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ALTERATIONS IN LIPID TURNOVER IN DEVELOPING MUSCLE

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SUMMARY

The incorporation and turnover of [^{3}H] glycerol into skeletal muscle cell cultures derived from embryonic chickens was studied. Both rates of incorporation and turnover of specific lipids were dependent on culture age and lipid species. The pattern of glycerol incorporation showed that prefusion myoblasts primarily synthesized both phosphatidylcholine and triglycerides whereas postfusion myotubes primarily synthesized phosphatidyl choline. This pattern could be modified in postfusion but not prefusion cells by briefly incubating the cells with unilammelar phosphatidyl choline vesicles. Analysis of major lipid species revealed that muscle triglycerides and phospholipids turned over at a higher rate in prefusion cultures compared to the postfusion state. These findings are discussed in light of the marked shift in lipid metabolism which occurs during myogenesis.

The metabolism of lipids has been reported to undergo marked alteration during myogenesis *in vitro*. Although myoblasts and myotubes have a similar phospholipid and acyl chain composition (1, 2) the prefusion myoblasts incorporate radiolabeled palmitic acid into triglycerides at a level 6-7 fold greater than do postfusion myotubes (2). Since endogenous triglyceride levels are similar in the two developmental stages(2), an increased triglyceride turnover in myoblasts has been postulated. This developmentally regulated control of muscle lipid was studied in the present report by analyzing turnover of lipid in embryonic chick muscle *in vitro*. The results demonstrate: 1) differential incorporation of [^{3}H] glycerol and its turnover in the various lipid classes, and 2) an overall decrease in turnover at postfusion compared to prefusion stages of myogenesis. The differential incorporation of lipids could be altered by preincubating postfusion cells with phospholipid vesicles.

Abbreviations: PC, phosphatidylcholine; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; PI, phosphatidyl inositol.

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Materials and Methods

Cells and Cell Cultures

Normal (strain 454) fertile chicken eggs were purchased from the University of California, Davis, Ca. The eggs were incubated for 12 days at 38° at which time myogenic cells were isolated from the breast or leg according to the methods of Konigsberg (3) and Stockdale and O'Neill (4). The muscle was minced, trypsinized for 5 min. at 37°C in 0.25% trypsin solution and the digestion was stopped by the addition of an equal volume of cold culture medium. The suspension was filtered through three layers of cheesecloth and centrifuged at 800 xg for 5 min. The cell pellet was then resuspended in complete culture medium which contained Minimal Essential Medium with Hanks' salts, 10% horse serum, and 2% chick embryo extract, and filtered through mesh bolting silk. The cells were plated onto 35 mm collagen-coated plastic petri dishes at a density of 5×10^5 cells per dish. Cultures were kept in a humidified atmosphere of 5% CO_2 in air. Culture medium was changed every other day. Results are based on duplicate cultures and at least three separate experiments. Standard error are within 10%.

Labeling experiments

Prefusion (24hr.) and post fusion (120hr.) muscle cultures were radiolabeled by the addition of fresh medium containing 5μCi/ml of [2-^{3}H] glycerol (200/mCimmol, New England Nuclear). After the labeling period, the cells were washed three times with phosphate buffered saline before extraction and analysis of lipids. In pulse-chase experiments, nonradiolabeled glycerol (10mM) containing medium was added to the culture dish after the radiolabeled medium was rinsed out with saline solution. After the chase period, the cultures were again washed and processed for lipid extraction.

Lipid Extraction and Analysis

After washing with saline solution, ice-cold methanol was added to cultured cells. The cells were scraped into glass tubes, dried under argon, and extracted overnight in chloroform-methanol (2:1;v/v) according to the method of Robbins and MacPherson (5). Aliquots of the extract were analyzed by thin layer chromatography using silica gel G plates. Two solvent systems (isopropyl ether: heptane: acetic acid 60:40:2 and chloroform: methanol: water 65:25:4) were sequentially run to separate the neutral lipids from the phospholipids. With the aid of appropriate lipid standards which were visualized by staining with iodine vapor, the spots corresponding to triglycerides (TG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine + phosphatidylinositol (PS + PI) were scraped into scintillation vials and counted.

Noncollagen Protein Determination

Noncollagen protein was determined by the method of Lowry et al (6) using bovine serum albumin as standard.

Phospholipid Vesicle Preparation

Unilammelar lipid vesicles were prepared from purified egg yolk phophatidyl choline by sonication as previously described (7, 8). Vesicles were applied to cultures washed 6 times in Hank's Minimal Essential Medium at 1 mg/ml for 1 hr at 37°C. Cultures were then washed free of excess vesicles (6 times) and replenished with [^{3}H] glycerol-containing medium for 24 hr and analyzed as above

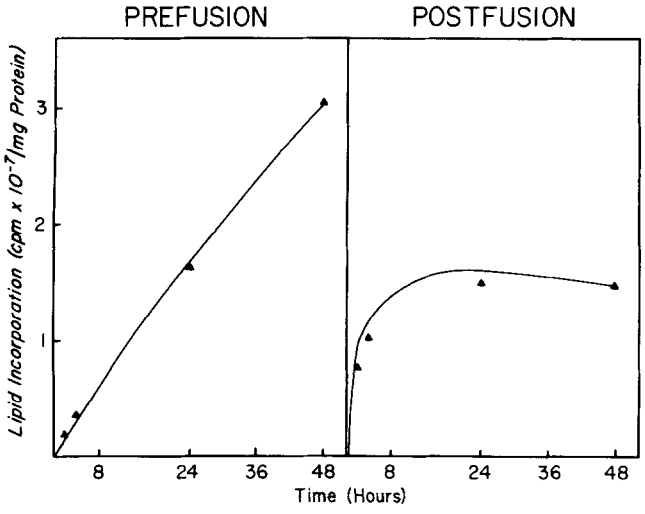


Figure 1. Incorporation of [^{3}H] glycerol into total lipid into chick muscle. Labeling of prefusion cultures began at day 1 and postfusion cultures at day 5 in vitro.

RESULTS

Total uptake of [^{3}H] glycerol into chloroform-methanol extractable material was greater in myoblasts than in myotubes (Fig. 1). The incorporation of [^{3}H] glycerol by myoblasts into the various lipid species indicates that labeling of triglycerides predominates. Labeling of 1-day old cultures tended to level off after 48 hr. in vitro, (Fig. 2). When postfusion (5 day) cultures

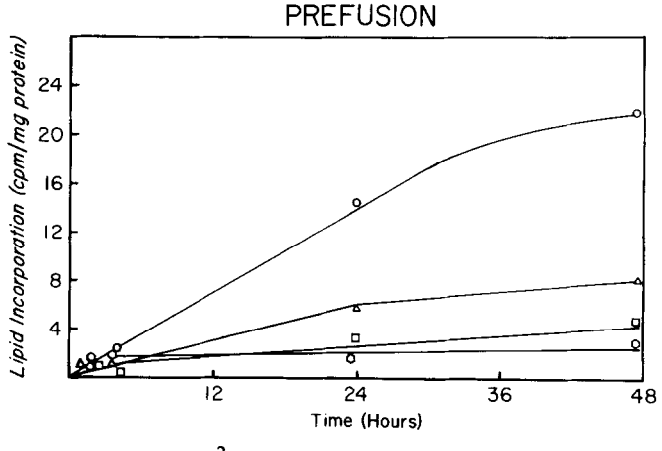


Figure 2. Incorporation of [^{3}H] glycerol into muscle lipids in prefusion (1 day) cultures as a function of time. (○) PC; (Δ_5) PE; (□) PS & PI; (0) TG. PC and TG are expressed as cpm x 10^{-4}/mg protein, and PE and PS & PI as cpm x 10^{-4}/mg protein.

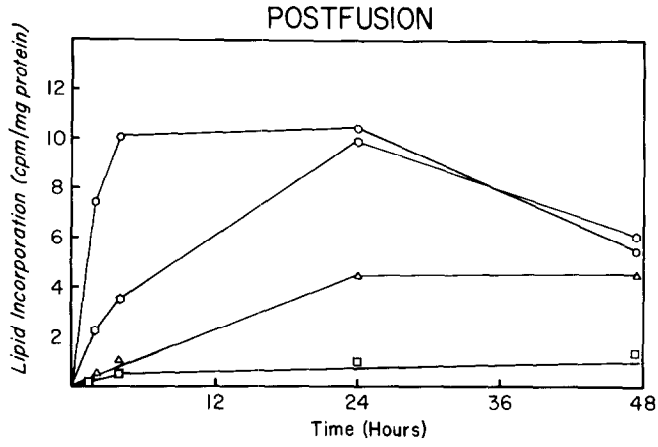
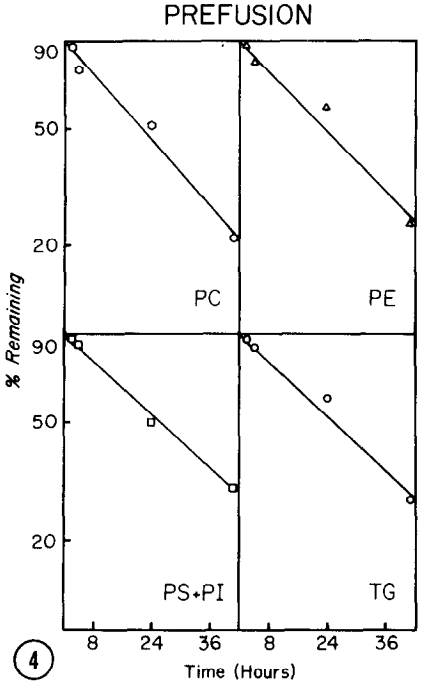


Figure 3. Incorporation of [^{3}H] glycerol into muscle lipids in postfusion (5 day) cultures as a function of time. (○) PC; (Δ) PE; (□) PS & PI: (0) TG. Results are expressed as described in Fig. 2.

were exposed to [^{3}H] glycerol, the pattern of precursor uptake into cellular lipids shifted (Fig. 3). Triglycerides were initially rapidly labeled and soon reached a steady state level. PC was labeled at levels comparable to triglycerides at subsequent times and the remaining lipids which were analyzed, PC and PS & PI, reached steady levels by 24 hr. of labeling.

When pre-and post fusion muscle cells were labeled for 48 hr. with [^{3}H] glycerol and subsequently chased using 10 mM glycerol, the rate of turnover varied with developmental state. In addition, the different lipid species themselves appeared to turnover at different rates (Fig. 4). In prefusion cultures, half-time of overall lipid turnover was much faster (∿24 hr.) than in postfusion cultures (∿4-5 days). PC demonstrated the highest rate of turnover in postfusion cultures followed by PS & PI, triglycerides, and PE (Fig. 5).

In order to determine whether or not the marked shift in lipid incorporation could be altered by exogenously supplied specific lipid, egg yolk phosphatidylcholine vesicles were interacted with the cells in situ for 1 hr at 37°C. After the cells were washed, and replenished with fresh medium, [^{3}H] glycerol was added to the cultures for 24 hr. Labeled lipids were extracted, separated by thin layer chromatography, and analyzed for



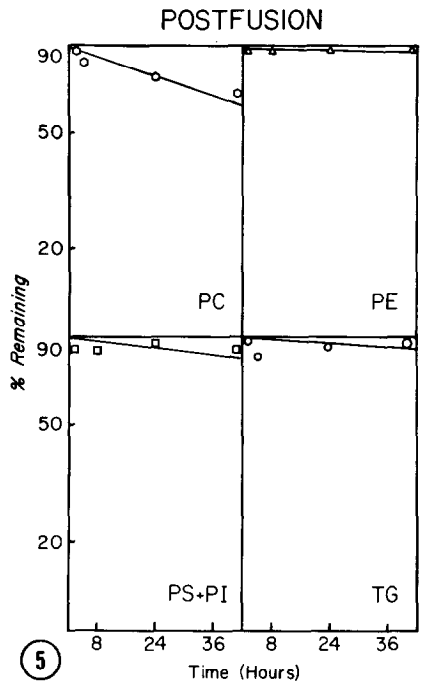


Figure 4. Percent of specific lipid remaining in prefusion cultures (day 1) after labeling for 48 hr with [^{3}H] glycerol and chasing at time zero with 10 mM glycerol. Results are expressed as % lipid remaining per mg protein.

Figure 5. Percent of specific lipid remaining in postfusion cultures (day 5) after labeling for 48 hr with [^{3}H] glycerol and chasing at time zero with 10 mM glycerol. Results are expressed as % lipid remaining per mg protein.

radioactivity. The results (Table 1) show that liposome treatment did not effect the incorporation of glycerol into phospholipids relative to triglycerides in prefusion cells, but sharply decreased relative incorporation into PC of postfusion cells.

DISCUSSION

The shift in [^{3}H] glycerol incorporation during myogenesis from primarily triglycerides in myoblasts to phospholipids in myotubes appears to be correlated with a faster triglyceride turnover in the earlier stage of muscle development. This dramatic shift in metabolism occurs without a significant change in cellular content of these lipids throughout myogenesis (2) and may be related to

Table 1. Effect of PC vesicles on ^{3}[H] glycerol incorporation in developing muscle cells (cpm x 10^{-4}/mg protein)

Lipid	Prefusion		Postfusion	
	Control	Liposome-treated	Control	Liposome-treated
PC	22	21	100	36
TG	150	145	92	90
PC/TG	0.1	0.1	1.1	0.4

Pre- and postfusion cells were treated with vesicles as described in Materials and Methods. Values are the average of three experiments.

the development of appropriate metabolic pathways required for the utilization of carbohydrates in mature muscle. These would include, for example, a hormonally-sensitive hexose transport system (9, 10) and a glycogen phosphorylase complex (11). Such a shift appears to be specific for muscle and not fibroblasts which make up 30-40% of the total muscle cell population in vitro. Pure fibroblast cultures derived from chick skin in our experiments do not demonstrate this shift and maintain a rather constant triglyceride-phospholipid ratio at both early and late stages (results not shown).

The turnover of radiolabeled phospholipids has been estimated at 15 hr. (12) and 20-24 hr. (13) using precursors other than glycerol. These values are similar to those obtained in the present study with prefusion myoblasts. The different species of lipids labeled with glycerol have also been shown to turn over at different rates. Lipids of fibroblasts derived from hamster and chick embryos turned over in the descending order: PC, PS & PI, PE (14). This is the same order of rate of lipid turnover which was observed in the present study using postfusion cultures. In prefusion cultures, a similar order was observed, although overall turnover rate was 4-5 times greater. The markedly enhanced turnover rate observed in prefusion cultures also appears to be muscle specific. In chick fibroblasts no difference in turnover rate was observed in sparse, fast growing cells compared to stationary, confluent ones (14).

The metabolism of lipids in cell culture has been the subject of extensive study (for reviews, see 15, 16). This work has generally concluded that serum containing medium provides the immediate source of cellular lipids. Upon withdrawal of serum, cultured cells utilize endogenous metabolic pathways for the synthesis of new lipid. The addition of radioactive precursors of lipid metabolism such as acetate or glucose lipid enter all major lipid classes and the extent of incorporation into each class appears to be about equally effected by the presence or absence of serum. Besides serum lipids, liposomes introduced into the medium have also been shown to effect cellular lipid metabolism. Bruckdorfer et al (17, 18) reported that cholesterol content of low density lipoproteins and red blood cell membranes was altered by incubation with cholesterol-containing liposomes. Incubations of red blood cells with a cholesterol-containing dispersion also elevated cholesterol: phospholipid ratios (19, 20), suggesting the alteration is specifically effecting one class of lipids.

Because more complex cells such as muscle regulate their lipids metabolically, the addition of exogenous PC may differentially effect metabolism depending on the metabolic demands of the cell. Thus, prefusion myoblasts which depend largely on oxidative metabolism appear to metabolize glycerol primarily into triglycerides and this pattern is not markedly effected by exogenous PC. Postfusion cells on the other hand, are primarily glycolytic and convert a much larger portion of glycerol into phospholipid, presumably to meet the demands of membrane synthesis. The cellular PC level in mature muscle may be more sensitive to exogenous phospholipid and thus the metabolic shift may account for the differential response of pre- and post fusion myoblasts to PC vesicle.

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REFERENCES

1. Kent, C., Schimmel, S., and Vagelos, P.R. (1974) Biochim. Biophys. Acta 360:312-321.
2. Smith, P.B. and Finch, R.A. (1979) Biochim. Biophys. Acta 572:139-145.
3. Konigsberg, I.R. (1971) Dev. Biol. 26:133-152.
4. Stockdale, R.E. and O'Neill, M.C. (1972) In Vitro 8:212-227.
5. Robbins, P.W. and MacPherson, I.A. (1971) Proc. Royal Soc. Lond. (Biol.) 177:48-58.
6. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193:265-275.
7. Huang, L. and Pagano, R.E. (1975) J. Cell Biol. 67:38-48.
8. Sandra, A. (1980) Exp. Cell Res. (In press).
9. Sandra, A. and Przybylski, R.J. (1979) Dev. Biol. 68:546-556.
10. Schudt, C., Gaertner, V. and Pette, D. (1976) Eur. J. Biochem. 68:103-111.
11. Wahrmann, J.P., Recouvreur, M. and Favard-Sereno, C. (1977) J. Cell Sci. 26:77-91.
12. Pasternak, C.A. and Bergeron, J.J. (1970) Biochem. J. 119:473-480.
13. Plagemann, P.G. (1971) J. Lipid Res. 12:715-724.
14. Hirschberg, C.B. (1977) Exp. Cell Res. 110:355-361.
15. Rothblat, G.H. (1969) Adv. Lipid Res. Vol 7, pp. 135-164, Academic Press, New York.
16. Bailey, J.M. (1977) Lipid Metabolism in Mammals, Vol 2, pp. 323-352, Plenum Press,New York.
17. Bruckdorfer, K.R., Edwards, P.A. and Green, G. (1968) Eur. J. Biochem. 4:506-511.
18. Bruckdorfer, K.R., Demel, J., DeGier, J. and van Deenen, L.L.M. (1969) Biochim. Biophys. Acta 183:334-345.
19. Cooper, R.A. (1977) N. Eng. J. Med. 297:371-377.
20. Cooper, R.A., Arner, E.C., Wiley, J.S. and Shattil, S.J. (1975) J. Clin.