Regulation of pyruvate carboxylase in 3T3-L1 cells

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When 3T3-L1 fibroblasts differentiate to adipocytes, the specific activity of pyruvate carboxylase (PC) increases about 25-fold in parallel with its intracellular protein concentration. The increase in PC protein concentration is accompanied by a 9–10-fold increase in the relative abundance of 4.2 kb PC mRNA measured by Northern-blot analysis using a cDNA probe encoding a segment of the PC gene of 3T3-L1 adipocytes. The effects of cyclic AMP (cAMP) alone and together with insulin on levels of cellular protein, PC activity, PC protein and on the relative abundance of PC mRNA were examined in mature 3T3-L1 adipocytes. Adipocytes exposed to cAMP for 24 h exhibited a 25 % decrease in cellular protein and marked decreases in enzyme activity (88 %) and PC mRNA abundance (98 %) compared with untreated adipocyte controls. After 48 h of exposure to cAMP, PC activity and PC mRNA diminished to levels approaching

their detection limits. When exposed to medium containing cAMP plus insulin, adipocyte enzyme activity and PC mRNA declined more slowly during the first 24 h exposure (about 20 % decrease) but after 48 h fell to values comparable with those of adipocytes exposed to cAMP alone. Despite these decreases in enzyme activity, the PC protein content of adipocytes treated with cAMP alone or cAMP plus insulin are nearly identical with that of control adipocytes. The inactivation of PC in cAMP-treated adipocytes does not involve loss of the prosthetic group from the holoenzyme. Cross-linking experiments suggest that the spatial arrangement of protomers in inactive PC may differ from that in the active tetrameric enzyme. Data presented suggest that, in addition to inducing inactivation, cAMP may also regulate adipocyte PC by decreasing transcription of the PC gene and/or enhancing the rate of degradation of PC mRNA.

INTRODUCTION

Pyruvate carboxylase (PC) (EC 6.4.1.1) is a key lipogenic enzyme that provides acetyl-CoA and NADPH for the *de novo* biosynthesis of fatty acids in adipocytes (Mackall and Lane, 1977; Freytag and Utter, 1980). The *in vitro* differentiation of 3T3-L fibroblasts ('preadipocytes') into cells possessing the characteristics of adipocytes has been used as a model system to study biochemical changes occurring during cell differentiation (Ailhaud et al., 1992; Spiegelman et al., 1993). The degree and rate of conversion of resting 3T3-L fibroblasts into adipocytes can be enhanced by insulin (Green and Kehinde, 1975), 1-methyl-3-isobutylxanthine (MIX) (Russell and Ho, 1976) and glucocorticoids (Rubin et al., 1978) and it is suppressed by adrenaline and cyclic AMP (cAMP) (Green and Kehinde, 1974). Therefore these cells provide a suitable model system for investigation of the molecular mechanisms of regulation of PC by insulin and cAMP.

The hormonal mechanisms that regulate the total amount of PC activity in liver (or kidney) during starvation, refeeding, diabetes, cortisol administration or manipulation of thyroid status are not completely understood in molecular terms. It is known only that alterations in enzyme activity, as observed during 3T3-L cell differentiation, are associated with parallel changes in the total amount of PC protein (Weinberg and Utter, 1979, 1980). Preliminary results of Freytag and Utter (1980) indicated that addition of cAMP to cultures of 3T3-L1 adipocytes caused a marked fall in PC activity but how cAMP elicited this effect remained to be defined.

We have recently reported the complete covalent structure of adipocyte PC as deduced from the nucleotide sequence of overlapping cDNA clones (Zhang et al., 1993), and there is

evidence suggesting that control of PC gene expression during the conversion of 3T3-L1 fibroblasts into adipocytes may be exerted at a pretranslational level (Angus et al., 1981; Freytag and Collier, 1984). Our use of specific cDNA clones as probes to investigate the molecular mechanisms of hormonal regulation of PC gene expression in 3T3-L1 cells shows that the relative abundance of PC mRNA increases 9–10-fold with a concomitant 23-fold increase in PC protein during the conversion of 3T3-L1 fibroblasts into adipocytes. The decrease in PC activity in adipocytes after their exposure to cAMP does not result from a decrease in PC protein but is accompanied by a precipitous fall in the relative abundance of PC mRNA. Thus cAMP not only exerts its effects on adipocyte PC by enzyme inactivation but may also affect PC gene transcription and/or stability of the encoding mRNA.

MATERIALS AND METHODS

Materials

Pyruvate, phenylmethanesulphonyl fluoride (PMSF), acetyl-CoA, 8-bromo-cAMP, ATP, NADH, malate dehydrogenase (EC 1.1.1.37), BSA (essentially fatty acid free), rabbit anti-goat IgG-alkaline phosphatase conjugate, guanidinium isothiocyanate, lauryl sarcosine, bovine insulin, MIX, dexamethasone (DEX), penicillin, streptomycin, Mops, sucrose, antifoam A, salmon sperm DNA, dithiothreitol and prestained molecular-mass standards were purchased from Sigma. All reagents for SDS/PAGE, immunoblotting and DNA and RNA ladders were purchased from Bio-Rad Laboratories. Ethylene glycolbis(succinimidyl succinate) (EGS) was purchased from Pierce. All other chemicals were of the highest purity commercially available.

Abbreviations used: cAMP, adenosine 3',5'-phosphate; DEX, dexamethasone; EGS, ethylene glycolbis(succinimidyl succinate); MIX, 1-methyl-3-isobutylxanthine; PMSF, phenylmethanesulphonyl fluoride.

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Cell culture

Growth of cells

3T3-L1 cells obtained from ATCC were grown in Dulbecco's modified Eagle's medium containing 100 units of penicillin/ml, 100 μ g of streptomycin/ml and 10 % fetal calf serum (Green and Kehinde, 1974). The cells were cultured in a 37 °C humidified incubator with an atmosphere of 95 % air/5 % CO₂ and were subcultured every 3 days.

Induction of adipose conversion

At 2 days after confluence of monolayers, the medium was replaced with a similar medium containing 0.5 mM MIX, 0.25 μ M DEX and 10 μ g/ml insulin for 3 days in order to initiate maximum and uniform lipogenic differentiation. After this treatment, the cells were fed with growth medium supplemented with 1 μ g/ml insulin.

Preparation of cell lysate

Cells were rinsed twice with 5 ml portions of cold washing buffer (0.25 M sucrose, 0.02 M Mops, 3 mM EDTA, 0.5 mM PMSF) and then harvested in 0.8 ml of EDTA/Mops solution (3 mM EDTA, 0.02 M Mops, 0.5 mM PMSF) by scraping with a plastic policeman. Cells were frozen and thawed twice in liquid N₂. After sonication of the cell suspension, acetyl-CoA was added immediately to the cell lysate to a final concentration of 0.8 mM (Freytag and Utter, 1980).

Enzyme assays

PC activity was measured at 37 °C by a radioisotopic CO₂-fixation assay in the presence of Triton X-100 (Atkin et al., 1979) as described (Ahmad et al., 1986); 1 munit of enzyme activity represents the synthesis of 1 nmol of oxaloacetate/min and the specific activity is defined as munits/mg of protein.

Protein determination

Protein was determined by the method of Lowry et al. (1951). Samples were centrifuged at 10000 *g* for 20 min at 0 °C after colour development to remove fat (Mackall et al., 1976).

Preparation of total RNA from cultured cells

Cell monolayers were rinsed twice with cold PBS solution and RNA was isolated by the guanidinium isothiocyanate technique (Chirgwin et al., 1979). The RNA pellet obtained after centrifuging cell lysate through 5.7 M CsCl solution [115000 *g*_{max}. (32000 rev./min) using an SW39 rotor in a Spinco Ultracentrifuge 2B for 22 h at 18 °C] was dissolved in TES buffer (10 mM Tris/HCl, pH 7.4, 5 mM EDTA, 1 % SDS). RNA was precipitated by the addition of sodium acetate and ethanol. It was then dissolved in water and its concentration determined by measuring *A*₂₆₀.

Analysis of RNA by Northern-blot hybridization

RNA was separated by electrophoresis using denaturing agarose gel containing 2.2 M formaldehyde. The separated RNA was transferred to a nitrocellulose membrane. Hybridization of baked filter was performed in solution containing 50 % deionized formamide, 25 mM sodium phosphate, pH 6.8, 5 × SSC (where 1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0), 5 × Denhardt's solution [where 1 × Denhardt's solution contains

0.02 % Ficoll, 0.02 % BSA and 0.02 % poly(vinyl pyrrolidone)], 10 % dextran sulphate, 100 μ g/ml salmon sperm DNA, 0.1 % SDS and 1 × 10⁶ c.p.m./ml ³²P-labelled double-stranded cDNA probe at 42 °C overnight. The filters were washed extensively in 1 × SSC, 0.25 × SSC and 0.1 % SDS at 65 °C.

The relative abundance of PC mRNA was estimated by measuring the density of PC mRNA bands on films using a Soft Laser Scanning Densitometer (model S L-504TL; Biomed Instruments). Films were scanned with the gain adjusted such that the peak height for the most prominent mRNA band was between 85 and 95. The peaks were traced on high-quality graph paper. The tracings were cut out and weighed. A linear relationship was obtained between the total RNA applied to the gel and the area under the peak in the densitometric scan over the range 0.5–10 μ g for mature adipocytes.

Uneven loading of RNA samples analysed was corrected by densitometry of ethidium bromide-stained 28 S ribosomal bands on photographic negatives (Sandouk et al., 1993). The observed variation in PC mRNA signals quantified in three independent experiments was approx. 10 %.

Western-blot analysis

Antibodies against homogeneous rat liver PC were raised in goats. The immune serum was processed and stored as described previously (Ahmad et al., 1979). Rat liver PC was covalently coupled to Sepharose for the purification of anti-PC antibodies. Affinity-purified antibodies were used in Western blotting.

PC (130 kDa) content of 3T3-L1 cells was estimated by Western-blot analysis as described previously (Ahmad et al., 1986).

Detection of apo- and holo-forms of PC in cell extracts

Cell homogenate (40 μ g of protein/reaction) was reduced and denatured by boiling in a solution containing 0.8 % SDS and 1 % 2-mercaptoethanol for 10 min. After addition of 10 μ g of avidin, the mixture was incubated at 37 °C for 15 min and then applied to a 7.5 % polyacrylamide slab gel. Samples were electrophoresed and subjected to Western-blot analysis.

Cross-linking of PC in cell extracts

A portion (11 μ l) of cell homogenate (30–50 μ g of protein) was mixed with 8 μ l of 2.5 × reaction buffer (where 1 × reaction buffer is 20 mM sodium phosphate plus 150 mM NaCl, pH 7.5). Freshly prepared solution of EGS (20 mM in dimethyl sulphoxide; 1 μ l) was added to the cell homogenate and the mixture incubated at room temperature for 2.5 h. Reaction was stopped by adding 5 × stop solution (where 1 × stop solution is 50 mM Tris/HCl; pH 8.0 plus 22.5 mM glycine). Protein samples were reduced and denatured and then subjected to Western-blot analysis.

All the results given were obtained from triplicate determinations on three to seven independent experiments. Variations between triplicate culture flasks were within ±10 % whereas variations between independent experiments were within ±20 %.

RESULTS AND DISCUSSION

The conversion of confluent non-differentiated 3T3-L1 cells into mature adipocytes is accompanied by a 320-fold increase in PC activity (Table 1). Depending on the ability of 3T3-L1 cells to differentiate, the cellular protein content of adipocytes increased 5–10-fold in comparison with preadipocytes (Katsuri and Wakil, 1983). We observed a 14-fold increase in the soluble protein content of differentiated cells in comparison with corresponding

Table 1 Protein content, PC activity and content of confluent and differentiated 3T3-L1 cells

Values are for cells present in a T-25 flask and are the mean of three independent determinations. Variations between independent experiments were within $\pm 15\%$. PC protein content of confluent and differentiated cells was determined by immunoblotting as described in the Materials and methods section.

Expt.	Protein (mg)	PC		
		Activity (munits)	Specific activity (munits/mg of cell protein)	Relative content of PC protein
1. Confluent cells	0.34	3	8	1
2. Adipocytes	4.8	960	200	23

undifferentiated 3T3-L1 cells (Table 1). This may be related to a greater number of fibroblasts converting into adipocytes under our experimental conditions. Even with a large increase in cellular protein content, the specific activity of PC increased about 25-fold on differentiation of 3T3-L fibroblasts to adipocytes (Table 1). A similar increase in the specific activity of PC in this cell line has been observed by others (Mackall and Lane, 1977; Freytag and Utter, 1980). Previous studies have shown that fluctuations in PC activity occurring under a variety of nutritional and hormonal perturbations in animals and experimental cell lines are the result of changes in the steady-state levels of enzyme protein (Weinberg and Utter, 1979, 1980; Ahmad and Ahmad, 1990). This is directly supported by the 23-fold increase in carboxylase protein content during cell differentiation as determined by Western blotting (Table 1). Western blotting provides a more reliable estimate of PC protomer content than does immunoprecipitation or immunotitration because cross-reacting protein(s) of molecular mass other than 130 kDa are removed during SDS/PAGE. In addition, it allows visualization of cross-reacting proteins and degradation products that may arise from PC breakdown during sample handling.

Effects of 8-Br-cAMP, insulin and 8-Br-cAMP plus insulin on PC activity and intracellular protein content of 3T3-L1 adipocytes

cAMP, a known lipolytic agent, down-regulates adipocyte expression of genes coding for the lipogenic enzymes, fatty acid synthase, lipoprotein lipase and glycerophosphate dehydrogenase (Spiegelman and Green, 1981; Paulauskis and Sul, 1988; Antras et al., 1991). Because PC provides essential precursors for lipogenesis in adipocytes, experiments were performed to determine if expression of the PC gene was controlled by the cyclic nucleotide.

Initial experiments were performed to define conditions under which a reproducible response would be obtained when adipocytes were exposed to cAMP, insulin or a combination of cAMP and insulin. Fully differentiated cells fed with serum-free medium for 24 h responded predictably to these biological agents for a period of 48 h, and the data reported here were obtained using adipocytes fed with serum-free medium to which cAMP, insulin or a combination of cAMP and insulin were subsequently added. None of these agents affected cell viability during the course of these experiments.

In control adipocytes, the carboxylase activity decreased 20% during the first 24 h with little further decline over the next 24 h

Table 2 Effect of insulin, cAMP and insulin plus cAMP on cellular protein and PC activity of 3T3-L1 adipocytes

After 10 days of withdrawal of inducing agents, cells (grown in T-25 flasks) were fed with serum-free medium for 1 day. This time point is considered day 0. The cells were then fed with serum-free medium containing insulin, cAMP or cAMP plus insulin for the length of time shown. These results are means of three independent determinations. Variations between independent experiments were within $\pm 15\%$.

Expt. no.	Additions	Duration of treatment (h)	Protein (mg)	PC activity (munits)	Specific activity (munits/mg)
1.	None	0	4.8	962	200
		24	4.2	777	185
		48	4.5	758	168
2.	Insulin (10 μ g/ml)	24	5.4	940	174
		48	5.3	831	157
3.	cAMP (1 mM)	24	3.6	8	2.2
		48	3.5	1.4	0.4
4.	cAMP (1 mM) + insulin (10 μ g/ml)	24	5.4	766	142
		48	3.9	20	5.1

of incubation (Table 2, Expt. no. 1). On the other hand, a 24 h exposure to 8-Br-cAMP (hereafter referred to as cAMP), a permeant cAMP analogue, caused a sharp decline in PC activity of adipocytes to levels barely detectable by the sensitive radioisotopic enzymic assay used (Table 2, compare Expt. nos 1 and 3). This confirms an earlier preliminary observation showing that addition of cAMP to cultures of 3T3-L1 adipocytes caused a marked decrease in PC activity (Freytag and Utter, 1980). Despite almost complete loss of carboxylase activity, the soluble protein content of adipocytes declined by only 25%. Thus cAMP does not induce global protein breakdown in adipocytes but affects specific adipocyte enzymes, including PC.

cAMP also produced morphological changes readily observed by microscopic examination. For example, the obscured cell borders became distinct, and intracellular lipid droplets assumed irregular shape and acquired a darker form. This dissolution of lipid droplets resulted in a rapid fall in pH of the medium (because of accumulation of end products of lipolysis).

PC activity in adipocytes treated with insulin only also decreased with incubation time (14% lower in adipocytes treated for 48 h with the hormone) but was slightly higher than in age-matched control cells (Table 2, compare Expt. nos. 1 and 2). A 10–15% increase in the soluble protein content of adipocytes fed with insulin-containing medium probably reflects the ability of insulin to promote a generalized increase in protein synthesis in mammalian cells (Alexander et al., 1985).

No appreciable change in PC activity occurred in adipocytes exposed for 24 h to the combined actions of cAMP and insulin, the carboxylase activity of treated cells remaining close to that of control cells (Table 2, compare Expt. nos. 1 and 4, the 24 h values). The increase in intracellular protein content of cells treated with cAMP plus insulin was similar to that of adipocytes fed with insulin-containing medium (Table 2, compare Expt. nos. 1, 3 and 4), resulting in a corresponding decrease in the specific activity of PC. Further exposure of adipocytes to cAMP plus insulin led to a sharp decrease (98%) in the carboxylase activity with a more modest decrease (20%) in the intracellular protein content. PC activity in adipocytes treated with cAMP plus insulin for 48 h was about 2.5% of control cells (Table 2, compare Expt. nos. 1 and 4, 48 h values) but was significantly higher than that of age-matched adipocytes treated for 48 h with cAMP alone (Table 2, compare Expt. nos. 3 and 4, 48 h values). Thus insulin almost completely abrogates the effects produced

Figure 1 Estimation of the relative abundance of PC mRNA of confluent and differentiated 3T3-L1 cells

Equal amounts (10 μ g) of total RNA isolated from mature adipocytes (lane 1) and confluent cells (lane 2) were applied to agarose gel and electrophoresed as described in the Materials and methods section. PC mRNA transcript was measured by Northern-blot hybridization with 32 P-labelled cDNA of clone no. 2-9 (Zhang et al., 1993) as detailed in the Materials and methods section. A representative autoradiogram of three independent determinations is presented.

on PC activity and soluble protein content during the first 24 h of exposure of adipocytes to cAMP but is ineffective in countering the effects produced during longer exposure.

Effects of cAMP, insulin and cAMP plus insulin on PC mRNA of 3T3-L1 adipocytes

The relative abundance of PC mRNA was measured in RNA isolated from confluent undifferentiated 3T3-L1 cells, in mature adipocytes and in adipocytes treated with insulin, cAMP or combination of cAMP and insulin. Northern-blot analysis was used for these measurements because it excludes cross-hybridizing RNAs of various size likely to be detected in dot-blot. The probe used was made by labelling the 2.3 kb cDNA encoding a segment of the 3T3-L1 PC gene. This clone hybridizes to a single species of RNA of 4.2 kb that encodes adipocyte pre-PC (Zhang et al., 1993). Compared with confluent undifferentiated 3T3-L1 fibroblasts, a 9–10-fold increase in the relative abundance of PC mRNA was observed in differentiated cells (Figure 1). Thus the magnitude of increase in the steady-state levels of PC mRNA is 2–3-fold lower than the increase in the specific activity of the PC protein that accompanies the differentiation process. This difference (fold increase in specific activity compared with hybridizable mRNA) is consistent with insulin inhibiting the degradation of PC more than it inhibits breakdown of total cellular adipocyte protein (Chandler and Ballard, 1983). In addition, as observed with fatty acid synthase of cultured chick hepatocytes (Wilson et al., 1986), insulin may accelerate the translation of PC without significantly changing the carboxylase mRNA levels (see below).

Like enzyme activity, the relative abundance of PC mRNA in adipocytes exposed to cAMP decreased precipitously to approx. 12% of control values within 24 h of treatment (Figure 2, compare lanes 2 and 3) and was barely detectable in cells treated with cAMP for 48 h (Figure 2, compare lanes 6 and 7).

Treatment of adipocytes with a mixture of cAMP and insulin produced a biphasic response in PC mRNA similar to that seen for enzyme activity. Compared with untreated cells, adipocyte PC mRNA declined approx. 20% in the first 24 h of exposure to cAMP plus insulin (Figure 2, compare lanes 2 and 5). After 48 h of incubation, PC mRNA declined to low levels (Figure 2, compare lanes 6 and 9) but its abundance was nearly 4-fold higher than that of adipocytes fed with medium containing cAMP alone (Figure 2, lanes 7 and 9). Thus changes in the relative abundance of PC mRNA occur in parallel with the carboxylase activity but this was not the case for PC protein content which did not decrease (see below).

Figure 2 Effect of cAMP, insulin and cAMP plus insulin on the relative abundance of PC mRNA of 3T3-L1 adipocytes

Total RNA isolated from control and treated adipocytes was denatured and electrophoresed as described in the Materials and methods section. The relative abundance of PC mRNA was measured by Northern-blot hybridization with 32 P-labelled cDNA of clone no. 2-9 as given in Figure 1. The amounts of RNA, in μ g, applied to different slots are shown in parentheses. Lane 1, zero-time control (10); lane 2, 24 h control (10); lane 3, 24 h with cAMP (15); lane 4, 24 h within insulin (10); lane 5, 24 h with cAMP plus insulin (10); lane 6, 48 h control (10); lane 7, 48 h with cAMP (30); lane 8, 48 h with insulin (10); lane 9, 48 h with cAMP plus insulin (15). A representative autoradiogram of three independent determinations is presented.

The relative abundance of PC mRNA in control adipocytes did not change significantly during the 48 h incubation period. Likewise, only modest diminution was observed in the relative abundance of PC mRNA in adipocytes that had been fed with insulin-containing medium for 24 and 48 h (Figure 2, compare lane 2 with lanes 4 and 8). This result indicates that insulin may not modulate PC mRNA of mature adipocytes in contrast with the observed insulin-induced increases in steady-state levels of mRNAs encoding lipoprotein lipase (Semenkovich et al., 1989), fatty acid synthase (Paulaukis and Sul, 1988) and glyceraldehyde-3-phosphate dehydrogenase (Alexander et al., 1985) in 3T3-L1 adipocytes.

The carboxylase activity of differentiating adipocytes is related directly to their PC protein content, as was found in tissues of animals subjected to various nutritional and hormonal perturbations (Weinberg and Utter, 1979, 1980). A similar relationship between activity and PC protein content was expected to be observed in adipocytes fed with cAMP-containing medium but these cells, although almost completely devoid of PC activity, contained PC protein at levels close to that in control cells. Figure 3 illustrates the patterns obtained when equal amounts of cell protein from control and treated adipocytes were analysed by Western blotting. As in control samples, only a single main band migrates with the molecular mass of PC protomer (130 kDa). Independent experiments established that the amount

Figure 3 Estimation of PC content of 3T3-L1 adipocytes treated with cAMP, insulin or cAMP plus insulin

Adipocytes were fed with medium and medium containing cAMP, insulin or cAMP plus insulin as described. PC protein content of cell homogenates was estimated by Western-blot analysis as given in Table 1. Equal amounts of cell protein (50 μ g) were applied to different gel slots. Lane 1, 24 h control; lane 2, 24 h with cAMP; lane 3, 24 h with insulin; lane 4, 24 h with cAMP plus insulin; lane 5, 48 h control; lane 6, 48 h with cAMP; lane 7, 48 h with insulin; lane 8, 48 h with cAMP plus insulin. Prestained proteins in lane S were of the following molecular mass (kDa in descending order): β -galactosidase, 116.5; phosphofructokinase, 96; pyruvate kinase, 80; only the β -galactosidase band is shown. A representative blot of five independent experiments is presented.

Figure 4 Detection of apo- and holo-forms of PC in 3T3-L1 adipocytes treated with cAMP, insulin or cAMP plus insulin

Cell homogenates were reduced, denatured and after incubation with 10 μ g of avidin were analysed by Western blotting. Equal amounts of cell protein (50 μ g) were applied to different gel slots. Lane 1, 48 h control; lane 2, 48 h with cAMP; lane 3, 48 h with insulin; lane 4, 48 h with cAMP plus insulin. The lane marked S shows the mobilities of the prestained proteins. These were, in order of descending molecular mass (kDa): myosin, 205; β -galactosidase, 116.5; BSA, 80. A representative blot of three independent experiments is presented.

of anti-PC antibodies used was more than sufficient to complex the amount of PC protein present in the samples.

We considered the possibility that PC of cAMP-treated adipocytes may be inactive because of the subtraction or lack of incorporation of its biotin prosthetic group. In previous studies we showed that apo- and holo-PC and holo-PC-avidin complex react similarly with anti-PC antibodies (Ahmad et al., 1986). This procedure provided a convenient method for the simultaneous detection and quantification of apo- and/or holo-forms of PC in cell homogenates. Control and treated adipocytes were reduced, denatured and, after the addition of avidin, analysed by Western blotting. The results depicted in Figure 4 show that the immunoreactive protein from control cells and those treated with cAMP and cAMP plus insulin migrates with the mobility of a 200 kDa polypeptide. This value is close to that expected for the avidin-PC complex ($68 + 130 = 198$ kDa). Almost all of the carboxylase (from control and treated adipocytes) must possess the biotin prosthetic group because only a small fraction migrated like the free PC protomer (130 kDa). Therefore a lack of holo-PC is not responsible for the apparent inactivation of PC in adipocytes treated with cAMP and cAMP plus insulin.

The similar amounts of PC-avidin complex in control and treated adipocytes (Figure 4) validates the data of Figure 3 showing that the carboxylase protein contents of adipocytes treated with cAMP and cAMP plus insulin are similar to that of control cells.

Vertebrate PC exists predominantly as a tetramer in solution (Taylor et al., 1978). The quaternary structure of PC in cell extracts of control and treated adipocytes was probed by chemical cross-linking, using the homo-bifunctional reagent, EGS, with the bridge span length 1.2 nm (12 Å) (Conn, 1983) in order to gain some insight into the association state of the species pre-existing in solution (Jarvis et al., 1988). Cell homogenates were incubated with EGS, and the cross-linked products separated by SDS/PAGE were detected with anti-PC antibodies. The Western blot is shown in Figure 5. Most of the enzyme in homogenates of control (Figure 5, lanes 1 and 5), insulin-treated (Figure 5, lane 3 and 7) and 24 h cAMP-plus-insulin-treated adipocytes (Figure 5, lane 4) migrated as a single cross-linked molecular species of 500 kDa. This molecular mass agrees with the value estimated for the tetrameric form of PC ($130 \times 4 = 520$ kDa). On the other hand, a cross-linked product of 500 kDa was not formed when

Figure 5 Cross-linking of cell proteins with the homo-bifunctional reagent, EGS

Equal amounts of protein (40 μ g) of control and treated adipocytes were incubated with EGS for 150 min. Cross-linked proteins were analysed by Western blotting as described in the Materials and methods section. Lane 1, 24 h control; lane 2, 24 h with cAMP; lane 3, 24 h with insulin; lane 4, 24 h with cAMP plus insulin; lane 5, 48 h control; lane 6, 48 h with cAMP; lane 7, 48 h with insulin; lane 8, 48 h with cAMP plus insulin. Crossed-linked phosphorylase b SDS molecular-mass markers (hexamer 584 kDa; pentamer, 487 kDa; tetramer, 390 kDa; trimer, 292 kDa; dimer, 195 kDa; monomer, 97 kDa) were included during the run to enable estimation of the molecular mass of cross-linked proteins. Arrows indicate migration distances of polypeptides of 500 (upper) and 205 kDa (lower). A representative blot of four independent experiments is presented.

homogenates of cAMP-treated (Figure 5, lanes 2 and 6) or 48 h cAMP-plus-insulin-treated adipocytes (Figure 5, lane 8) were incubated with EGS. Intraprotomer cross-linking occurred only in cell extracts containing catalytically active PC. The protomers in the active tetramer are spatially (intramolecular distance about 12 Å) arranged such that they are readily and completely cross-linked by EGS. This close proximity is supported by electron-microscopic analysis of different PCs. Negatively stained preparations of vertebrate and yeast PC reveal images resembling a tetrahedron-like structure which consists of two pairs of identical subunits arranged so that the opposing pairs interact with each other in the tetramer (Mayer et al., 1980).

Neither the 500 kDa cross-linked product nor the 130 kDa PC protomer was detected in extracts of adipocytes treated with cAMP or cAMP plus insulin (48 h) (Figure 5, lanes 2, 6 and 8) and cross-linked with EGS. In cAMP-treated adipocytes, the absence of the cross-linked tetramer suggests an unfavourable spatial position of the reactive groups on adjacent subunits in the inactive PC. The absence of the 130 kDa PC band is puzzling but may be the result of intermolecular cross-linking (catalysed by EGS) of protomers (or of inactive tetramer) with neighbouring proteins to form large aggregates that do not enter the gel. Further studies are required to provide definitive evidence in support of this postulate.

In summary, our results indicate that cAMP regulates the cellular content of PC mRNA. The observed changes in PC mRNA levels caused by chronic exposure of mature adipocytes to cAMP may result from an alteration in the rate of gene

transcription or mRNA degradation or a combination of the two. In addition, our results suggest that, in cAMP-treated adipocytes, PC undergoes a yet to be defined modification causing it to become catalytically inactive. Preliminary experiments performed to establish whether phosphorylation inactivates PC in cAMP-treated adipocytes have been unsuccessful.

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