ROLE OF PYRUVATE CARBOXYLASE IN FATTY ACID SYNTHESIS: ALTERATIONS DURING PREADIPOCYTE DIFFERENTIATION

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SUMMARY:

Transport of mitochondrial acetyl units to the cytoplasm for fatty acid synthesis via the citrate cleavage pathway requires replenishment of mitochondrial oxaloacetate. Pyruvate carboxylase is thought to fulfill this role although compelling evidence has been lacking. During lipogenic differentiation of 3T3-L1 preadipocytes, pyruvate carboxylase activity rises 18-fold in close coordination with fat accumulation and the activity of ATP-citrate lyase, an established lipogenic enzyme. The activities of enzymes less directly related to lipogenesis rise only 3-5-fold while other unrelated enzymes do not increase significantly. These results indicate that pyruvate carboxylase is in fact a lipogenic enzyme.

Pyruvate carboxylase, once considered primarily a gluconeogenic enzyme, is now thought to play a significant role in fatty acid synthesis. The action of this enzyme appears particularly important in adipose tissue where a substantial proportion of the NADPH required for lipogenesis arises via the malate transhydrogenation cycle. As illustrated in Fig. 1, the generation of NADPH is coupled to the transport of mitochondrial acetyl units into the cytoplasm for fatty acid synthesis. Acetyl-CoA is transported to the cytoplasm as citrate where it is cleaved to acetyl-CoA and oxaloacetate by ATP-citrate lyase. It was proposed some time ago (1, 2) that cytoplasmic oxaloacetate formed in this manner is reduced to malate by malate dehydrogenase with NADH produced by glycolysis and that malate is then decarboxylated by malic enzyme to form pyruvate and NADPH. The operation of this pathway requires continual replenishment of mitochondrial oxaloacetate, a function attributed to pyruvate carboxylase (3), but never substantiated. Compelling evidence establishing the role of this enzyme in the synthesis of fatty acids in preadipocytes is presented in this communication.

3T3-L1 preadipocytes, derived from the original 3T3 mouse fibroblast line (4), differentiate in culture into cells having morphological and biochemical characteristics of adipocytes (4, 5, 6). Associated with adipocyte conversion is a dramatic coordinated rise in the activities of lipogenic enzymes (6). The 3T3-L1 subline therefore provides an ideal system for assessing the relationship of pyruvate carboxylase to lipogenesis.

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METHODS

3T3-L1 and 3T3-C2 cells obtained from Dr. Howard Green (Massachusetts Institute of Technology) were plated at a density of 10^4 cells per 5 cm culture dish and maintained in Dulbecco's Modified Eagle's Medium containing 10% calf serum (Microbiological Associates) as previously described (6). Cells were removed by scraping in 1.0 ml of 0.25 M sucrose, disrupted by treatment with 0.5 ml digitonin (7) (3 mg per ml) in 0.25 M sucrose containing 3 mM EDTA for 90 sec at 4° and centrifuged in a microfuge (Beckman Model B) for 5 min. Supernates were brought to 5 mM citrate for ATP-citrate lyase assays, adjusted to 0.1 M potassium phosphate (pH 8.0) for phosphofructokinase assays and used without modification for assays of other soluble enzymes. Mitochondrial pellets were washed and resuspended in 0.5 ml of 0.25 M sucrose. Triton X-100 (0.5%) was added to assay reaction mixtures when enzyme activities of these suspensions were determined.

ATP-citrate lyase activity was determined as previously described (6). Pyruvate carboxylase assays were conducted by modification of the method of Scrutton et al.(8). Bicarbonate fixation was measured by following [^{14}C] incorporation into malate in the presence of malate dehydrogenase at 37° in an assay mixture containing $KH^{14}CO_3$ (300 cpm per nmole), 20 mM; Tris (Cl^-), 250 mM, pH 7.8; ATP, 2.5 mM; $MgCl_2$, 12.5 mM; NADH, 0.75 mM; pyruvate, 10 mM; acetyl-CoA, 1 mM; and malate dehydrogenase, 6 units. Aliquots were removed at 2 min intervals, quenched in 6 N HCl and evaporated to dryness at 90° for 60 min. Water and scintillation fluid were added and fixed [^{14}C] determined. Citrate synthase activity was determined by following the reaction of CoA with 5,5'-dithiobis-(2-nitrobenzoate) spectrophotometrically at 30° (9). Lactate dehydrogenase activity was determined by the method of Pesche et al. (10). Aldolase, glutamate dehydrogenase, the mitochondrial NAD-dependent isocitrate dehydrogenase and phosphofructokinase were assayed fluorometrically at 30° with standard spectrophotometric assays in which the nucleotide concentrations were adjusted to the levels indicated: aldolase (11), 20 μM NADH; glutamate dehydrogenase (12), 100 μM NAD; isocitrate dehydrogenase (13), 100 μM NAD; and phosphofructokinase (14), 5 μM NADH. 1 unit is that amount of enzyme which catalyzes formation of 1 μmole of product per min.

RESULTS AND DISCUSSION

Differentiation of 3T3-L1 cells is accompanied by coordinate rises in de novo lipogenesis and the activities of the enzymes which comprise the cytoplasmic pathway for fatty acid synthesis, i.e. ATP-citrate lyase, acetyl-CoA carboxylase and fatty acid synthetase (6). To examine the possible role of pyruvate carboxylase in lipogenesis, the activity of this enzyme was followed during adipocyte conversion and compared directly with that of ATP-citrate lyase, an enzyme known to undergo lipogenic differentiation.

For determination of pyruvate carboxylase activity mitochondria were isolated by disruption of cells with digitonin (7) followed by centrifugation. Digitonin which interacts specifically with 3-β-hydroxysterols, including cholesterol, damages the plasma membrane and leaves the inner mitochondrial membrane and matrix intact. In all cases this procedure released >95% of the cytoplasmic marker enzyme, lactate dehydrogenase, while >95% of the mitochondrial marker enzyme, citrate synthase, was

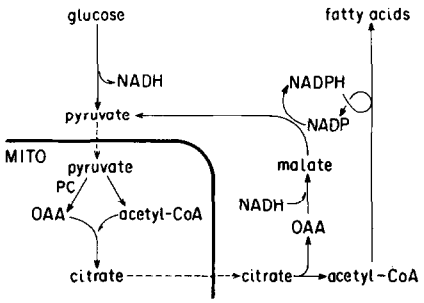

Fig. 1 Pathway of fatty acid synthesis from glucose

recovered in the mitochondrial fraction. Virtually all, >95%, of the cellular pyruvate carboxylase activity was localized within the mitochondrial matrix. Earlier reports indicating the occurrence of cytoplasmic pyruvate carboxylase in adipose tissue are considered to be the consequence of leaky mitochondria (15, 16).

The activities of mitochondrial pyruvate carboxylase and cytoplasmic ATP-citrate lyase were measured in 3T3-L1 cultures from confluence through the course of lipogenic differentiation. During the first 12 days post-confluence pyruvate carboxylase activity remains low (~ 5 mUnits per mg of cellular protein) and less than 1% of the cells exhibit cytoplasmic triglyceride accumulation (Fig. 2). Carboxylase activity begins to rise about 14 days after confluence when 5-10% of the cells have accumulated lipid. A level of carboxylase activity (~ 90 mUnits per mg of cellular protein) 18 times that of the original fibroblasts is reached 21 days after confluence when > 60% of the cells express the fatty phenotype. In contrast, pyruvate carboxylase activity in 3T3-C2, a control cell line which does not undergo adipocyte conversion, remains at a low level throughout the course of the experiment. Thus, the dramatic rise of pyruvate carboxylase activity in 3T3-L1 cells is a consequence of differentiation rather than a non-specific effect of culture conditions on 3T3 cells. Even more striking is the close correlation between the rise of pyruvate carboxylase activity and that of ATP-citrate lyase during adipocyte conversion (Fig.2). Thus, pyruvate carboxylase activity levels appear to be tightly coupled to those of the other lipogenic enzymes (6). In addition, the final differentiated level of pyruvate carboxylase activity is more than adequate to regenerate mitochondrial oxaloacetate at a rate sufficient to support the elevated rate of fatty acid synthesis in the differentiated state (6).

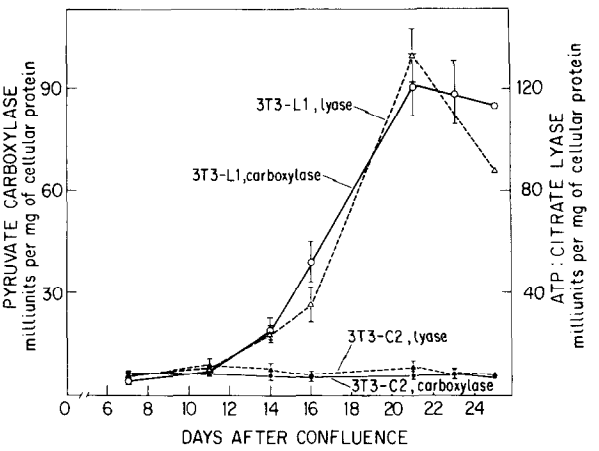

Fig. 2 Changes in the activities of pyruvate carboxylase (circles) and ATP-citrate lyase (triangles) during differentiation in 3T3-L1 cells (open symbols) and 3T3-C2 cells (closed symbols).

To place the findings involving pyruvate carboxylase within the overall metabolic framework of de novo lipogenesis, the activities of several other enzymes were determined before and after differentiation. Most of these enzymes are involved in the conversion of glucose to fatty acids, while others are thought to be unrelated to fatty acid synthesis. Citrate synthase plays a key role in the transport of mitochondrial acetyl units to the cytoplasm for fatty acid biosynthesis (Fig. 1). Unlike pyruvate carboxylase and ATP-citrate lyase, however, citrate synthase also has an important role in the normal energy metabolism of the undifferentiated fibroblast and as a result is present in rather large amounts (127 mUnits per mg of cellular protein) prior to differentiation (Table I). Following differentiation the level of citrate synthase has predictably risen only about 3-fold. The final level is, nevertheless, more than adequate to supply sufficient citrate to support fatty acid synthesis in the differentiated 3T3-L1 cell. Glycolytic enzymes which are involved in both the formation of pyruvate for fatty acid synthesis and normal energy metabolism, also increase nominally during differentiation of 3T3-L1 cells. Phosphofructokinase, aldolase, and lactate dehydrogenase increase 5-, 4-, and 3-fold, respectively (Table I). On the other hand, enzymes not known to be involved in fatty acid synthesis, such as glutamate dehydrogenase and the mitochondrial NAD-dependent isocitrate dehydrogenase, do not increase significantly during the adipocyte conversion process (Table I).

The striking rise in pyruvate carboxylase activity accompanying

Table I. Differentiation-linked Enzyme Activity Changes in 3T3-L1 Preadipocytes

Enzyme	Enzyme activity		
	Undifferentiated cells[1]	Differentiated cells[2]	Differentiated / Undifferentiated
	mUnits per mg of cellular protein		
Pyruvate carboxylase[3]	5	92	18.4
ATP-citrate lyase[3]	7.6	134	17.6
Citrate synthase	127	341	2.7
Phosphofructokinase	13.8	72	5.2
Aldolase	11.7	49	4.2
Lactate dehydrogenase	1250	3620	2.9
Isocitrate dehydrogenase (mitochondrial NAD-linked)	1.7	2.2	1.3
Glutamate dehydrogenase	0.12	0.24	2.0

[1] < 1% of cells exhibiting triglyceride accumulation

[2] > 60% of cells exhibiting triglyceride accumulation

[3] From Fig. 2, this paper

expression of fatty acid synthesis in 3T3-L1 cells demonstrates its role as a lipogenic enzyme in the adipocyte. In fact, there appear to be two classes of "lipogenic" enzymes whose expression is altered during preadipocyte differentiation. One group referred to as primary lipogenic enzymes appear to function exclusively (or primarily) in de novo lipogenesis. These include pyruvate carboxylase, ATP-citrate lyase, acetyl-CoA carboxylase and fatty acid synthetase. The activities of the primary lipogenic enzymes rise dramatically (> 15-fold) during adipocyte conversion. Another group which may be referred to as secondary lipogenic enzymes appear to have multiple metabolic functions only one of which is the support of de novo lipogenesis. Enzymes such as phosphofructokinase, aldolase, lactate dehydrogenase and citrate synthase, which have constitutive roles in energy metabolism, in both the undifferentiated and differentiated state, fall into this category. These enzymes, which rise only 3-5-fold during lipogenic

differentiation, appear to be required to support increased fatty acid synthesis. Although it is not clear by what mechanism(s) expression of primary and secondary lipogenic enzymes is controlled, any mechanism must account for the different extents of amplification of the two classes of enzymes. The fact that expression is coordinate (6) and that cellular enzyme concentration *per se* is elevated in at least one case, acetyl-CoA carboxylase (6), is consistent with control of expression exerted at the level of transcription.

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