Modulation by dexamethasone of the pyruvate dehydrogenase-complex activity in 3T3-L1 adipocytes

Mulchand S. PATEL, Cindy RAEFSKY, Chii-Whei C. HU and Lap HO
Department of Biochemistry, Case Western Reserve University School of Medicine,
Cleveland, OH 44106, U.S.A.

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Chronic exposure of 3T3-L1 pre-adipocytes to dexamethasone plus 3-isobutyl-1-methylxanthine (IBMX) with or without insulin caused a significant increase in the specific activity of 'total' pyruvate dehydrogenase complex (PDC) and in the percentage of the 'active' form of the complex compared with cells exposed to a chronic insulin treatment or an acute treatment (2 days) with dexamethasone plus IBMX. In acute-drug-switch-over experiments, dexamethasone also caused an increase in the percentage of 'active' PDC in 3T3-L1 adipocytes. The results show that, in 3T3-L1 adipocytes, dexamethasone, even in the absence of insulin, increases the proportion of PDC in its 'active' form. The mechanism of the dexamethasone effect remains to be investigated.

PDC is regulated by its reaction products, acetyl-CoA and NADH, and by a phosphorylation-dephosphorylation mechanism (Reed, 1974; Denton *et al.*, 1975). Phosphorylation of the α -subunit of the PDH component of PDH kinase renders it inactive, whereas dephosphorylation of phospho-PDH by PDH phosphatase restores the catalytic activity of PDH and hence PDC (Linn *et al.*, 1969; Reed, 1974). Acute regulation of PDH by phosphorylation-dephosphorylation is also under insulin control, although the precise mechanism by which insulin increases the proportion of PDC in the 'active' form is not well understood. A putative mediator released from the plasma membrane in response to insulin (Seals & Jarett, 1980; Seals & Czech, 1981; Kiechle *et al.*, 1981; Saltiel *et al.*, 1982) appears to act at the level of PDH phosphatase (Kiechle *et al.*, 1981).

We recently demonstrated that, during drug-induced differentiation of 3T3-L1 pre-adipocytes to 3T3-L1 adipocytes, the specific activity of 'total' PDC increased approx. 6-fold without any significant alteration in the percentage of the 'active' form of PDC (Hu *et al.*, 1983). Differentiation was initiated by combined treatment with insulin, dexamethasone and IBMX (Hu *et al.*, 1983). We now report individual action of these drugs at the level of 'total' activity and the percentage of the

'active' form of PDC in 3T3-L1 cells during differentiation.

Methods

Cell culture and differentiation

3T3-L1 pre-adipocytes were grown in a supplemented Eagle's minimal essential medium as described previously (Hu *et al.*, 1983). The medium was changed every day, and when monolayer cultures reached confluence (designated as 'day 0'), the cells were treated with the following drugs, either singly or in various combinations: insulin (10 μ g/ml), dexamethasone (0.25 μ M) and IBMX (0.5 mM). When the pre-adipocytes were treated with drugs, the medium was supplemented with biotin (8 μ g/ml) (Hu *et al.*, 1983). To examine the possible influence of biotin on 'total' PDC activity in confluent 3T3-L1 pre-adipocytes, cells were maintained in the presence or absence of biotin (8 μ g/ml) for 7 days. This treatment had no significant effect on 'total' PDC activity (5.83 ± 0.14 and 5.03 ± 0.57 munits/mg of protein in the absence and presence of biotin in the medium respectively; means $\pm$ S.E.M.; three dishes per treatment). We also studied the lack of biotin on 'total' PDC activity in differentiating 3T3-L1 cells. Although the addition of avidin (0.1 μ M) to the medium renders biotin unavailable to cells and abolishes the increase in pyruvate carboxylase activity in 3T3-L1 adipocytes (Freitag & Utter,

Abbreviations used: PDC, pyruvate dehydrogenase complex; PDH, pyruvate dehydrogenase; IBMX, 3-isobutyl-1-methylxanthine.

1980; C.-W. C. Hu & M. S. Patel, unpublished work), this treatment had a small effect (12% inhibition) on the specific activity of 'total' PDC activity (21.2 ± 0.7 and 18.7 ± 0.6 munits/mg of protein in cells treated with drugs and drugs-plus-avidin respectively). Biotin was present in all drug treatments. Therefore any effect observed on 'active' and 'total' PDC activity was due to added drugs and not due to biotin.

PDC assay

To maintain the phosphorylation-dephosphorylation state of the complex *in situ*, cell monolayers were washed twice with phosphate-buffered saline (Dulbecco & Vogt, 1954) containing 5mM-dichloroacetate (PDH kinase inhibitor) (Whitehouse *et al.*, 1974), 100mM-NaF (to inhibit phosphatase) and 10mM-EDTA, and then scraped into the same buffer (Buxton & Olson, 1982). After centrifugation, cells were suspended in the same buffer devoid of EDTA (to minimize chelation of Mg^{2+} and Ca^{2+} in the assay system). A portion of this suspension was immediately frozen and used later to assay for 'active' PDC activity. The remainder was centrifuged and cells were resuspended in phosphate-buffered saline containing 5mM-dichloroacetate, 10mM- $MgCl_2$ and 1mM- $CaCl_2$ to remove NaF and to provide Mg^{2+} and Ca^{2+} for phosphatase activity. Cells were washed once again with this solution and were suspended in this solution and incubated for 15min at 37°C to activate (dephosphorylate) PDC completely, and then frozen immediately. All cell preparations were freeze-thawed three times before enzyme activity was assayed by monitoring the decarboxylation of [$1-^{14}C$]pyruvate to $^{14}CO_2$ as described by Sheu *et al.* (1981). The PDC activity measured in dichloroacetate-treated cells represents 'total' activity of the complex in the present study. The percentage of 'active' PDC is calculated as (active PDC/total PDC activity) $\times 100$ for a given cell sample. One munit of activity represents the decarboxylation of 1 nmol of [$1-^{14}C$]pyruvate/min at 37°C. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Statistical analysis was performed by using the Student's *t* test.

Dichloroacetate is a potent inhibitor of PDH kinase (Whitehouse *et al.*, 1974), whereas NaF, a phosphoprotein phosphatase inhibitor, is widely used in the range of 50–100mM to inhibit PDH phosphatase in rat tissues (Kiechle *et al.*, 1981; Seals & Czech, 1981; Buxton & Olson, 1982). To demonstrate that the dichloroacetate/NaF treatment was adequate to maintain the phosphorylation-dephosphorylation state of the complex *in situ* by inhibiting both PDH kinase and phosphatase activities, 3T3-L1 adipocytes suspended in phos-

phate-buffered saline containing 5mM-dichloroacetate and 100mM-NaF were either frozen immediately, maintained at 4°C for 10min or incubated at 37°C for 15min before freezing. 'Active' PDC activity in cells with these treatments respectively was 100% (5.24 ± 0.57 munits/mg of protein; three dishes), $104.2 \pm 3.4\%$ and $91.5 \pm 3.3\%$. The results show that, in the presence of these two inhibitors, the phosphorylation state of 'active' PDC remained unaltered in cells even during an extended period of handling. When these inhibitors were not added to phosphate-buffered saline, the phosphorylation state of PDC was significantly increased, as judged by marked reduction in 'active' PDC activity (results not shown).

In another set of experiments, NaF-mediated inhibition of PDH phosphatase was monitored. In differentiated 3T3-L1 cells, PDC was first dephosphorylated in the presence of 5mM-dichloroacetate for 15min ('total' PDC activity 35.7 ± 4.7 munits/mg of protein; three dishes). Portions of these cell suspensions were then washed twice with phosphate-buffered saline to remove dichloroacetate, suspended in buffer containing 100mM-NaF and 10mM- $MgCl_2$ and incubated for 15min at 37°C to inhibit phosphatase and to allow kinase to phosphorylate, and hence inactivate, the complex. We observed approx. 90% inhibition (4.1 ± 1.5 munits/mg of protein; three dishes) of PDC activity, showing that NaF treatment was effective in inhibiting PDH phosphatase.

To establish that the dichloroacetate treatment completely inhibited kinase and allowed endogenous phosphatase to dephosphorylate the complex before the measurement of 'total' PDC activity, we compared this treatment with exogenously added PDH phosphatase. Portions of one set of 3T3-L1 adipocyte cell suspensions were treated with 5mM-dichloroacetate as described above, whereas another set of portions were freeze-thawed once and homogenized to disrupt cells before the addition of highly purified bovine kidney PDH phosphatase (0.88 unit/ml of incubation medium). After 15min incubation at 37°C, 'total' PDC activity was 17.1 ± 1.8 and 17.3 ± 1.0 munits/mg of protein ($n=4$) in the dichloroacetate- and PDH phosphatase-treated cells respectively, showing the adequacy of the dichloroacetate treatment for the measurement of 'total' PDC activity in 3T3-L1 cells.

Results and discussion

When confluent 3T3-L1 pre-adipocytes are differentiated into 3T3-L1 adipocytes by exposing them to insulin, dexamethasone and IBMX for 2 days and then to insulin alone for an additional 6 days, the 'total' PDC activity increases approx.

6-fold (Hu *et al.*, 1983). However, the percentage of 'active' PDC is unaffected (Hu *et al.*, 1983). These findings are confirmed in the present study (Table 1, treatments 1 and 2). Exposure of confluent 3T3-L1 pre-adipocytes to insulin alone had little effect on the specific activity of 'total' PDC during the first 12 days, but thereafter an induction (approx. 6-fold) of 'total' PDC activity occurred by day 17 (Fig. 1). Exposure of confluent 3T3-L1 pre-adipocytes to dexamethasone plus IBMX for only the first 2 days caused a significant increase in the specific activity of 'total' PDC as early as day 4, but the maximum stimulation was only 4-fold on day 12 (Fig. 1). In contrast, chronic exposure of 3T3-L1 cells to dexamethasone plus IBMX in the presence or absence of insulin (Table 1, treatments 3 and 4 respectively) caused a larger induction of 'total' PDC activity, and also significantly increased the percentage of the 'active' form of PDC on days 9 and 15 compared with a brief exposure to insulin, dexamethasone and IBMX followed by a prolonged exposure to insulin alone (Table 1, treatment 2).

To examine further the effect of dexamethasone plus IBMX on the proportion of PDC in the 'active' form, two different types of drug-switch-over experiments were performed. In the first experiment, confluent 3T3-L1 pre-adipocytes were grown in the medium containing insulin, dexamethasone and IBMX for the first 2 days, and grown in medium containing insulin alone for an additional 5 days. On day 7 of the experiment, one set of culture dishes were continued in this medium, while the other set received medium

Fig. 1. Effect of insulin, dexamethasone and IBMX on the specific activity of 'total' PDC in 3T3-L1 pre-adipocytes. Monolayer cultures of 3T3-L1 pre-adipocytes at confluence were subjected to one of the following treatments: ○, no drug added; ●, insulin (10 µg/ml); △, 0.25 µM-dexamethasone and 0.5 mM-IBMX for the first 2 days only; ▲, insulin (10 µg/ml), 0.25 µM-dexamethasone and 0.5 mM-IBMX for the first 2 days and then insulin (10 µg/ml) alone for the duration of the treatment. Unless indicated, drugs were present in the medium for the duration of the treatment. 'Total' PDC activity was measured in cells at various time periods as indicated. Results are the means ± S.E.M. for three separate dishes.

Table 1. Effect of drug treatments on the activity of 'active' and 'total' PDC in 3T3-L1 adipocytes. Monolayer cultures of 3T3-L1 pre-adipocytes at confluence were subjected to one of the four treatments as described in the text. Drug concentrations were: insulin, 10 µg/ml; dexamethasone, 0.25 µM; and IBMX, 0.5 mM. Unless indicated, drugs were present in the medium for the duration of the treatment. '% Active' was calculated on the basis of individual observations before calculation of the mean value. The results are means ± S.E.M. for four or five separate dishes.

Treatment		Type of activity	Period of treatment (days)	PDC activity (munits/mg of protein)		
No.	Drugs			0	9	15
1	None	Active		0.6 ± 0.1	—	—
		Total		4.8 ± 0.5	—	—
		% Active		13.6 ± 2.1	—	—
2	Insulin + dexamethasone + IBMX for the first 2 days and then insulin alone	Active		—	3.0 ± 0.4	7.5 ± 0.4
		Total		—	24.2 ± 2.2	32.0 ± 3.7
		% Active		—	12.4 ± 1.2	22.6 ± 1.9
3	Insulin + dexamethasone + IBMX	Active		—	12.6 ± 0.4	18.5 ± 1.3
		Total		—	35.6 ± 2.2	39.6 ± 3.7
		% Active		—	36.0 ± 2.9	53.4 ± 3.8
4	Dexamethasone + IBMX	Active		—	17.5 ± 1.4	—
		Total		—	34.2 ± 3.2	—
		% Active		—	41.4 ± 3.7	—

containing insulin, dexamethasone and IBMX for 2 days. On day 9 there were no significant differences in 'total' PDC activity (Table 2, expt. 1). However, 'active' PDC activity increased significantly in the cells exposed to insulin, dexamethasone and IBMX (Table 2, expt. 1), resulting in a 2-fold increase in the percentage of 'active' PDC (Table 2, expt. 1).

In the second experiment, confluent 3T3-L1 pre-adipocytes were grown in medium containing insulin, dexamethasone and IBMX for 7 days. At that point one set of culture dishes was further maintained with the same drug treatment for 2 additional days. The medium from the other set of dishes was removed and the dishes were rinsed once with sterile phosphate-buffered saline to remove unbound drugs. The cells in these dishes were then maintained in medium containing insulin alone for 2 days. A small, but significant, increase was observed in 'total' PDC activity in the cells exposed to insulin alone compared with those subjected to the combined drug treatment of insulin, dexamethasone and IBMX during the last 2 days. 'Active' PDC activity decreased significantly in the cells exposed to insulin alone compared with those exposed to the combined drug treatment (Table 2, expt. 2). This decrease was also significant when the results were expressed as the percentage of 'active' PDC (Table 2, expt. 2).

Our results provide two significant information on PDC activity in 3T3-L1 adipocytes. First, chronic exposure of cells to dexamethasone plus IBMX increases the specific activity of 'total' PDC to a level greater than that observed with an acute exposure (2 days) of cells to these two compounds and insulin (Table 1). Secondly, the proportion of PDC in its 'active' form (percentage of 'active' PDC) increases in 3T3-L1 adipocytes exposed chronically to dexamethasone plus IBMX with or without insulin compared with those briefly ex-

posed to dexamethasone plus IBMX (Table 1). Acute exposure (2 days) of confluent 3T3-L1 pre-adipocytes to dexamethasone plus IBMX in the presence or absence of insulin increases the insulin receptor concentration in 3T3-L1 adipocytes (Reed & Lane, 1980; Ronnett *et al.*, 1982). Binding of insulin to its receptor at the plasma membrane is reported to generate a putative mediator that causes an increase in the percentage of 'active' PDC in the mitochondria (Seals & Jarett, 1980; Seals & Czech, 1981; Kiechle *et al.*, 1981; Saltiel *et al.*, 1982) by stimulating PDH phosphatase (Kiechle *et al.*, 1981). In the present study, as in previous studies on 3T3-L1 pre-adipocyte differentiation (Reed & Lane, 1980; Ronnett *et al.*, 1982; Hu *et al.*, 1983), a very high concentration of insulin (10 µg/ml) was used. Since insulin causes down-regulation of the insulin-receptor concentration in differentiating 3T3-L1 adipocytes (Ronnett *et al.*, 1982), a reduction in the receptor concentration may, in part, explain a decrease in the percentage of 'active' PDC in 3T3-L1 adipocytes exposed to high insulin concentrations for 2 days (Table 2, expt. 2). Alternatively, it may be that higher concentrations of insulin cause a release of an inhibitory effector of PDC, as has been shown with adipocyte plasma membranes (Seals & Czech, 1981; Saltiel *et al.*, 1982).

On the other hand, dexamethasone increases insulin-binding capacity of cultured hepatocytes by stimulating the synthesis of insulin receptors (Salhanick *et al.*, 1983). Differentiation of 3T3-L1 pre-adipocytes after exposure to dexamethasone plus IBMX for 2 days caused an increase in insulin-binding capacity of 3T3-L1 adipocytes (Rubin *et al.*, 1978). In the present study, when confluent 3T3-L1 pre-adipocytes were chronically exposed to dexamethasone plus IBMX (in the absence of insulin), the 'active' PDC represented approx. 50% of 'total' PDC activity. Since, in this experiment, insulin present in the medium [derived from 10%

Table 2. Effect of a drug-switch-over treatment on 'active' and 'total' PDC activity and percentage of 'active' PDC in 3T3-L1 adipocytes

Monolayer cultures of 3T3-L1 pre-adipocytes at confluence were exposed to drugs for 7 days, then for the last 2 days of the experiment the drug treatment was changed in some dishes as indicated. The results are the means $\pm$ S.E.M. for four or five separate dishes. *P* values are shown in parentheses; ns, not significant (*P* > 0.05).

Expt.	Drug treatment		PDC activity		
	Day 0-day 7	Day 7-day 9	Active	Total	% Active
1	Insulin + dexamethasone + IBMX for the first 2 days and then insulin alone for 5 days	Insulin alone	3.0 $\pm$ 0.4	24.2 $\pm$ 2.2	12.4 $\pm$ 1.2
		Insulin + dexamethasone + IBMX	5.9 $\pm$ 0.5 (<i><</i> 0.005)	26.4 $\pm$ 1.8 (ns)	24.9 $\pm$ 2.0 (<i><</i> 0.001)
2	Insulin + dexamethasone + IBMX for the first 7 days	Insulin + dexamethasone + IBMX	10.8 $\pm$ 0.7	44.0 $\pm$ 1.0	26.0 $\pm$ 1.3
		Insulin alone	8.0 $\pm$ 0.3 (<i><</i> 0.01)	49.7 $\pm$ 1.8 (<i><</i> 0.05)	15.4 $\pm$ 1.2 (<i><</i> 0.001)

(v/v) fetal-calf serum] was negligible (A. Caliendo & M. S. Patel, unpublished work), the increase in the percentage of 'active' PDC was not caused by the binding of insulin to its receptor and generation of a putative stimulator of PDH phosphatase.

Administration of dexamethasone to rats (50 µg/day) for 4 days has been reported to cause a reduction in both 'total' and insulin-stimulated 'active' PDC activity in adipose tissue, and a decrease in the capacity of adipose-tissue plasma membrane to generate PDH activator in response to insulin (Begum *et al.*, 1984). In contrast, exposure of 3T3-L1 pre-adipocytes to dexamethasone plus IBMX resulted in an increase in the concentration of insulin receptors and not in insulin resistance (Reed & Lane, 1980; Ronnett *et al.*, 1982). Furthermore, 3T3-L1 adipocytes exposed to dexamethasone plus IBMX had higher levels of 'total' PDC activity and also exhibited a higher percentage of the 'active' form of PDC than did cells exposed to insulin. Since PDC activity is modulated by a kinase and a phosphatase specific for PDH, a change in the balance of these regulatory enzymes may be expected to alter the proportion of PDC in its 'active' form in cells grown in medium containing dexamethasone and IBMX. Additional studies are needed to explore potential effects of dexamethasone on the activities of PDH kinase and PDH phosphatase in 3T3-L1 adipocytes.

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