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Changes in oleic acid oxidation and incorporation into lipids of differentiating L6 myoblasts cultured in normal or fatty acid-supplemented growth medium

Victor S. SAURO and Kenneth P. STRICKLAND*
Department of Biochemistry, Health Science Centre, University of Western Ontario, London, Ontario, Canada N6A 5C1

L6 myoblasts accumulate large stores of neutral lipid (predominantly triacylglycerol) when cultured in fatty acid-supplemented growth medium. No accumulation of neutral lipid was evident in myotubes (differentiated myoblasts) when treated similarly. Triacylglycerol accumulation was rapid and dependent on exogenous fatty acid concentration. Triacylglycerol content in myoblasts cultured in fatty acid-supplemented growth medium was approx. 3-fold higher than that in myotubes treated similarly and 2–3-fold higher than that in myoblasts cultured in normal growth medium. Incorporation studies using [1-^{14}C]oleic acid showed that myoblasts and myotubes take up exogenous fatty acid at similar rates. However, cells cultured in fatty acid-supplemented growth medium remove more exogenous fatty acid than do cells cultured in normal growth medium. Over 90% of the incorporated label was found in phospholipid and triacylglycerol fractions in all situations studied. Myoblasts incorporated a more significant proportion ($P < 0.001$) of label into triacylglycerol compared with that of myotubes. No differences in fatty acid oxidation rates were detected when differentiating L6 cells cultured in normal growth medium were compared with those cultured in fatty acid-supplemented growth medium. However, fatty acid oxidation rates were observed to increase 3–5-fold upon myoblast differentiation. We conclude that there is a marked change in the pattern of lipid metabolism when myoblasts (primarily triacylglycerol-synthesizing cells) differentiate into myotubes (primarily phospholipid-synthesizing cells). Understanding these changes, which coincide with normal muscle development, may be important, since a defect in this natural switch could explain the observed accumulation of lipid in muscle characteristic of some of the muscular dystrophies and other lipid-storage myopathies.

INTRODUCTION

We have previously shown that L6 myoblasts accumulate large stores of neutral lipid when cultured in medium supplemented with exogenous fatty acid. A similar accumulation could not be demonstrated in myotubes treated in the same manner, nor in myoblasts nor myotubes cultured in normal (unsupplemented) medium (Sauro *et al.*, 1985). Similar observations of lipid accumulation in response to exogenous fatty acids were reported in a variety of cell types by Geyer (1967), Moskowitz (1967) and Mackenzie *et al.* (1967). Blaise-Smith & Finch (1979), using cultured primary chick muscle, showed that palmitic acid was incorporated predominantly into myoblast triacylglycerols and myotube phospholipids, which suggests a switch in the pattern of lipid metabolism during muscle-cell differentiation. This pattern was confirmed in similar studies by Sandra & Ionasescu (1980). In support of their studies, we observed a significant increase in triacylglycerol lipase activity in L6 myotube extracts compared with myoblast extracts (Sauro *et al.*, 1985). These observations are indicative of a differentiation-dependent switch in lipid metabolism in muscle cells. The present project was undertaken to characterize further the change in lipid metabolism that coincides with myoblast differentiation. This study may be important in understanding the manifestation of not only the muscular dystrophies but a number of other lipid-storage myopathies, since one characteristic of these diseases is an accumulation of neutral lipid in the affected muscle (Engel & Angelini, 1973; Slavin *et al.*, 1975; Chanarin *et al.*, 1975; Miranda *et al.*, 1979). Indeed, a study by Ionasescu *et al.* (1981) led them to propose that the primary defect in Duchenne muscular dystrophy may be involved in the switching mechanism that coincides with myoblast differentiation.

MATERIALS AND METHODS

Materials

α-Minimal Essential Medium, Dulbecco's phosphate-buffered saline A, pH 7.8, and horse serum were obtained from Flow Laboratories (Rockville, MD, U.S.A.). Lipids were purchased from Serdary Research Laboratories, London, Ontario, Canada. [1-^{14}C]Oleic acid (sp. radioactivity 56 mCi/mmol) was obtained from Amersham–Searle (Arlington Heights, IL, U.S.A.). Protosol and Formula 963 liquid-scintillation-counting 'cocktail' were obtained from New England Nuclear (Lachine, Quebec, Canada). Tween 20 and Sudan Black B were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Linear K 5D silica-gel G t.l.c. plates were purchased from Whatman Chemical Separation (Clifton, NJ, U.S.A.). Kontes rubber seals and centre wells were purchased from Mandel Scientific Co. (Rockwood, Ontario, Canada). All other chemicals and solvents were of reagent grade.

* To whom reprint requests should be addressed.

Cells and cell culture

L6 myoblasts are a permanent cell line derived from rat skeletal muscle by Yaffe (1968). They were routinely cultured in α-Minimal Essential Medium supplemented with 50 μg of gentamycin/ml of medium, 10% (v/v) horse serum and 16 mM-glucose (normal growth medium) at 37 °C and in an atmosphere of 5% (v/v) CO_2 in water-saturated air. Culture medium was removed and fresh medium added after 3 days in culture. Cells were seeded at an initial density of 390000 cells/100 mm-diam. culture dish in 10 ml of normal growth medium. After the initial seeding, myoblasts proliferated until confluent (after 3 days in culture), at which point the cells fused into multinucleate myotubes (which represent rudimentary muscle fibres). This differentiation process was usually complete 6 days after the initial seeding, with over 90% of the myoblasts fused. At stated times, normal growth medium was supplemented with 0.1 mg of oleic acid/ml of medium [dissolved in 0.15% (w/v) Tween 20 to emulsify the oleic acid], which we term 'fatty acid-supplemented growth medium'; at the same time, 0.15% Tween 20 only was added to normal growth medium to serve as a control. Subculture, cell-fusion estimation (used as an index of myoblast differentiation) and cell harvest were performed by the procedures stated by Sauro *et al.* (1985).

Lipid separation and triacylglycerol determination

Lipids were extracted by the procedure outlined by Bligh & Dyer (1959). Samples of the lipid extracts were spotted on Linear K 5D silica-gel G t.l.c. plates along with neutral-lipid standards. Neutral lipids were separated by developing the plates in a unidirectional solvent system, which was light petroleum (b.p. 40–60 °C)/diethyl ether/acetic acid (70:30:1, by vol.). Triacylglycerol contents were estimated after scraping the silica gel containing the triacylglycerol, extracting the lipid from the gel three times in chloroform/methanol/diethyl ether (1:1:1, by vol.) by the procedure of Kates (1972), and determining the ester content by the method of Goddu *et al.* (1955). Standards were separated, isolated and extracted in the same manner, to correct for any losses during the procedure. Results are expressed as nmol of triacylglycerol/mg of protein.

[1-^{14}C]Oleic acid: depletion from the medium and incorporation

[1-^{14}C]Oleic acid (approx. 0.05 μCi/ml of medium) was added to cells cultured in normal and fatty acid-supplemented growth medium, on day 3 (myoblasts) and day 6 (myotubes) of culture. For the study of oleic acid depletion from the medium, small samples (0.05 ml) of the growth medium were removed immediately and at successive time points. They were added to 10 ml of liquid-scintillation 'cocktail' and the radioactivity was counted (all liquid-scintillation counting was performed in a Beckman LS 7500 scintillation counter). Results are expressed as μmol of oleic acid removed/plate after correction for differences in the specific radioactivity of the substrate. For studying incorporation of the radiolabel, the growth medium was aspirated off at various times and the cells were washed twice in ice-cold Dulbecco's phosphate-buffered saline A, pH 7.8, before cell harvest. The lipids were extracted and neutral lipids separated as stated above. Lipids were detected with iodine vapour, and the spots scraped and counted for radioactivity in 10 ml of liquid-scintillation 'cocktail'. Results are expressed as μmol of oleic acid incorporated/mg of protein after correction where necessary for differences in the specific radioactivity of the substrate owing to dilution by the added oleic acid (3.7-fold).

[1-^{14}C]Oleic acid oxidation

The rate of oleic acid oxidation was determined essentially by the procedure outlined by Angelini *et al.* (1980). $^{14}CO_2$ trapped in the growth medium was released by acidifying the medium [final concn. 10% (w/v) trichloroacetic acid] and capturing the $^{14}CO_2$ on filter paper in a centre well containing 0.2 ml of Protosol. Cells were cultured in 75 cm² flasks, but otherwise all culture conditions were as stated above. The flasks were fitted with Kontes rubber seals, through which a Kontes centre-well/filter-paper assembly was fitted. The assay was initiated at various stages of cell differentiation by the addition of 0.05 μCi of [1-^{14}C]oleic acid/ml of growth medium. The reaction was stopped after 3 h (unless otherwise stated) by injection of trichloroacetic acid

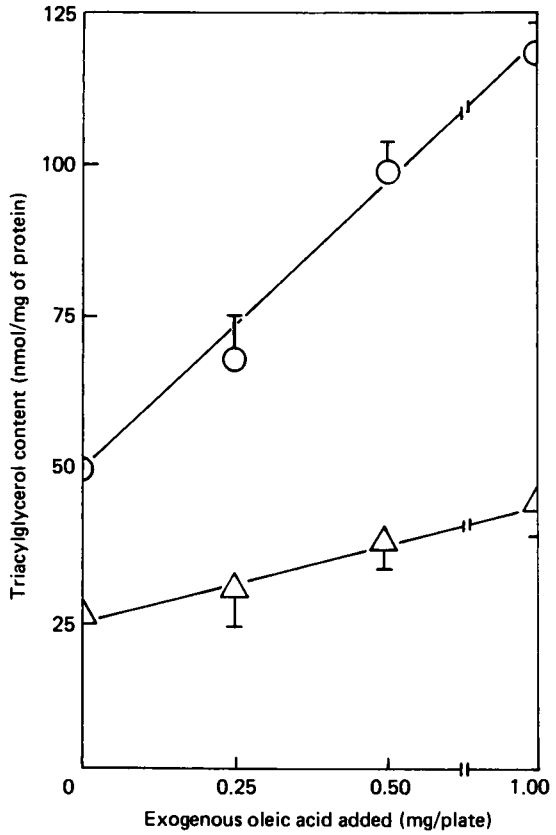

Fig. 1. Effect of increasing exogenous fatty acid concentrations on triacylglycerol contents of myoblasts and myotubes

L6 myoblasts (3 days in culture; ○) and myotubes (6 days in culture; △) were incubated for 12 h with increasing concentrations (0, 0.25, 0.50, 1.00 mg of oleic acid/plate; 10 ml of growth medium/plate) of exogenous fatty acid. Cells were harvested, lipids extracted and triacylglycerols isolated and quantified by the procedures stated in the Materials and methods section. Results, expressed as nmol of triacylglycerol/mg of protein, are means ± S.D. for five separate experiments.

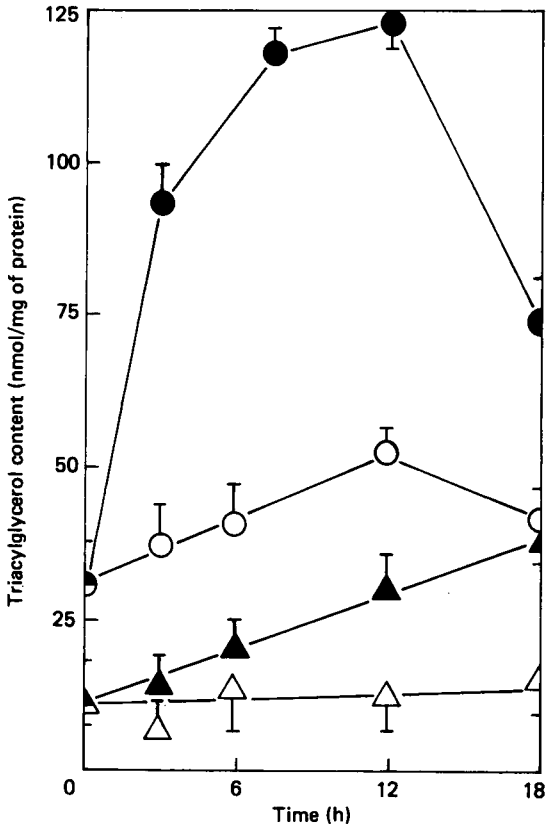

Fig. 2. Effect of time of exposure to exogenous fatty acid on triacylglycerol contents of myoblasts and myotubes

L6 myoblasts (3 days in culture; ○, ●) and myotubes (6 days in culture; △, ▲) were cultured in normal (○, △) or fatty acid-supplemented (0.1 mg of oleic acid/ml of medium; ●, ▲) growth medium for increasing periods of time (up to 18 h). Cells were harvested, lipids extracted and triacylglycerols isolated and quantified by the procedures stated in the Materials and methods section. Results, expressed as nmol of triacylglycerol/mg of protein, are means ± S.D. for six separate experiments.

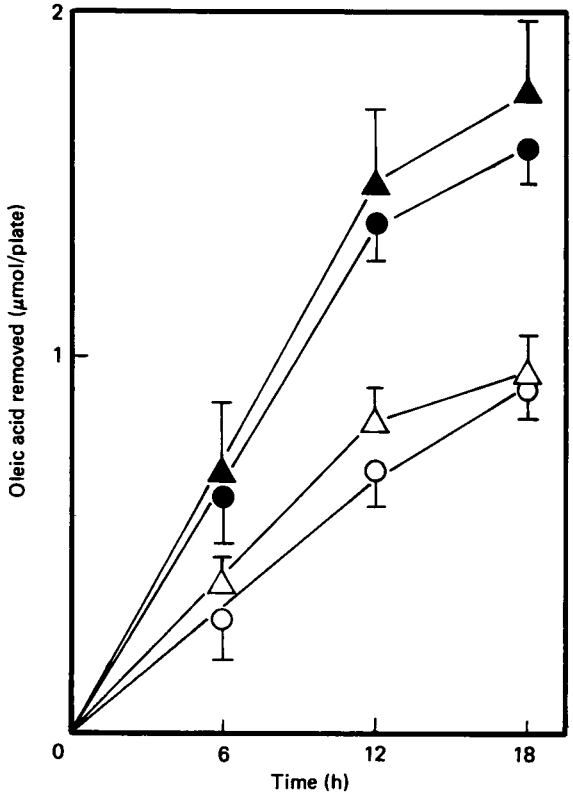

Fig. 3. Growth-medium depletion of exogenous [1-^{14}C]oleic acid by myoblasts and myotubes

L6 myoblasts (3 days in culture; ○, ●) and myotubes (6 days in culture; △, ▲) were cultured in normal (○, △) or fatty acid-supplemented (0.1 mg of oleic acid/ml of medium; ●, ▲) growth medium for various incubation times (up to 18 h). To start the experiment (zero time), 0.05 μCi of [1-^{14}C]oleic acid/ml of medium was added. A small sample (0.05 ml) was removed immediately and then others were taken at successive time points from the growth medium. The sample was used to determine the radioactivity present in the medium as described in the Materials and methods section. Results are expressed as μmol of oleic acid removed from the culture medium/plate, and are means ± S.D. for eight separate experiments.

through the rubber seal with a Hamilton syringe. At this point, the Protosol was added to the centre well and the flask was left for 1 h (to ensure maximum capture of $^{14}CO_2$). The centre-well/filter-paper assembly was then removed and added to 10 ml of scintillation 'cocktail' and the radioactivity counted. Results are expressed as nmol of oleic acid oxidized/mg of protein or nmol of oleic acid oxidized/h per mg of protein, after correction for differences in the specific radioactivity of the substrate.

Protein determination

Protein contents were measured by the method described by Bradford (1976), with bovine γ-globulin (Bio-Rad Laboratories, Richmond, CA, U.S.A.) as standard.

RESULTS AND DISCUSSION

Triacylglycerol content

We have previously reported that only L6 myoblasts, and not L6 myotubes, accumulate neutral lipid in response to exogenous oleic aicd (Sauro *et al.*, 1985). We have now shown that this neutral-lipid accumulation in myoblasts is a result of an increase in triacylglycerol content only. Although oleic acid was the only fatty acid used to supplement growth medium in these studies, preliminary studies have shown that a wide range of fatty acids varying in chain length and degree of saturation resulted in this similar accumulation of triacylglycerols in myoblasts only (as judged by Sudan Black B staining); oleic acid was the fatty acid of choice, since it appeared to stimulate the largest accumulation of triacylglycerol. This significant ($P < 0.001$) increase in triacylglycerol content in myoblasts is directly dependent on the exogenous fatty acid concentration, as shown in Fig. 1. The exogenous fatty acid supplement did result in an increase in triacylglycerol content in myotubes, but this change was minor compared with the effect in myoblasts. Fig. 2 shows that myoblasts accumulate triacylglycerols rapidly [significant ($P < 0.001$) after 3 h and optimal after 12 h incubation] when cultured in fatty acid-supplemented growth medium. On the basis of these

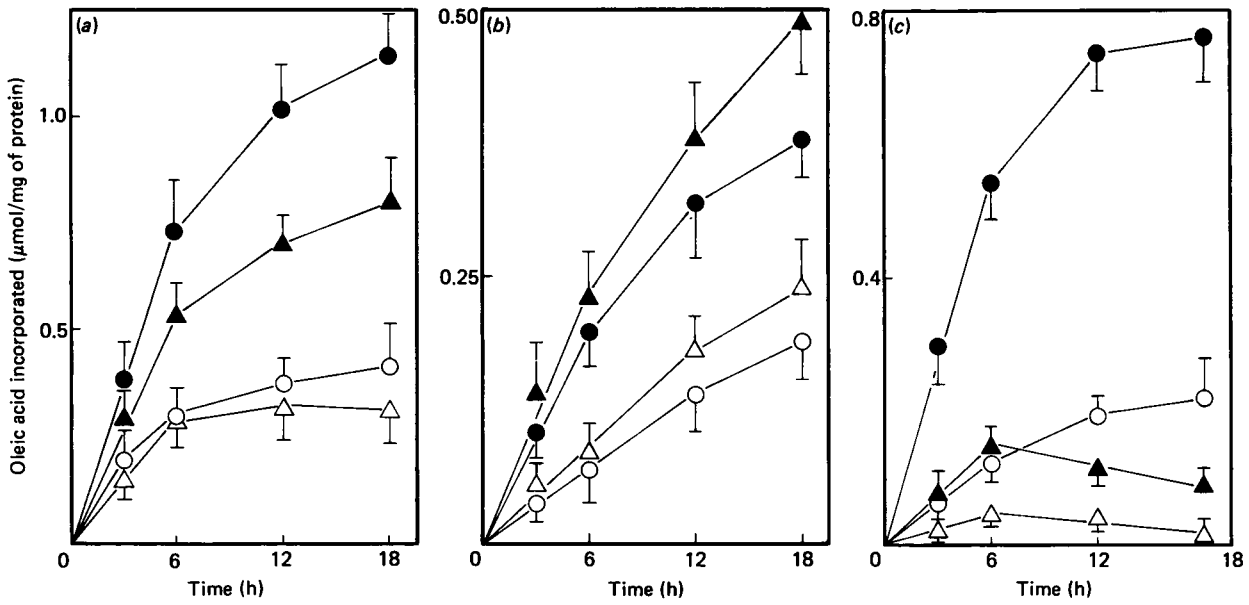

Fig. 4. Incorporation of [1-^{14}C]oleic acid into the total lipid (*a*), phospholipids (*b*) and triacylglycerols (*c*) of myoblasts and myotubes

L6 myoblasts (3 days in culture; ○, ●) and myotubes (6 days in culture; △, ▲) were cultured in normal (○, △) and fatty acid-supplemented (0.1 mg of oleic acid/ml of medium; ●, ▲) growth medium for various periods of time; 0.05 μCi of [1-^{14}C]oleic acid/ml of medium was added to initiate the incorporation. Cells were harvested, lipids extracted and separated, and radioactivity/lipid fraction was measured by the procedures stated in the Materials and methods section. Results, expressed as μmol of oleic acid incorporated/mg of protein, are means ± S.D. for four separate experiments.

results, all further studies were completed by using normal growth medium (the equivalent of 130 ± 20 μM-oleic acid) or growth medium supplemented with 0.1 mg of oleic acid/ml of medium (fatty acid-supplemented growth medium with the equivalent of 480 ± 30 μM-oleic acid). Therefore there is an approx. 3.7-fold increase in exogenous fatty acid concentration when fatty acid-supplemented growth medium is used instead of normal growth medium. This increase in exogenous fatty acid closely parallels the increase in triacylglycerol content observed in myoblasts cultured in fatty acid-supplemented growth medium compared with that cultured in normal growth medium.

Oleic acid medium depletion and incorporation

Fig. 3 clearly shows that both myoblasts and myotubes are able to deplete the medium of labelled oleic acid when cultured in normal and fatty acid-supplemented growth medium. No difference in depletion of exogenous oleic acid from the medium is evident when myoblasts are compared with myotubes. Cells cultured in fatty acid-supplemented growth medium, however, remove significantly more exogenous fatty acid from the medium. This observation was expected, since it is known that fatty acids are taken up by muscle-cell cultures by passive diffusion (Sallee & Dietschy, 1973; Spector *et al.*, 1965). In all studies, the rate of depletion from the medium is linear for up to 12 h. Similar studies using [1-^{14}C]oleic acid and [1-^{14}C]palmitic acid bound to fatty acid-free bovine serum albumin (1:1 molar ratio) show identical rates of medium depletion. Since both myoblasts and myotubes appear to remove fatty acid at similar rates, any differences in the patterns of incorporation must reflect alterations in lipid metabolism only (i.e. not uptake).

Fig. 4(*a*) shows that the rates of incorporation into total lipids are dependent on the cell type (myoblasts or myotube) and the choice of growth medium (normal or fatty acid-supplemented). No difference in oleic acid incorporation into total lipids was observed when myoblasts and myotubes cultured in normal growth medium were compared. Myoblasts and myotubes cultured in fatty acid-supplemented growth medium also have similar rates of oleic acid incorporation into total lipids. However, cells cultured in fatty acid-supplemented growth medium show a 2–3-fold stimulation in incorporation into total lipids as compared with cells cultured in normal growth medium. These results reflect the increased oleic acid uptake observed in cells cultured in fatty acid-supplemented growth medium as shown in Fig. 3. When total lipids are separated into the various lipid fractions, over 90% of the label distributes into phospholipids and triacylglycerols in all situations studied. Oleic acid incorporation into the phospholipids is shown in Fig. 4(*b*), and that into triacylglycerols in Fig. 4(*c*). Myotubes incorporate oleic acid into phospholipid at a slightly greater rate than that observed in myoblasts when cells are cultured in normal or fatty acid-supplemented growth medium (Fig. 4*b*). There is, however, an approx. 2-fold stimulation in oleic acid incorporation into phospholipids of cells cultured in fatty acid-supplemented growth medium compared with those cultured in normal growth medium. Fig. 4(*c*) shows that when myoblasts are cultured in fatty acid-supplemented growth medium there is a 4–5-fold increase in the incorporation of fatty acid into triacylglycerols over that of myoblasts cultured in normal growth medium. These results indicate a marked stimulation in fatty acid incorporation into triacylglycerols when myoblasts are cultured in fatty acid-supplemented growth medium. Only minor differences were observed in fatty acid incorporation into myoblast

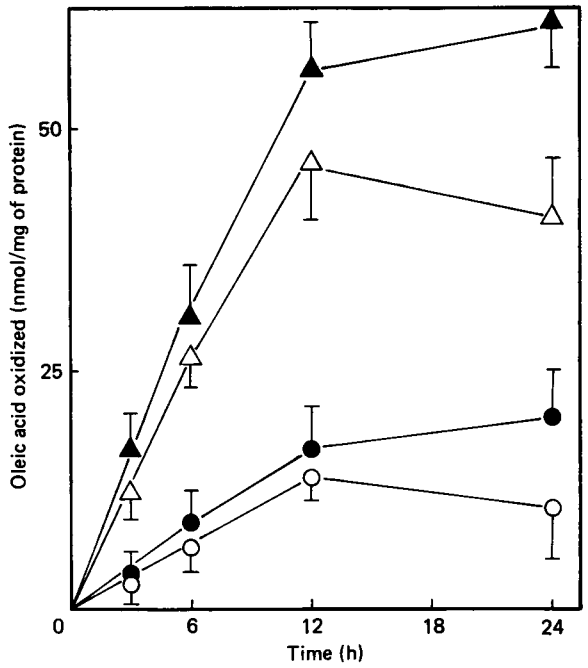

Fig. 5. Time-course characterization of [1-^{14}C]oleic acid oxidation in myoblasts and myotubes

L6 myoblasts (3 days in culture; ○, ●) and myotubes (6 days in culture; △, ▲) were cultured in normal (○, △) or fatty acid-supplemented (0.1 mg of oleic acid/ml of medium; ●, ▲) growth medium for various periods of time in 75 cm^2 flasks. [1-^{14}C]Oleic acid oxidation was assayed by the procedure stated in the Materials and methods section. The assay was initiated by addition of 0.05 μCi of [1-^{14}C]oleic acid/ml of medium in a sealed flask. Released $^{14}CO_2$ was trapped on Protosol-treated filter paper for 1 h after the reaction was stopped with 10% trichloroacetic acid. Results, expressed as nmol of oleic acid oxidized/mg of protein, are means±S.D. for four separate determinations.

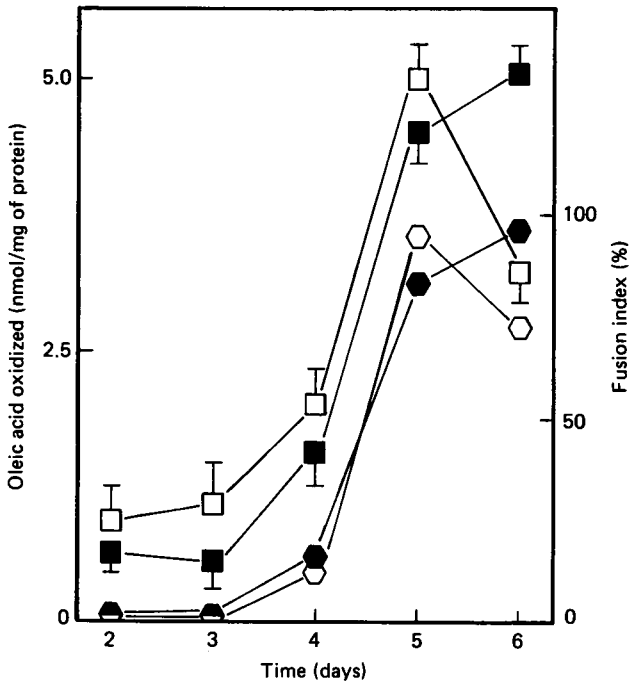

Fig. 6. [1-^{14}C]Oleic acid oxidation in myoblasts and its changes on differentiation

L6 myoblasts were cultured in normal (□, ⬡) or fatty acid-supplemented (0.1 mg of oleic acid/ml of medium; ■, ⬢) growth medium in 75 cm^2 flasks. At various times in culture (2, 3, 4, 5 and 6 days), cells were assayed for [1-^{14}C]oleic acid oxidation (□, ■; expressed as nmol of oleic acid oxidized/h per mg of protein) by the procedure described in the Materials and methods section, with a 3 h incubation period. Duplicate flasks were fixed and stained for estimation of fusion (⬡, ⬢; expressed as fusion percentage) by the procedure stated in the Materials and methods section. Results are means±S.D. for six separate determinations.

and myotube triacylglycerols when cultured in normal growth medium, and into myotube triacylglycerols when cultured in fatty acid-supplemented growth medium. The fraction of fatty acid incorporated into triacylglycerol relative to that into total lipid was markedly different for myoblasts compared with myotubes. Approx. 30% and 60% of the incorporated radiolabel distributes into myoblast triacylglycerols when cells are cultured in normal and fatty acid-supplemented growth medium respectively. Only 5% and 20% of the incorporated radiolabel is found in myotube triacylglycerols when cells are cultured in normal and fatty acid-supplemented growth medium respectively. These results suggest that, like protein synthesis (Sanwal, 1979), lipid turnover increases in synchrony with myoblast differentiation. The increased turnover probably reflects an increase in membrane lipid (mostly phospholipid) synthesis. This increase has been verified by measuring the lipid-soluble phosphate extracted from myoblasts and myotubes. There is 2.6-fold increase in lipid-soluble phosphate extracted from myotubes over that of myoblasts, which parallels the increase in protein content described previously (Sauro *et al.*, 1985).

Again, in order to show that the observed patterns of [1-^{14}C]oleic acid incorporation are representative of fatty acid incorporation in general, similar experiments were repeated with [1-^{14}C]palmitic acid as the marker. Identical patterns of incorporation were observed, with similar but somewhat lower rates of incorporation. Cook *et al.* (1982) have also observed an accumulation of label in the triacylglycerols of neuroblastoma cells when similar experiments were done. They hypothesized that fatty acids are temporarily stored by esterification to triacylglycerol before being used for the synthesis and turnover of membrane phospholipid. The results reported here support this model.

Changes in fatty acid oxidation with age of cultured cells

Fatty acid oxidation rates were measured in myoblasts and myotubes in order to determine (1) if an increased energetic demand could be correlated with differentiation and (2) the probability that an increase in oxidation, if it exists, could be responsible for the inability of myotubes to accumulate neutral lipid. Fatty acid oxidation was first characterized in 3-day (myoblast) and 6-day (myotube) cultures and then assessed in cell cultures at various stages of cell differentiation. The optimal incubation period for capture of $^{14}CO_2$ was 1 h. Fatty acid oxidation rates in myoblasts and myotubes

cultured in normal and fatty acid-supplemented growth medium were characterized with respect to time (Fig. 5). In all situations studied, oxidation was linear with respect to time for up to 12 h. Fig. 5 also shows that myotubes have a much higher rate of fatty acid oxidation (3–5-fold greater) than myoblasts. Fig. 6 shows the fatty acid oxidation rates in cells cultured in normal or fatty acid-supplemented growth medium at various stages of differentiation; these results suggest that there is no difference in the oxidation rate when cells cultured in normal growth medium are compared with cells cultured in fatty acid-supplemented growth medium. There is, however, a highly significant increase in fatty acid oxidation with time ($P < 0.001$) when fatty acid oxidation rates are compared at 3 and 6 days in culture. This increase was synchronous with cell differentiation, as indicated in Fig. 6 by cell fusion. Again, a similar pattern of fatty acid oxidation was seen when [1-^{14}C]-palmitic acid was used as substrate, but with noticeably lower rates of oxidation. It is probable that the increase in fatty acid oxidation observed to coincide with myoblast diffentiation reflects the increased energetic demands on the cell (increase in both protein and lipid synthesis), which is observed during myoblast differentiation. The nature of this increase, whether due to an increase in fatty acid substrate, oxidative enzymes or a combination of both, remains to be determined. Since the addition of exogenous carnitine (1 mg/ml of growth medium) stimulates fatty acid oxidation 2–3-fold in both myoblasts and myotubes (results not shown), the carnitine content does appear to be rate-limiting in fatty acid oxidation, but does not explain the observed increase in oxidation that coincides with cell differentiation.

Our results show that the capacity to oxidize fatty acid increases significantly during normal myoblast differentiation. A defect in dystrophic muscle preventing the normal changes in lipid metabolism that occur simultaneously with myoblast differentiation (specifically, increased fatty acid oxidation and triacylglycerol turnover) could explain the noted accumulation of neutral lipid in the diseased muscle, as postulated by Lin *et al.* (1969). However, the rates of fatty acid incorporation are at least 100-fold greater than those of fatty acid oxidation in L6 cells. Therefore it is unlikely that the increase in oxidation is responsible for the inability of L6 myotubes to accumulate neutral lipid under conditions which produce this effect in L6 myoblasts.

In summary, we have shown that fatty acid uptake by myoblasts and myotubes is similar, but only myoblasts accumulate neutral lipid (primarily triacylglycerols) in response to exogenous fatty acid. Incorporation studies have shown that myoblasts synthesize predominantly triacylglycerols and myotubes predominantly phospholipids. Fatty acid oxidation was significantly greater in myotubes than in myoblasts, but it is unlikely that this is responsible for the inability of myotubes to accumulate neutral lipid. This study suggests that myotubes have a greater requirement for fatty acid than do myoblasts, primarily owing to an increased demand for fatty acid as substrate for phospholipid synthesis.

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