III. ANALYSIS OF THE FUNCTIONING OF SKELETAL MUSCLE LIPIDS DURING FASTING*

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SUMMARY

The effect of fasting on skeletal muscle lipids has been studied. The amount of phospholipid per g of muscle remained constant throughout prolonged fasting, although when death is imminent it appears that a small fall in phospholipid concentrations may occur. The distribution of lipid phosphorus among the various phospholipid subclasses was not found to be influenced by fasting. Triglyceride tended to increase in muscle during the early stages of fasting but was ultimately depleted when the period of fasting was quite prolonged. Muscle free fatty acids (FFA) were markedly increased during fasting, and evidence is presented that the changes in muscle FFA were not merely a reflection of the changes occurring in plasma FFA.

The conclusion is drawn that the phospholipids of muscle are structural-functional elements that are not depleted during fasting for purposes of energy metabolism. It is also concluded that muscle triglyceride does not serve as an energy reservoir to be drawn upon rapidly during caloric deficiency, although if caloric restriction is sufficiently prolonged the triglycerides are then drawn upon to the point of nearly total depletion.

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In recent years, there has been a growing belief (1-6) that the intracellular skeletal muscle lipids serve as a reservoir of ready energy to be used during periods of high energy expenditure, such as vigorous contractile activity, and during periods of caloric deficiency, such as fasting. Presumably these lipid stores are then replenished during rest and refeeding. On the bases of respiratory quotient and plasma free fatty acid turnover and oxidation studies in exercising dog and man, Isselkutz et al. (6) and Havel et al. (3-5) suggested that intracellular muscle lipid esters supply fatty acid fuel for the contracting muscle in the postabsorptive state. Furthermore, from the work of Neptune et al. (1, 2), it has been concluded that intracellular skeletal muscle lipids serve as an energy reservoir to be used during periods of restricted caloric supply, this conclusion being based on the disappearance of some of the triglyceride and most of the phospholipid from rat diaphragm during a 48-hour fast.

In the last paper reported from our laboratory (7), however, the concept that skeletal muscle lipids serve as an energy reservoir to be used during contractile activity was not borne out. Rather, it was established that the concentration of various classes of phospholipid and triglyceride in the skeletal muscle is not influenced by prolonged contractile activity, even in postabsorptive animals. The conclusion was drawn that intracellular muscle lipids are not used as a net source of fuel for the increased energy metabolism of contracting muscle. Since it seemed paradoxical that intracellular muscle lipids would serve as an energy reservoir for ready use during fasting but could not be utilized for contractile activity, the effect of fasting on intracellular muscle lipid was investigated further. The following is a report of this study.

METHODS

Male rats of the Charles River strain were used. Data on the weights and the fasting periods are recorded in the various tables. The rats were killed by decapitation; the muscles were then excised and chilled on ice before the adipose tissue was carefully removed by dissection.

The phospholipid, neutral lipid fatty acid esters, and cholesterol of the muscle were quantitatively extracted and purified (8). Small aliquots of the extract were used for the determination of lipid phosphorus (9). In the experiments in which plasmalogens were measured, a small aliquot of the extract was assayed by the method of Gottfried and Rapport (10).

In the experiments in which the neutral lipids and phospholipids were subfractionated into various components, the column and thin layer chromatographic methods described previously (8) were used; the phospholipids, however, were eluted from the thin layer plates by a recently described modification (7). Cholesterol was assayed as previously described (8). Triglyceride was measured by the modified (8) hydroxylamine method of Lands (11). The lipid phosphorus of the various phospholipid subfractions was determined as described previously (8, 9).

In many experiments only the following components were measured: lipid phosphorus, neutral lipid fatty acid ester, and FFA. To measure neutral lipid ester, the lipid extract was

1 The abbreviation used is: FFA, free fatty acid or acids.
Comparison of hydroxylamine and infrared methods of assay of fatty acid ester content of neutral lipids of rat gastrocnemius

<table>
<thead>
<tr>
<th>Nutritional state</th>
<th>Hydroxylamine assay</th>
<th>Infrared assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µeq fatty ester/g</td>
<td>µeq fatty ester/g</td>
</tr>
<tr>
<td>Fed</td>
<td>9.6</td>
<td>9.3</td>
</tr>
<tr>
<td>Fed</td>
<td>6.9</td>
<td>6.6</td>
</tr>
<tr>
<td>Fed</td>
<td>10.5</td>
<td>10.5</td>
</tr>
<tr>
<td>24-hr fasted</td>
<td>7.5</td>
<td>7.2</td>
</tr>
<tr>
<td>72-hr fasted</td>
<td>13.5</td>
<td>12.0</td>
</tr>
<tr>
<td>72-hr fasted</td>
<td>30.9</td>
<td>29.7</td>
</tr>
</tbody>
</table>

* Each value is an average of duplicate determinations from a single extract of muscle. In no instance did duplicate determinations vary from each other by more than 5%.

† Each value is an average of duplicate determinations from a single extract of muscle. In no instance did duplicate determinations vary from each other by more than 2%.

TABLE II
Effect of 72 hours of fasting on lipids of adult rat leg muscle

The results shown are average values from four rats ± S.E.M. The fed rats weighed 487 ± 49.9 g, and the fasted rats, 450 ± 34.6 g before fasting and 389 ± 31.0 g after the 72-hour fast.

<table>
<thead>
<tr>
<th>Lipid Fraction</th>
<th>Concentration units (µg per g of muscle wet wt)</th>
<th>Fed rats</th>
<th>Fasted rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lipids</td>
<td>mg</td>
<td>13.5 ± 0.28</td>
<td>14.6 ± 0.93</td>
</tr>
<tr>
<td>Lipid phosphorus</td>
<td>µmoles</td>
<td>12.1 ± 0.28</td>
<td>12.8 ± 0.06</td>
</tr>
<tr>
<td>Total plasmalogens</td>
<td>µmoles</td>
<td>1.7 ± 0.06</td>
<td>1.7 ± 0.08</td>
</tr>
<tr>
<td>Plasmalogens to lipid phosphorus ratio...</td>
<td>0.14 ± 0.03</td>
<td>0.13 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Triglyceride</td>
<td>µmoles</td>
<td>2.9 ± 0.10</td>
<td>4.6 ± 0.13</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>µmoles</td>
<td>1.4 ± 0.13</td>
<td>1.4 ± 0.08</td>
</tr>
<tr>
<td>Polyglycerophosphatide</td>
<td>µeqP</td>
<td>0.74 ± 0.045</td>
<td>0.70 ± 0.076</td>
</tr>
<tr>
<td>Phosphatidyethanolamine</td>
<td>µmoles</td>
<td>3.0 ± 0.06</td>
<td>3.1 ± 0.18</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>µmoles</td>
<td>0.39 ± 0.069</td>
<td>0.35 ± 0.057</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>µmoles</td>
<td>0.75 ± 0.032</td>
<td>0.88 ± 0.056</td>
</tr>
<tr>
<td>Lecithin</td>
<td>µmoles</td>
<td>6.5 ± 0.07</td>
<td>6.7 ± 0.08</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>µmoles</td>
<td>0.35 ± 0.030</td>
<td>0.34 ± 0.024</td>
</tr>
</tbody>
</table>

* Difference between fed and fasted rats was analyzed with Student’s t test; p value was calculated as < 0.001.

The relationship between absorbance at 5.85 µ and the concentration of triolein (Applied Science Laboratories) in chloroform was determined. This relationship is linear in the concentration range of 1.2 to 10.8 µeq of fatty acid ester per ml. With a 1-mm light path cuvette, the range of absorbance was 0.048 to 0.642.

The good agreement between the two methods is related to the following facts: (a) the amount of FFA compared to neutral fatty acid ester is quite small, and thus FFA adds little absorbance at 5.85 µ above that due to the esters; (b) cholesterol esters, which have a slightly lower absorbance per eq of fatty acid ester than do glycerides, are present in small amounts compared to glycerides and therefore influence total absorbance at 5.85 µ to a small extent. Indeed, since monoglyceride and diglyceride are present in muscle only in trace quantities, the infrared assay of neutral lipid ester is an approximate measure of triglyceride fatty acid ester.

To measure FFA of muscle, an aliquot of the phosphorus-free chloroform solution described above was combined with a similar aliquot of a phosphorus-free chloroform solution derived from an acid extract of the muscle residue. The FFA of this combined solution was measured by the colorimetric copper salt method of Itaya and Ui (13). The preparation of the acid extract of muscle was found to be necessary since, on the basis of three tests, the extraction system (8) used for quantitative extraction of the lipid phosphorus, cholesterol, and neutral lipid esters was found to extract only 66 to 83% of the FFA. The following procedure was used.

The residue remaining after the extraction of the lipid phosphorus, cholesterol, and neutral lipid esters was homogenized in chloroform-methanol-hydrochloric acid (66:33:0.25). The material was then quantitatively filtered through a sintered glass funnel. The extract was purified by adding an appropriate amount of 0.1 M KCl (aqueous solution) (14) and shaking vigorously in a separatory funnel. An aliquot of the lower phase was brought to dryness in a rotary evaporator under reduced pressure and dissolved in chloroform. An aliquot of the chloroform solution was treated with silicic acid by the method of Jover (12). An aliquot of the resultant solution was combined with the one from the neutral extraction for FFA analysis. The Jover method removes 10% of the FFA from the chloroform solutions. Since this loss is small and reproducible, it was felt that, for our purposes, no correction need be made.

Plasma FFA was measured by the method of Itaya and Ui (13).

RESULTS

Data from the detailed analyses of the lipid content of the hind leg muscles of fed and 72-hour fasted adult rats are recorded in Table II. Neither the total amount of lipid phosphorus per g of muscle nor the distribution of lipid phosphorus among the phospholipid subclasses is influenced by 72 hours of fasting. Nor are plasmalogens or cholesterol per g of muscle influenced by this period of fasting; however, there is a significant increase in triglyceride per g of muscle.

The time course of changes in lipid phosphorus, neutral lipid fatty acid ester, and FFA in the gastrocnemius during a 72-hour fast of adult rats was studied (Table III). On a per g of muscle basis, phospholipid remained constant, neutral lipid fatty acid ester tended to increase but not statistically significantly, and the levels of FFA were markedly elevated by 24 hours of fasting and remained so for the total 72 hours of fasting.

The effect of fasting on the mass of the gastrocnemius was also measured (Table IV). The gastrocnemius made up the same percentage of the body mass in fed rats as it did in rate fasted for various time intervals. Since about 10% of the body weight...
of rat was lost by the end of the 72-hour fast, 10% of the mass of the gastrocnemius was also lost. Since the concentration of phospholipid in muscle during 72 hours of fasting did not change, it is clear that muscle phospholipid disappeared at the same rate as muscle mass. Neutral lipid fatty acid ester and FFA, however, tended to accumulate during the 72-hour fast.

The finding of Neptune et al. (1, 2) that phospholipid disappears from muscle during fasting was based on work with rat diaphragm. Therefore, the effect of a 72-hour fast on the lipids

Table III

Effect of varying periods of fasting on lipids of gastrocnemius of adult rats

The results shown are average values for the number of rats listed in each case, except for the FFA of fed rats, which is the average value for five rats ±S.E.M. The fed rats weighed 550 ± 17.6 g; the 24-hour fasted rats weighed 639 ± 56.0 g before fasting and 502 ± 55.5 g after fasting; the 48-hour fasted rats weighed 576 ± 45.1 g before fasting and 530 ± 54.3 g after fasting; and the 72-hour fasted rats weighed 600 ± 48.2 g before fasting and 552 ± 45.4 g after fasting. The fed rats weighed 550 ± 28.2 g, and the fasted rats weighed 547 ± 24.0 g before and 486 ± 24.6 g after fasting.

The results shown are average values for the number of rats listed in each case, except for the FFA of fed rats, which is the average value for five rats ±S.E.M. The fed rats weighed 143 ± 4.7 g.

Table IV

Effect of fasting on mass of gastrocnemius

The results shown are average values for the number of rats listed, ±S.E.M.

Table V

Effect of 72-hour fasting on lipids of diaphragm of adult rats

Five rats were used in each case. The results shown are average values for each group of animals, ±S.E.M. The fed rats weighed 552 ± 28.2 g, and the fasted rats weighed 547 ± 24.0 g before and 486 ± 24.6 g after fasting.

Table VI

Effect of fasting on lipids of diaphragm of young rats

The results shown are average values for each group of rats, ±S.E.M., expressed per g of muscle, wet weight.

Table VII

Effect of prolonged fasting on phospholipids of rat leg muscle

Each entry represents a different animal.
phospholipid, a tendency of neutral lipid fatty acid ester to rise, and a marked increase in FFA per g of muscle.

However, the rats used by Neptune et al. (1, 2) were very young and, for this reason, it was decided to repeat the diaphragm work with young rats (Table VI). Neither a 48-hour fast nor a 72-hour fast caused the amount of phospholipid per g of diaphragm to change. By 48 hours of fasting, however, there was some tendency for the neutral lipid fatty acid ester per g of diaphragm to fall, and by 72 hours of fasting there was almost a complete disappearance of neutral lipid fatty acid ester from the diaphragm of young rats.

The young rats lost from 29 to 36% of body weight during the 72-hour fast, the average being 33%. In order to learn if these rats were near death because of starvation, six rats of the same initial weight were fasted until death. All deaths occurred during an interval of 80 to 180 hours of fasting, three rats dying in the interval between 120 and 132 hours of fasting. At the time of death the percentage of body weight lost ranged from 36 to 44%, with an average of 41%. To learn if fasting until death or near death could cause a marked depletion of phospholipid per g of muscle, three young rats were fasted for 94 to 96 hours (Table VII). One rat died in 94 to 94½ hrs, another was comatose after 96 hours, and the third was alert and vigorous after this period of fasting. It appears that even such prolonged periods of fasting as these did not cause a precipitous fall in the concentration of phospholipid in muscle.

In Fig. 1 the relationship between plasma FFA and muscle FFA is plotted. It is evident that, at similar plasma FFA concentrations, diaphragm had a much higher concentration of FFA than did gastrocnemius. In the case of both diaphragm and gastrocnemius, the FFA in muscle of fasted rats was much higher than that found in fed rats. However, within a specific group, e.g. gastrocnemius of 72-hour fasted rats, no relationship could be established between the concentration of plasma FFA and that of muscle FFA. This was also true of 24-hour and 48-hour fasted rats, data not included in Fig. 1. Indeed, the most striking results were obtained with fed rats exposed to 2₉ for 1 day. In this case, the level of FFA in the gastrocnemius was similar to that reported in Fig. 1 for the gastrocnemius of fed rats, but the plasma FFA was elevated to the level seen in fasting rats.

DISCUSSION

The concept that intracellular lipid esters of skeletal muscle serve as an energy source to be drawn upon during exercise is based on studies with exercising dogs (6) and men (3-5). In these studies, measurements of the respiratory quotient, plasma FFA turnover, and plasma FFA oxidation provided evidence that strongly suggests a net utilization of intracellular muscle lipids as fuel for contractile activity. However, direct measurements in the monkey, comparing lipid content of quiescent right gastrocnemius and soleus muscles and left gastrocnemius and soleus muscles that were undergoing prolonged vigorous contraction, showed that contractile activity causes no net disappearance of either triglyceride or phospholipid (7).

The belief that muscle lipids serve as an energy reservoir to be drawn upon during fasting is based on direct evidence; Neptune et al. (1, 2) found an almost total disappearance of phospholipid and a moderate loss of triglyceride from rat diaphragm during a 48-hour fast. However, these findings are not confirmed in the present investigation. Phospholipid of muscle is maintained at normal concentrations during prolonged fasting; indeed, even when death occurs or is imminent, no marked fall in muscle lipid phosphorus occurs. This finding, coupled with earlier work on contracting muscle (7), strongly suggests that the phospholipids of muscle are structural-functional elements that are not depleted for the purposes of energy metabolism. Of course, muscle mass is, to some extent, decreased during fasting, and the muscle phospholipid does disappear at the same rate as muscle mass; such phospholipid is undoubtedly used for energy purposes. However, this use of phospholipid cannot be considered as evidence to support the concept that muscle phospholipid serves as a readily energy reservoir of muscle to be depleted and replaced with the changing energy requirements of the organism.

The neutral lipid fatty acid ester of muscle, i.e., primarily the triglyceride, remains at or above normal levels in adult animals for at least 72 hours of fasting. Therefore, these lipid esters do not serve as a reservoir to be drawn on rapidly during periods of caloric need. This finding is in accord with work done on monkeys (7), which showed that prolonged vigorous contractile activity does not cause net depletion of muscle triglyceride. However, it is clear that, when fasting is sufficiently prolonged, muscle neutral lipid fatty acid ester (i.e., triglyceride) ultimately serves as a reservoir, undergoing net utilization to a point at which it almost totally disappears.

The FFA content of muscle increases markedly during fasting. That this increase in muscle FFA is not merely a reflection of plasma FFA levels can be seen from the data in Fig. 1. Moreover, there are conditions under which plasma FFA increases markedly and muscle FFA not at all, e.g., in the case of rats acutely exposed to cold. It seems clear, therefore, that muscle FFA levels are regulated more by mechanisms residing within the muscle metabolic system than by plasma FFA levels.

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REFERENCES
