Leucine Catabolism during the Differentiation of 3T3-L1 Cells

EXPRESSION OF A MITOCHONDRIAL ENZYME SYSTEM*

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Leucine can be utilized efficiently as a precursor for lipid biosynthesis by adipose tissue, especially in the presence of glucose or insulin. During the differentiation of 3T3-L1 fibroblasts to adipocytes, the rate of lipid biosynthesis from 1-[U-14C]leucine increases at least 30-fold and lipogenesis, with [U-14C]acetate as the precursor, increases by 10- to 15-fold. The specific activities of two mitochondrial dehydrogenases in the leucine oxidative pathway, the branched chain α-ketoacid dehydrogenase and isovaleryl-CoA dehydrogenase, as well as of leucine α-ketoacid transaminase, increase at least 20-fold during the adipose conversion. Isovaleryl-CoA dehydrogenase was assayed in crude extracts using a specific fluorimetric method employing electron transfer flavoprotein as the electron acceptor for the flavoprotein dehydrogenase. The specific activity of 3-hydroxy-3-methylglutaryl-CoA lyase, the mitochondrial enzyme catalyzing the terminal reaction in the leucine degradation pathway, increases 4-fold during differentiation. The increases in the specific activities of the mitochondrial enzymes occur without a change in the specific activity of cytochrome oxidase, indicating that the increases do not simply reflect proliferation of mitochondria. The biosynthesis of at least 20 soluble mitochondrial polypeptides is enhanced during the adipose conversion of the fibroblasts as determined by polyacrylamide gel electrophoresis following incubation of the cells with [35S]methionine. The results provide a conservative estimate of the extent of changes in mitochondrial soluble proteins during the adipose conversion. They also establish that differentiated 3T3-L1 adipocytes metabolize leucine like mature adipose tissue and illustrate the roles of the branched chain α-ketoacid dehydrogenase and isovaleryl-CoA dehydrogenase in lipogenesis.

The differentiation program for the conversion of 3T3-L1 fibroblasts into adipocytes (1, 2), involves qualitative and quantitative changes in cellular proteins (3, 4). Some of these changes, which occur in a definite temporal sequence (5-7), have been directly shown to result from alterations in the rates of de novo synthesis of these proteins (8-11). In several instances, the changes in synthetic rate of proteins during adipose conversion have been shown to result from activation or inhibition of gene transcription (11-13). Changes in the enzymatic activities for de novo fatty acid (8, 9, 14) and triglyceride biosynthesis (4, 15) and in the responses to lipogenic and lipolytic hormones (16-19) have been studied extensively in these cells. However, relatively little is known about changes in mitochondrial composition and metabolism during the differentiation process.

The enzymes of the leucine catabolic pathway, with the possible exception of some fraction of leucine α-ketoacid transaminase activity (20), are compartmentalized exclusively in mitochondria (21-23). In adipocytes, unlike other tissues, leucine is a significant precursor for fatty acid and sterol biosynthesis, especially in the presence of glucose with or without insulin (24-26). Furthermore, leucine apparently serves as a source of nitrogen for synthesis of glutamine and, to lesser extents, glutamate and alanine, which are released by adipocytes (27) and can serve as substrates for gluconeogenesis in liver and kidney.

In the experiments reported here, we show that the acquisition of the fatty phenotype by 3T3-L1 cells is accompanied by the enhanced capacity to utilize leucine as a lipid precursor. We further demonstrate that adipocytes couple leucine catabolism to glutamine biosynthesis (27).

EXPERIMENTAL PROCEDURES

Materials

ETF was purified from pig liver mitochondria as described by McKeen et al. (29). The A260nm/A440nm ratios of the ETF preparations were 6.07 and 6.20. The concentration of ETF was determined spectrophotometrically using the extinction coefficient, ε260nm = 1.34 × 104 M-1 cm-1 (30). CoA, 3-hydroxy-3-methylglutaryl-CoA, and isovaleryl-CoA were obtained from P-L Biochemicals. Bovine serum albumin (essentially fatty acid-free), NAD, α-ketoacid, L-leucine, α-ketoisocaproate, thiamin pyrophosphate, cytochrome c (equine heart, Type VI), L-α-phenylalanine, glucose oxidase, citrate synthase, and malate dehydrogenase were purchased from Sigma. Crystalline bovine insulin was a gift from Lilly Research Laboratories. Succinyl-CoA was synthesized from succinic anhydride and CoA by the method of Simon and Shemin (31). Succinic anhydride was recrystallized before use. [U-14C]Acetate (54 mCi/mmol) was obtained from American-Schwarz. L-[U-14C]Leucine (340 mCi/mmol), L-[1-14C]leucine (58 mCi/ mmol), and L-[35S]methionine (995 mCi/mmol) were obtained from New England Nuclear. L-Amino acid oxidase was obtained from Worthington Biochemical Corporation and 3,5-diaminobenzazole was obtained from Aldrich. Percoll was purchased from Pharmacia Fine Chemicals.

α-[1-14C]Ketoisocaproate was prepared enzymatically by oxidation of 1-[1-14C]leucine with L-amino acid oxidase in the presence of catalase as described by Rudiger et al. (32). The α-ketoacid was

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1 The abbreviations used are: ETF, electron transfer flavoprotein; MIX, 3-isobutyl-1-methylxanthine; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.
purified by chromatography on a 1 × 10 cm column of AG-50W (Bio-Rad) and was about 93% pure as judged by thin layer chromatography (33). The ketoacid was stored in acidic solution at −70 °C until used. Aliquots of the ketoacid were neutralized with 0.5 M KPO buffer, pH 8.0, immediately before the assays were performed.

All other chemicals were reagent grade and were obtained from commercial sources.

Cell Growth and Differentiation

Mouse 3T3-L1 cells were obtained from the American Type Culture Collection, Washington, D. C. The cells were grown and maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml of penicillin, and 100 µg/ml of streptomycin. Cell cultures were split weekly at a ratio of 1:15 and were not used after more than six passages out of the original stocks frozen at −70 °C.

The cells were plated at an initial density of 10^4.5 ml of culture medium in 60-mm plastic dishes. The culture medium was changed 2 days after plating. When the cultures reached confluence after about 5 days, the cells were stimulated to differentiate by the addition to the culture medium of 10 µg/ml of insulin, 0.5 mM MIX, 0.6 µM dexamethasone, and 8 µg/ml of biotin. After 2 days, the medium containing this mixture was removed, and the cells were maintained in medium with insulin and biotin. The medium was changed three times per week. Cultures were generally 90% differentiated 6 to 8 days after reaching confluence when stimulated as described above.

In control experiments, lipid biosynthesis and the specific activities of the enzymes were also determined in unstimulated cultures, in cultures to which only insulin was added, and cultures exposed to MIX and dexamethasone for 2 days and then maintained in the standard medium (10% fetal bovine serum in Dulbecco's modified Eagle's medium with biotin). When compared with cultures stimulated to differentiate with MIX, dexamethasone, and insulin, only the cultures exposed to MIX and dexamethasone for 2 days showed a small degree of adipose conversion; the level of conversion was generally 20-30% at the time when fully stimulated cultures were at least 90% differentiated.

Enzyme Assays

Cells were rinsed twice with 4 ml of phosphate-buffered saline (10 mM NaPO4, buffer, pH 7.4), scraped from the dishes with a rubber policeman and sedimented at 1000 × g for 5 min. The cells were washed twice with sediment containing 10 ml of Scintiverse (Fisher) and 0.75 ml of H2O. Samples

Isovaleryl-CoA dehydrogenase was assayed by following the decrease in fluorescence due to the reduction of ETF flavin, essentially as described by Beckmann et al. (36). The 1.5 ml incubation mixtures contained 20 mM Tris HCl, pH 8, 18 mM glucose, 0.1 mM isovaleryl-CoA, and 1 µM ETF flavin. The reaction mixtures were made anaerobic by 15 cycles of alternate evacuation and purging with argon; 30 units of glucose oxidase and 5 units of catalase were added, and the reaction mixtures were equilibrated at 30 °C. The reactions were initiated by addition of 5-90 µg of the enzyme preparation. Assays were carried out at 30 °C with three different concentrations of protein. ETF flavin was excited at 340 nm and fluorescence was measured at 496 nm. Under the conditions of the assay, the rate of decrease in fluorescence was proportional to enzyme concentration. During the assays, 6-10% of the ETF flavin was reduced to the semiquinone.

Ferrocytochrome c oxidase activity was assayed at 30 °C by the method of Yonetani (37). The frozen and thawed preparations were routinely treated with 0.3 mg of Lubrol WX/ml of protein as described by Schimke and Greenwald (38) prior to assaying oxidative activity with 10-100 µg of protein. Results are expressed as cytochrome c oxidase activity-sensitive to inhibition by 10 µM KCN. Cyanide-insensitive cytochrome c oxidase activity was 2-8% of the total activity.

CoA lyase was assayed fluorometrically at 30 °C in 1.5 ml reaction mixtures containing 0.2 mM Tris-HCl, pH 8.5, 10 mM MgCl2, 1.5 mM NAD, 5 mM dithiothreitol, 2.5 mM L-malate, 20 units of malate dehydrogenase, 20 units of citrate synthase, and the enzyme preparation (50-150 µg of protein). The reaction mixtures were preincubated for 10 min at 30 °C before the reactions were initiated by the addition of 0.9 nM HMG-CoA (39). Excitation was at 340 nm and fluorescence was measured at 460 nm.

CoA transferase was assayed spectrophotometrically at 30 °C in 1 ml in incubation mixtures containing 67 mM Tris-HCl, 5 mM MgCl2, 2.0 mM l-malate, and 20 µM succinyl-CoA. The reaction was initiated by the addition of 2 mM acetoacetate and the formation of the Mg2+-enolate of acetoacetyl CoA was followed at 310 nm (ε20,000 = 11.9 × 103 M−1).

L-Leucine-a-ketoglutarate transaminase activity was assayed at 30 °C in incubation mixtures (1.5 ml) containing 35 mM potassium pyrophosphate, pH 8.2, 18 mM 2-mercaptoethanol, 3.3 mM a-ketoglutarate, 0.07 mM pyridoxal-5'-phosphate, 20 mM L-leucine and the crude extract (0.05-0.25 mg of protein). The reactions were initiated by the addition of L-leucine after preincubation at 30 °C for 5 min. The reactions were carried out for 15 and 30 min and were terminated by the addition of 1.5 ml of 10% trichloroacetic acid and centrifuged. The amount of a-ketoisocaproate formed was then quantitated spectrophotometrically at 440 nm after formation of the 2,4-dinitrophenylhydrazone as described by Aki and Ichihara (41). The 2,4-dinitrophenylhydrazide of α-ketoisocaproate was used as the standard.

In all cases, 1 unit of enzyme activity is equal to 1 µmol of substrate consumed or product generated per min at 30 °C.

Analytical Methods

Protein was estimated by the method of Lowry et al. (42) as modified by Miller (43) except in the experiments on lipid biosynthesis, when the method of Sedmak and Grossberg was employed (44). Bovine serum albumin was the standard in both protein assays. DNA was assayed fluorometrically with 0.5-diaminobenzene as described by Himgruber (45) with calf thymus DNA as the standard.

Quantitation of a-[14C]ketocaprate released by cells into the culture media was performed by gas-liquid chromatography of the trimethylsilyl derivative of the oxime of the ketoacid (46). The Hewlett-Packard gas chromatograph was equipped with an effluent splitter and a Nuclear Chicago radioactivity monitor. The monitor was calibrated with [U-14C]acetate to give specific radioactivity. The trimethylsilyl derivative of a-ketoisocaprate oxime was prepared from authentic a-ketoisocaprate and was employed as the standard. The analyses were kindly performed for us by Dr. Stephen Goodman, University of Colorado Health Sciences Center, Denver, Colo.

Neutral lipids were separated on Rhodamine 60-impregnated thin layers of silica gel by the method of Rock and Grossberg (47). The lipids were located on the plates by visualization with a long wavelength ultraviolet lamp and identified based on comparisons of Rf values of components with authentic standards. Zones of silica gel containing each neutral lipid class, as well as the origins, were scraped from the plates, and radioactivity was quantitated in a toluene-based
scintillation fluid. Thin layer chromatography of lipids was also conducted using a two-dimensional system: first dimension, CHCl₃, CH₂OH, 28% NH₃ (65:25:5); second dimension, CHCl₃, acetone, CH₂OH, acetic acid, H₂O (68:2:2:1). Lipid extracts were saponified by the procedure of Folch et al. (49). Radioactivity in the organic extracts was assayed as described above after drying the samples in scintillation vials with a stream of nitrogen.

Analysis of Lipid Biosynthesis—Lipid biosynthesis in the cell cultures was assayed by the incorporation of [U-¹³C]acetate or [U-¹³C]leucine into lipids. The cells in 1.5 ml of the medium in which they had been maintained were incubated with 0.67 μCi/ml of leucine or acetate for 4 h. At the end of the labeling period, the cultures were rinsed twice with 1.5 ml of 0.9% NaCl and 1 ml of 0.9% NaCl were added. The cells were removed from the plates by scraping with a rubber policeman and lipids were extracted by the method of Bligh and Dyer (48) or of Folch et al. (49). No difference was noted between the two procedures. The organic-soluble fraction was assayed for radioactivity by liquid scintillation spectrometry.

Radiolabeling and Preparation of Mitochondrial Proteins—Cells in 60-mm culture dishes were rinsed twice with methionine-free medium and incubated for 1 h with 1.5 ml of the methionine-free medium containing 0.5 mCi of L-[³⁵S]methionine. The medium was removed and the cells were rinsed three times with phosphate-buffered saline, pH 7.4. Mitochondria were isolated from fibroblasts by the procedure of Sun and Poole (50) and from adipocytes by the method of Jarrett (51). The mitochondria were further purified by centrifugation in Percoll density gradients generated in 1.5-ml tubes in an Eppendorf microcentrifuge according to the method of Mickelson et al. (52). The method for purification of the mitochondria resulted in a 10- to 12-fold purification of cytochrome c oxidase from the adipocytes and fibroblasts. The mitochondria were disrupted in 10 mM Tris-HCl containing 2 mM phenylmethylsulfonyl fluoride by brief sonication and the soluble mitochondrial protein fraction was obtained after centrifugation of the preparations at 50,000 × g for 1 h. Soluble mitochondrial proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 9% gels by the method of Laemmli (53). The gels were dried by suction on Whatman III filter paper and exposed to Kodak XAR-5 film.

RESULTS

To estimate the extent of changes in the rates of soluble mitochondrial polypeptide biosynthesis which occur during the adipose conversion of 3T3-L1 cells, 3T3-L1 fibroblasts and adipocytes were labeled on day 5 after confluence with L-[³⁵S]methionine in a methionine-free medium for 1 h. At this time, approximately 70% of the cells in the population treated with MIX, dexamethasone, and insulin had differentiated. Mitochondria were isolated and soluble proteins were prepared in the presence of 2 mM phenylmethylsulfonyl fluoride. The polypeptides were separated by electrophoresis and autoradiographed (Fig. 1). Equal amounts of radioactivity from each cell population were electrophoresed. Although the distribution of polypeptides on the gels is very similar, the data indicate that at least 20 polypeptides are synthesized at a higher rate in the 3T3-L1 adipocytes. One mitochondrial polypeptide (M, = 67,000) is synthesized at a higher rate in the fibroblasts.

Since the utilization of leucine carbon for lipogenesis is characteristic of adipocytes and the catabolic pathway for leucine is compartmentalized almost exclusively in mitochondria, we initiated a study of leucine catabolism in differentiating 3T3-L1 cells. Table I illustrates a basic finding of this study. During the differentiation of 3T3-L1 cells from a population of fibroblasts to a population which was about 90% adipocytes by day 6 after confluence, lipogenesis from acetate increased 10- to 15-fold, in agreement with previous work (1, 3, 8). The capacity to utilize leucine increased 30- to 40-fold during the adipose conversion. Only in these control cultures treated with MIX and dexamethasone for 2 days and then maintained in the standard medium for 3 days was there any appreciable increase in lipid biosynthesis. In these cultures, the -fold increase in lipid synthesis from both precursors reflected the proportion of cells with adipocyte morphology, i.e. those which had become enlarged and round and had accumulated lipid. The distribution of radioactivity among lipid classes in lipid extracts from differentiated cells (stimulated with MIX, dexamethasone, and insulin) and undifferentiated fibroblasts which were labeled with leucine or acetate was determined after thin layer chromatography. In the differentiated cells, approximately 60% of the radioactivity from both precursors was found in triglyceride and about 4% of the label migrated with the same Rf value as a cholesterol standard. In the chromatographic system used to separate neutral lipid classes, approximately 35% of the radioactivity in the lipid extracts from both acetate- and leucine-labeled differentiated cells remained at the origin. In the undifferentiated
cells, only 7% of the leucine label was incorporated into triglyceride and 40% of the label was found at the origin. Recoveries of radioactivity from the thin layer plates ranged from 84 to 97% and was usually about 94%. Using a two-dimensional thin layer chromatographic system in which most polear lipids migrate away from the origin, only 5% of the applied radioactivity from the lipid extracts of differentiated and undifferentiated cells labeled with either precursor remained at the origins. Finally, after saponification of the lipid extracts from differentiated cells and undifferentiated fibroblasts labeled with either precursors, 95 to 97% of the radioactivity was recovered with the fatty acid fraction, and 3 to 4% of the radioactivity was recovered with the nonsaponifiable lipids. These experiments document the distribution of radioactivity among lipid classes and also serve to show that the leucine label is present almost exclusively in fatty acids. This finding is important since leucine could also be incorporated into proteins which could partition into the organic phase of the extractions. The data also show that the 30-fold increase in lipid biosynthesis from leucine is due almost entirely to an increase in the rate of fatty acid biosynthesis. Considering the dilution of the specific radioactivity of the labeled leucine by unlabeled leucine (105 μg/ml in the medium, it is evident that leucine is utilized efficiently as a lipid precursor by the adipocytes.

It is possible that the increased rate of lipogenesis from leucine reflects a corresponding reduction in the amount of α-ketoisocaproate released by the adipocytes into the culture medium. The ketoacid is the major leucine catabolite released by muscle (54, 55) which does not utilize leucine as a lipid precursor (24). Also, α-ketoisocaproate production by epidydimal fat pads from fed rats decreases 35% in the presence of glucose which stimulates lipogenesis from leucine (27). The production of α-ketoisocaproate by undifferentiated fibroblasts and differentiated adipocytes was determined under the conditions used to assay lipid biosynthesis (see “Experimental Procedures” and Table 1) except that leucine-free medium was employed. Supernatant culture fluids were analyzed for α-ketoisocaproate by gas-liquid chromatography after oxime formation and synthesis of the trimethylsilyl derivative. A single radioactive peak with a retention time identical with the trimethylsilyl derivative of authentic α-ketoisocaproate was found in the culture fluids from both cell types. Ketoisocaproate production by the adipocytes (24,343 ± 222 cpm/h/mg of protein) was reduced by 38% compared to production of the α-ketoacid by the undifferentiated fibroblasts (39,124 ± 561 cpm/h/mg of protein). The values are the averages and ranges of two experiments.

Decreased α-ketoisocaproate release by 3T3-L1 adipocytes does not adequately account for the increased flux of leucine carbon into lipid. The results might be explained by increased activities of enzymes in the leucine catabolic pathway, especially the branched chain α-ketoacid dehydrogenase which catalyzes the rate-limiting reaction in the pathway (22). The activities of four enzymes in the leucine oxidation pathway were examined: L-leucine:α-ketoglutarate transaminase, the branched chain α-ketoacid dehydrogenase, isovaleryl-CoA dehydrogenase, and HMG-CoA lyase. The mitochondrial succinyl-CoA:aceoacetate-CoA transferase was also assayed, since the transferase activity could be involved in the utilization of acetoacetate generated in the lyase-catalyzed reaction for lipid synthesis. Isovaleryl-CoA dehydrogenase cannot be assayed accurately in crude extracts with routinely used dye-coupled reactions due to nonenzymatic reduction of the dye by endogenous reductants. Therefore, a direct fluorimetric assay of the dehydrogenase was used in which the decrease in fluorescence due to reduction of the ETF by isovaleryl-CoA was followed. Using this direct assay, the Kₘ for isovaleryl-CoA is 7.74 ± 1.04 μM, assayed with 1 μM ETF (flavin). This value is comparable to that obtained with a crude preparation of the enzyme from rat liver and a partially purified preparation from pig liver employing an assay which monitors the release of tritium from [2.3-3H]isovaleryl CoA (56). The Kₘ for ETF of the enzyme from the differentiated cells using the fluorimetric assay was determined to be 0.51 ± 0.14 μM, assayed with 100 μM isovaleryl CoA as the cosubstrate. The Kₘ for ETF of the dehydrogenase from the preadipocytes is essentially identical, 0.58 ± 0.13 μM. Over the period assayed, the reduced form of ETF generated (Fig. 2) was identified spectrophotometrically as the anionic semiquinone (57). The semiquinone form of ETF has been previously shown to be the kinetically significant reduced form of ETF generated by other ETF-linked flavoprotein dehydrogenases (36, 58, 59). Under the conditions of the experiment shown in Fig. 2, semiquinone formation was complete after about 30 min, and no ETF flavin hydroquinone was formed as indicated by the maintenance of isosbestic points at 475 nm, 402 nm, and 340 nm.

Fig. 3A shows that, during differentiation, the specific activities of the α-ketoacid dehydrogenase and isovaleryl-CoA dehydrogenase increase coordinately at least 20-fold. The increase in lipogenesis from L-leucine (Fig. 3B) parallels the increases in the specific activities of the two dehydrogenases. The protein:DNA ratio of the cells remained almost constant during the experiments (Fig. 3C). The specific activity of cytochrome c oxidase also remains relatively constant over this period (Fig. 3C), indicating that the increased specific activities of the two dehydrogenases do not merely reflect the proliferation of mitochondria in the developing adipocytes. Cells cultured for 7 days after confluence, in the presence of 10% fetal bovine serum alone, and cells treated with insulin alone from the time of confluence exhibited specific activities of the α-ketoacid dehydrogenase of 0.01 and 0.04 milliunits/mg of protein, respectively. This range of specific activities is characteristic of the confluent, undifferentiated fibroblasts. Phase-contrast microscopy showed that the extent of differentiation in these cultures was less than 5%. In cells exposed to MIX and dexamethasone for 2 days followed by maintenance in the standard medium for 5 days, the specific activity of the branched chain α-ketoacid dehydrogenase was 0.07 to 0.09 milliunits/mg of protein. Confluent cells exposed to MIX and dexamethasone for 2 days, followed by maintenance in the standard medium for 4 days, were about 20% differentiated and the specific activity of isovaleryl-CoA dehydrogenase was 5.86 milliunits/mg of protein; untreated cells exhibited a specific activity of 1.31 milliunits/mg of protein on day

![Fig. 2. Absorption spectra of ETF during reduction by crude isovaleryl-CoA dehydrogenase from 3T3-L1 adipocytes.](http://www.jbc.org/)

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the experiment resulted in less than 10% differentiation. The morphology and appearance of fat droplets in the cells. Treatment of the cells with only insulin throughout the 3T3-L1 fibroblasts to MIX and dexamethasone for 2 days followed by refeeding without insulin for 3 days resulted in the extent of differentiation was about 1% over the course of the experiment when the confluent cells were simply maintained in the standard medium. These results indicate that the comparatively small changes in HMG-CoA lyase and CoA transferase activities are related to the adipose conversion.

**Discussion**

The leucine catabolic pathway, a site of integration of nitrogen metabolism, lipid biosynthesis, and energy metabolism in adipocytes, is compartmentalized almost exclusively in mitochondria (22, 23). Leucine is an efficient precursor for fatty acid and steroid biosynthesis by adipocyte tissue in the presence of glucose with or without insulin (24-26). Our experiments clearly show an increased rate of lipogenesis from leucine when 3T3-L1 fibroblasts differentiate into adipocytes. Further, our results have implications regarding nitrogen metabolism in adipocytes, Tischler and Goldberg (27) showed that rat epididymal fat pad produces large amounts of glutamine and smaller amounts of glutamate and alanine without affecting the protein balance of the tissue. Of the three branched chain amino acids, leucine is transaminated most rapidly in adipocytes and only leucine specifically stimulates glutamine synthesis by adipose tissue (27). Tischler and Goldberg suggested that amino acid synthesis, primarily glutamine synthesis, serves to dispose of amino groups generated by catabolism of branched chain amino acids, especially leucine. In our experiments, the 30- to 40-fold increased rate of lipogenesis from leucine is not due to a correspondingly decreased rate of α-ketoisocaproate production which is reduced only 38% in differentiated 3T3-L1 adipocytes. These results are explained by the 20-fold increase of leucine transaminase activity when the fibroblasts differentiate. The fate of leucine nitrogen is very likely amino acid synthesis as proposed by Tischler and Goldberg (27). Indeed, Miller and Corrino (28) showed that 3T3-L1 adipocytes, but not 3T3-L1 fibroblasts, release large amounts of glutamine into the culture medium. Further, they showed that the specific activity and mass of glutamine synthetase increase at least 100-fold during the adipose conversion of 3T3-L1 cells. Since glutamine synthetase is not required for the adipose conversion, Miller and Corrino suggested that their results were related to the potentially important role of adipocytes in glutamine synthesis proposed by Tischler and Goldberg. Our results, considered with the findings of Miller and Corrino, support the hypothesis of Tischler and Goldberg that adipocytes couple leucine catabolism to glutamine synthesis. It is not currently known whether significant amounts of alanine and glutamine are produced by 3T3-L1 adipocytes.

**Table II**

| Treatment after confluence | Leucine transaminase | HMG-CoA lyase | CoA transferase |
|---------------------------|---------------------|---------------|----------------|
| None                      | 6.85               | 2.61          | 7.80           |
| Insulin                   | 9.80               | 3.10          | 10.17          |
| MIX + dexamethasone       | 40.9               | 4.32          | 18.8           |
| MIX + dexamethasone + insulin | 139.9              | 11.2         | 59.7           |

FIG. 3. Time course of the changes in A, branched chain α-ketoacid dehydrogenase (O) and isovuleryl-CoA dehydrogenase (C); B, the capacity to utilize leucine as a lipid precursor (A), and C, cytochrome oxidase (O) and protein:DNA ratio (X) during the adipose conversion of 3T3-L1 fibroblasts. Lipid biosynthesis from t-[U-14C]leucine was determined as described in the legend of Fig. 1. *DEX, dexamethasone.

6 after confluence and the extent of differentiation was less than 5%.

As shown in Table II, leucine:α-keto glutarate transaminase-specific activity also increases by about 20-fold during the adipose conversion induced by MIX and dexamethasone plus insulin. In this experiment, differentiation was about 90% complete on day 6 after confluence when the enzymes were assayed. Treatment with 8 µg/ml of insulin throughout the 5 days or exposure to MIX and dexamethasone for 2 days followed by refeeding the cells with the standard medium without insulin resulted in smaller increases in transaminase activity. In contrast to the relatively large increase in the specific activities of the two dehydrogenases and transaminase, adipocyte conversion resulted in a 4-fold increase in HMG-CoA lyase activity and a 7-fold increase in CoA transferase activity relative to unstimulated cells. Treatment with MIX and dexamethasone for 2 days after confluence or with 8 µg/ml of insulin results in smaller increases in the specific activities of HMG-CoA lyase and CoA transferase which correspond to the increases observed in transaminase activity and lipogenesis in these control cultures. Exposure of the 3T3-L1 fibroblasts to MIX and dexamethasone for 2 days followed by refeeding without insulin for 3 days resulted in about 30% differentiation of the population as judged by cell morphology and appearance of fat droplets in the cells. Treatment of the cells with only insulin throughout the 5 days of the experiment resulted in less than 10% differentiation. The
The increases in the specific activities of the two dehydrogenases occur in the absence of significant changes in specific activity of cytochrome oxidase. These data indicate that the increased dehydrogenase activities do not merely reflect a specific proliferation of mitochondria in 3T3-L1 adipocytes. Skeletal muscle can also oxidize large amounts of leucine; however, in contrast to adipocytes, the capacity for lipid biosynthesis from leucine is low in muscle (24). During the in vitro differentiation of proliferating chicken embryo myoblasts to multinucleate myotubes, there is a 4-fold increase in cytochrome oxidase (60) and mitochondrial DNA (61). Preliminary experiments in our laboratory show a 4-fold increase in the specific activities of cytochrome oxidase and branched chain α-ketoacid dehydrogenase in the chicken myoblast-myotube differentiation system. Thus, differentiated 3T3-L1 cells, unlike developing muscle, show increases in branched chain α-ketoacid dehydrogenase activity without general proliferation of mitochondria.

The increase in the specific activity of the 3T3-L1 branched chain α-ketoacid dehydrogenase probably reflects de novo synthesis rather than the activation via dephosphorylation (62-65). This conclusion is suggested by the following observations. First, the presence of Mg2+ during the preincubation step in the assay procedure fully activates the enzyme (66, 33). Therefore, it is reasonable that the maximal activities are assayed throughout the experiments. Second, the kinetics of the increase suggest de novo synthesis of this enzyme and the isovaleryl-CoA dehydrogenase which increases almost coordinately. Finally, the increase in the specific activity of the enzymes are comparable in magnitude to the increases in the specific activities of many other lipogenic enzymes in 3T3-L1 cells which increase in a differentiation-dependent fashion. The increases in several of these enzymes have been shown by immune precipitation to be the result of de novo synthesis (4, 11, 16, 28).

It is possible that utilization of the acetoacetate derived from leucine degradation involves activation of acetoacetate to acetoacetyl-CoA by a cytosolic acetoacetyl-CoA synthetase, as recently demonstrated in liver (67) rather than by the succinyl-CoA:acetoacetate-CoA transferase. Acetoacetyl-CoA synthetase activity permits efficient utilization of acetoacetate for fatty acid and sterol synthesis in cytosol, bypassing the unfavorable equilibrium of the thiolase reaction. We were unable to detect acetoacetyl-CoA synthetase activity in 3T3-L1 fibroblasts, 3T3-L1 adipocytes, or the soluble fraction of rat epididymal fat pad. We, therefore, conclude that acetoacetate, a product of leucine degradation, is probably utilized for lipid synthesis by a series of reactions catalyzed by the CoA transferase, thiolase, and citrate synthase in mitochondria and the cytosolic ATP-citrate lyase.

The localization of the leucine-α-keto glutarate transaminase activity which increases during the differentiation of 3T3-L1 fibroblasts is unknown. In most tissues, the greater percentage (60-80%) of leucine transaminase activity is reported to be mitochondrial (20), although there are conflicting reports for skeletal muscle (68) and liver (69). In some tissues, there are as many as three cytosolic isozymes, in addition to the mitochondrial isozyme, which transaminate branched chain amino acids with α-keto glutarate (20). In liver, only one of the cytosolic isozymes is specific for leucine (69); the remaining cytosolic and mitochondrial isozymes in liver and the mitochondrial enzymes from heart transaminate all three branched chain amino acids (70, 71). The synthesis of liver, kidney, and adipose leucine transaminases has been shown to be regulated hormonally (27, 69) and in response to diet (72), growth, and cell maturation (70). The synthesis of the hepatic leucine-specific isozyme is thought to be involved in cell maturation. The distribution of leucine-α-keto glutarate transaminase activity and the physiological responses of possible isozymes in adipocytes are unknown. The physiological functions of branched chain amino acid transaminases include provision of precursors for gluconeogenesis, catabolism of amino acids, and perhaps regulation of concentrations of branched chain amino acids for protein synthesis (73). All three of these functions are important in the differentiation of the preadipocytes.

The enzymes of the leucine catabolic pathway are part of an extensive modification of the composition of soluble mitochondrial proteins during the adipose conversion of 3T3-L1 fibroblasts indicated by the data shown in Fig. 1. From those data, it can be conservatively estimated that at least 20 soluble mitochondrial proteins are synthesized at greater rates in the adipocytes. At least one mitochondrial protein is synthesized at a lower rate or is absent in the adipocytes. Sidhu (3) has shown that the synthesis of at least 30 cytosolic proteins, 9 non-histone chromosomal proteins, and 24 membrane proteins is affected during the adipose conversion of the 3T3-L1 cells. Thus, the protein composition of the mitochondrial compartment is altered on a scale comparable to that observed in other cellular compartments and occur without mitochondrial proliferation.

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