehydrogenase complex is the first committed step in the catabolism of the branched-chain amino acids, the increased activity of the complex in 3T3-L1 adipocytes would enhance the flux through these catabolic pathways.

Although biochemical mechanisms underlying hormone-mediated differentiation of 3T3-L1 cells have not been elucidated, parallelism of magnitude and kinetics of induction of activities of the pyruvate dehydrogenase complex and the branched-chain 2-oxo acid dehydrogenase complex suggest that hormonal regulation of these two complexes may occur by the same or similar mechanism(s). The differentiating 3T3-L1 adipocyte may provide a useful model for studying hormonal modulation of 2-oxo acid dehydrogenase complexes. In this system, hormonal and nutritional perturbations can be precisely controlled during adipocyte differentiation.

This study was supported, in part, by NIH Grants AM-20478 and AM-26758 and by March of Dimes Birth Defects Foundation, Grant 1-796.

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