Intramuscular energy sources in dogs during physical work

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Abstract Three groups of dogs were run under different experimental conditions characterized by varying the work load or the running time. Lipid and glycogen analyses were carried out on biopsy specimens from the biceps femoris muscle before and after exercise. In addition, arterial and venous triglycerides and free fatty acids were determined on plasma samples from one group of dogs that had been previously catheterized. Under the conditions of these experiments, results revealed: (*1*) plasma triglycerides did not contribute significantly to the energy supply for muscle contraction; (*2*) plasma free fatty acid efflux into muscle was increased during mild exercise but significantly lowered during heavy exercise; (*3*) exercise did not affect the phospholipid level or its composition in the muscle; and (*4*) muscle triglyceride levels may increase, decrease, or remain unchanged, depending upon the work load imposed by the exercise.

Supplementary key words exercise · FFA A-V differences · plasma triglyceride · muscle glycogen · muscle triglyceride

It is generally agreed that the free fatty acids of blood plasma are the principal source of fat transported in the blood from body stores to the sites of oxidative metabolism (1–3). However, measurements of radioactivity in expired CO_2, after injection of ^{14}C-labeled fatty acids in man (4, 5) and in dogs (6–8), suggest that the oxidation of circulatory FFA accounts for less than 50% of ventilatory CO_2 originating from the oxidation of fat. This led to the assumption that intramuscular lipid must also be an important source of fuel for contracting muscle (9).

In contrast, Masoro et al. (10, 11) refuted the concept that intramuscular lipid is used as an energy source for muscular contraction. Working with anesthetized monkeys, they electrically stimulated the gastrocnemius and soleus muscles for 5 hr and compared their lipid content with that of the corresponding contralateral quiescent muscles. They found no change in any of the lipid fractions and concluded that intramuscular lipids are not used as a net source of fuel for the increased energy requirements of contracting muscle. More recently, Carlson, Ekelund, and Fröberg (12) obtained muscle tissue by needle biopsy from fasting men who were exercised to exhaustion on a bicycle. Analysis revealed that muscle triglyceride concentration decreased during the exercise.

In view of these apparent discrepancies, further studies of the metabolism of muscle lipids during exercise appeared warranted. Although the radiotracer techniques used by Issekutz and Paul (6–9) have contributed valuable information and have led to the assumption that muscle lipids participate in oxidative metabolism during exercise, they were unable to distinguish whether or not these lipids come from the muscle cells or adipose tissue dispersed throughout the muscle mass. It is conceivable that the mechanisms that increase mobilization of FFA from adipose tissue also operate in relation to fat cells within the muscle mass. This FFA could reach muscle cells by simple diffusion without entering the circulation. It was decided, therefore, to approach the problem by direct chemical analyses of muscle lipid before and after exercise performed at various work loads. In order to assess the relative work load imposed, as well as to compare the relative contribution of lipid and carbohydrate as muscular energy sources, the degree of muscle glycogen depletion was also determined.

Abbreviations: FFA, free fatty acids; RQ, respiratory quotient.

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METHODS

Sedentary male mongrel dogs weighing 22–27 kg were initially familarized with treadmill running during six sessions on the treadmill over a 2-wk period. At each session, the dogs ran at a speed of 125 m/min at 0° grade for 15 min. 2 days prior to the experimental test run, dogs were anesthetized initially with Surital (2 mg/kg), after which anesthesia was maintained by halothane administration. A cutdown was then performed on the right external jugular vein, and a no. 7 Cournard catheter was advanced to the right femoral vein under fluoroscopic control via the superior vena cava, right atrium, and inferior vena cava. The tip of the catheter was positioned 3 cm distal to the right inguinal ligament. PE160 tubing was then advanced to the central aorta following a cutdown on the left carotid artery.

While the dogs were still under anesthesia, an open muscle biopsy was performed on the left biceps femoris muscle (mean wt 150 mg). After the biopsies were obtained, the cutdown sites in the neck were closed with 4-0 nylon sutures, and the catheters were brought out in the posterior neck region, filled with saline, and sealed. The dogs were then allowed to recover over the next 36–48 hr, after which they underwent the test run on the treadmill.

Control arterial and venous blood samples were drawn 5 min prior to the onset of exercise while the dogs were standing quietly on the treadmill. Further serial samples were drawn at specific intervals while the dogs were running and again at the time of exhaustion. The latter was defined as an inability to maintain a normal stride on the treadmill despite vigorous coaxing on the part of the investigators. The blood was immediately transferred to precooled heparinized tubes and kept cold until the plasma was collected after centrifugation in a refrigerated centrifuge. At the time of exhaustion, a second muscle biopsy was performed on the right biceps femoris under local anesthesia with 2% lidocaine. These muscle samples were obtained within 5 min after the cessation of exercise.

Three groups of dogs were run under different experimental conditions characterized by varying the workload or varying the running time. Group I consisted of four animals that were run to exhaustion, as previously defined, at 125 m/min on a 10° grade. Group II consisted of four dogs that ran for a period of 30 min at 125 m/min at a 10° grade. None of these animals appeared exhausted at this end point. Group III consisted of three dogs who ran for 120 min at 125 m/min on a 0° grade. None of these dogs appeared to be near exhaustion. Dogs in groups II and III were not catheterized prior to study and only underwent control and postexercise muscle biopsies.

Lipid extraction

Visible fat and connective tissue were removed from the muscle, which was divided into two samples, one for lipid and one for glycogen determination. Muscle lipids were extracted by homogenizing 100–150 mg of tissue in an all-glass homogenizer with 5 ml of methanol. Chloroform (10 ml) was then added to the methanol homogenate, and nitrogen was bubbled through the mixture for 20 min. The lipid extract was then collected by filtration under positive nitrogen pressure. Plasma samples were lyophilized and total lipids were extracted with chloroform–methanol 2:1 (v/v). All solvents used for lipid extraction and analysis were freshly redistilled.

Lipid analysis

The chloroform–methanol extracts from either plasma or muscle were evaporated to near dryness in a rotary evaporator under vacuum. The residues were redissolved in a small volume of chloroform–methanol 2:1 (v/v). These samples were used for triglyceride, free fatty acid, and phospholipid analyses. Phospholipids were determined by phosphorus analysis of spots after two-dimensional thin-layer chromatographic separation of the phospholipid classes, as described by Rouser, Fleischer, and Yamamoto (13). The triglycerides and free fatty acids were first isolated by thin-layer chromatography in a solvent system consisting of petroleum ether–ethyl ether–acetic acid 160:40:2. They were visualized with iodine vapor, scraped from the plate into a small flask, and extracted from the silica gel with chloroform–methanol 2:1 (v/v). The triglycerides and free fatty acids were then determined by liquid scintillation counting of the methyl esters, after transesterification with [^{3}H]methanol, by a modification of the procedure described by Fischer and Kabara (14). The extracts were transferred to a glass tube fitted with a Teflon-lined screw cap. The solvent was evaporated under a stream of nitrogen, and the reaction mixture, consisting of 0.35 ml of hexane, 0.35 ml of 14% BF_3 in methanol, and 0.30 ml of [^{3}H]methanol (sp act about 30 $\times$ 10^3 dpm/μmole), was added. The reaction was carried out at 100°C for 20 min. After cooling in an ice bath, 1.0 ml of water and 4.0 ml of hexane were added and the tubes were shaken. The hexane phase was removed and washed twice with 40–50 ml of water in a separatory funnel. The washed hexane solutions were then transferred to counting vials, the solvent was evaporated under a stream of nitrogen, and the vials were placed in a vacuum oven for 2 hr at 60°C. The fatty acid methyl esters were then dissolved in 15 ml of toluene containing 8 g of butyl PBD (2-[4′-*t*-butylphenyl]-5-[4′′-biphenyl]-1,3,4-oxadiazole) and 0.5 g of PBBO (2-[4′-biphenyl]-6-phenyl benzoxazole) per liter. The samples were counted in a Beckman model LS-232 liquid scintillation counter. Appropriate

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blanks were carried through simultaneously, and accurately determined specific activity of the [^{3}H]-methanol was used in the computation of the amount of fatty acid.

Recovery and reproducibility studies of the radiochemical analyses for FFA and triglyceride were done on lipid extracts from 10 g of muscle and 25 ml of plasma. The lipid extracts were stored under nitrogen in a freezer. On four separate occasions, aliquots were taken from each extract and the analysis was carried out in quadruplicate. At the same time, recovery studies were done on separate aliquots from the same samples, to which measured amounts of pure oleic acid and triolein were added. The results are shown in Table 1.

Glycogen analysis

Muscle samples weighing 20–50 mg were homogenized within 10 min of removal in 1.6 ml of water. Protein was precipitated by adding 0.4 ml of 50% trichloroacetic acid. The homogenate was allowed to stand in an ice bath for at least 2 hr before centrifugation. The supernatant solution was transferred to a glass tube fitted with a Teflon-lined screw cap and glycogen was precipitated with 10 ml of cold absolute ethanol. The tubes were placed in the cold for at least 12 hr prior to centrifugation. The supernatant solution was decanted and the remaining glycogen was hydrolyzed in 0.4 ml of 2 N H_2SO_4 at 100°C for 2 hr. The resulting glucose was determined by the *o*-toluidine method described by Dubowski (15).

RESULTS

Table 2 presents the plasma triglyceride values from a representative dog in group I. Dogs in this group were first made to run at 125 m/min on a 0° grade. At the end of 15 min while the dogs were still running, the treadmill was elevated to a grade of 10°. Even though there were considerable variations in plasma triglyceride levels among the dogs, the arterial and venous concentrations remained essentially the same in each dog while at rest or during exercise at either of the two work loads.

TABLE 1. Replicate determinations of muscle and plasma FFA and triglyceride levels and recovery of added fatty acid and triglyceride standards

Muscle		Plasma	
Triglyceride	Free Fatty Acid	Triglyceride	Free Fatty Acid
μmoles/g		*μmoles/l*	
10.54 ± 0.46	2.14 ± 0.08	0.41 ± 0.03	0.73 ± 0.01
Recovery of Added Standard			
99.1%	100.2%	99.6%	99.9%

Each value is the mean ± SE of 16 determinations.

TABLE 2. Effects of exercise on plasma triglyceride levels from a representative dog in group I

Running Time	Arterial		Venous
min		*mmoles/l*[a]	
0	0.42 ± 0.05		0.41 ± 0.06
5	0.40 ± 0.06	0° grade	0.40 ± 0.04
15	0.39 ± 0.04		0.38 ± 0.02
20	0.37 ± 0.02	10° grade	0.38 ± 0.05
30	0.36 ± 0.06		0.37 ± 0.02
40	0.34 ± 0.03		0.35 ± 0.05
Exhaustion[b]	0.34 ± 0.02		0.34 ± 0.04

[a] Means ± SE of quadruplicate determinations.
[b] Mean exhaustion time was 60 min (range 50–95).

The small decrease in plasma triglyceride shown in Table 2, which is not statistically significant, was observed in only two of the four dogs studied. The plasma arteriovenous (A-V) free fatty acid differences are shown in Fig. 1. There is a slight rise during mild exercise. However, with the onset of heavy exercise the A-V difference decreases until, at the point of exhaustion, it is essentially abolished.

The results of a detailed analysis of the phospholipids in muscle from dogs in group I, before and immediately after cessation of exhaustive heavy exercise, are presented in Table 3. No changes were observed in the phospholipid concentration, nor were there changes in any of the phospholipid classes as a result of exercise. The results of muscle glycogen and triglyceride determinations from dogs in groups I and II are presented in Table 4. Muscle triglyceride and glycogen levels were

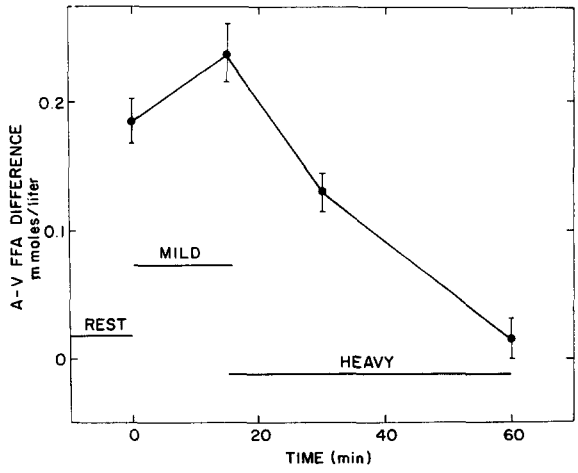

FIG. 1. Each point is the mean ± SE of values obtained from the four dogs in group I. The first point at time 0 is the resting A-V difference. The second point is after 15 min at 125 m/min at 0° grade. The third and fourth points are from samples obtained while the dogs were running at 125 m/min at 10° grade. Statistical significance compared with the preexercise value: second point, $P < 0.05$; third point, $P < 0.01$; and fourth point, $P < 0.001$ (Student's t test).

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TABLE 3. Phospholipid composition of muscle before and after heavy exercise in group I

	Rest	After Heavy Exercise[a]
	μmole/g tissue[b]	
Phosphatidylcholine	6.30 ± 0.26	6.16 ± 0.31
Phosphatidylethanolamine	3.29 ± 0.15	3.18 ± 0.12
Phosphatidylserine	0.30 ± 0.04	0.28 ± 0.05
Phosphatidylinositol	0.58 ± 0.07	0.63 ± 0.06
Diphosphatidylglycerol	0.70 ± 0.06	0.72 ± 0.04
Phosphatidic acid	tr	tr
Sphingomyelin	0.57 ± 0.09	0.67 ± 0.08
Lipid phosphorus	12.46 ± 0.39	12.37 ± 0.41

[a] Muscle samples were obtained within 5 min after exhaustion.
[b] Means ± SE of three dogs from group I. Samples from the fourth dog were inadvertently lost.

exercise is estimated to yield 1.7 times more ATP than did the oxidation of muscle glycogen.

The effects of mild exercise (group III), carried out over a period of 2 hr, on the triglyceride and glycogen content of muscle are shown in Table 6. The glycogen content decreased by 20%, and its rate of utilization was 8 μmoles of glucose/100 g of muscle/min. In contrast, the triglyceride level rose from 9.8 μmoles/g to 20.4 μmoles/g, an increase of 108%.

DISCUSSION

It was estimated from the depletion of muscle glycogen in dogs running on a 10° grade (Table 4) that the utilization of muscle glycogen was about 50 μmoles of

TABLE 4. Triglyceride and glycogen levels in muscle during heavy exercise

	Group II		Group I[a]	
	Triglyceride	Glycogen	Triglyceride	Glycogen
		μmoles/g tissue[b]		
Rest	11.3 ± 0.8	46.7 ± 2.6[c]	8.9 ± 0.8	42.6 ± 2.1[c]
After exercise	10.5 ± 0.9	35.0 ± 1.9	4.5 ± 0.3	12.8 ± 0.7
Individual differences	0.8 ± 0.7	11.7 ± 1.0	4.4 ± 0.5	29.8 ± 1.1
P[d]	>0.05	<0.001	<0.001	<0.001
% Decrease	7	25	50	70
Theoretical ATP yield	353	456	1954	1162

[a] Mean exhaustion time was 60 min (range 50–95).
[b] Means ± SE of four dogs.
[c] Values expressed as μmoles of glucose/g of tissue.
[d] Calculated using Student's *t* test.

significantly decreased in group I. Only glycogen was significantly decreased in group II. The rate of muscle glycogen depletion in dogs from group I was 50 μmoles of glucose/100 g of muscle/min. During this time, the muscle triglyceride level decreased 50%. In group II the rate of glycogen depletion was about the same as in group I, but muscle triglyceride levels were not significantly changed. Table 5 shows the similarity in triglyceride and glycogen levels in the contralateral muscles.

The relative contributions of muscle triglycerides and glycogen as energy sources for muscle contraction were calculated. These calculations were based on the assumption that each substrate was completely oxidized through the normal oxidative pathways. The complete oxidation of 1 mole of glucose derived from glycogen yields 39 moles of ATP, whereas, if it is assumed that C_{18} is the average chain length of the fatty acids in muscle triglycerides, the complete oxidation of the fatty acids from 1 mole of triglyceride yields 441 moles of ATP. The contribution of the glycerol moiety of triglyceride is neglected in this calculation. Using these values, the complete oxidation of the fatty acids derived from muscle triglycerides during this period of heavy glucose/100 g of muscle/min. This is close to the values reported by Bergström et al. (16) for men pedaling a bicycle to exhaustion at a work load corresponding to an 80% maximal oxygen uptake. It is, however, in definite contrast to the rate of muscle glycogen utilization seen in dogs during mild exercise in this study, 8 μmoles of glucose/100 g of muscle/min (Table 6).

TABLE 5. Triglyceride and glycogen levels in the left and right biceps femoris muscles before and after exercise[a]

	Before Exercise			After Exercise		
	L	R	L − R	L	R	L − R
Triglyceride (n = 8) (μmoles/g)						
Mean	9.98	10.31	−0.33	4.37	4.98	−0.61
± SE	0.88	1.01	0.98	0.39	0.44	0.35
P[b]			>0.05			>0.05
Glycogen (n = 8) (μmoles glucose/g)						
Mean	48.2	46.9	1.3	12.2	11.5	0.7
± SE	2.1	2.3	2.0	0.9	0.7	0.8
P			>0.05			>0.05

L, left; R, right; L − R, individual difference between left and right; n, number of dogs.
[a] Dogs were run to exhaustion at 125 m/min on a 10° grade.
[b] Calculated using Student's *t* test.

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TABLE 6. Concentration of triglycerides and glycogen in muscle before and after mild exercise (group III)[a]

	Triglyceride	Glycogen
	μmoles/g tissue	*μmoles glucose/g tissue*
Rest	9.8 ± 0.9	48.99 ± 3.0
After exercise	20.4 ± 1.2	38.9 ± 2.6
Individual differences	10.6 ± 0.6	10.0 ± 1.0
P[b]	<0.001	<0.001
% Change	+108	−20
Theoretical ATP yield		390

[a] Means ± SE of three dogs in group III; running time, 2 hr at 0° grade.
[b] Calculated using Student's *t* test.

Since studies by Havel and coworkers (4, 5) in man and by Issekutz, Paul, and coworkers (6–8) in dogs indicate that less than half the CO_2 originating from the oxidation of fat was derived immediately from the circulating plasma FFA, some investigators have suggested that plasma triglyceride fatty acids might be an alternative source of fatty acids available to the working muscle. Carlson and Mossfeldt (17) observed that plasma triglyceride levels in young men exercising at a moderate work load were not changed. However, in well-trained skiers participating in prolonged heavy exercise of cross-country racing, plasma triglyceride levels were reduced. Results of the present study show no evidence of an efflux of triglyceride fatty acid from the circulation into muscle during the 60 min of running. Arteriovenous differences are not the most sensitive method to measure the ability of muscle to extract lipid from the circulation. However, if there were significant changes in lipoprotein lipase activity at the muscle sites, they would be reflected in an increased A-V difference. These data by no means show that lipoprotein lipase activity is absent, but rather indicate that the activity is not significantly increased during exercise. More recently, Fröberg (18) reported that plasma triglyceride levels in untrained rats did not change, but in trained rats a decrease was observed. It may be that the state of training determines the source of extra muscular energy used during heavy work. However, under conditions of this study with untrained dogs, it is unlikely that plasma triglycerides contribute significantly to energy metabolism.

The other important source of fatty acids for muscle metabolism must be intramuscular. There are numerous observations which indicate that muscle cells can store lipids which may serve as energy sources. Neptune, Sudduth, and Foreman (19) discovered that if rat diaphragms were incubated in a substrate-free medium the total fatty acid content decreased. It has also been shown that ^{14}C-labeled palmitate is incorporated into both triglyceride and phospholipid of rat diaphragms (20–22). The lipid which is found in subcellular particles is phospholipid (23), and the question raised is whether increased muscle activity utilizes phospholipids for fuel. If phospholipid is broken down, this need not necessarily be reflected in a decreased phospholipid concentration. It is possible that the loss of fatty acid from phospholipids could result in the formation of lyso compounds or phosphatidic acid. Not only was the phospholipid concentration unchanged by exhaustive heavy exercise, but also the composition was not affected. There was no indication of the appearance of lyso compounds, or an accumulation of phosphatidic acid, leading us to conclude that phospholipids do not constitute a significant source of substrate for muscle activity. This interpretation is consistent with the observations of Carlson et al. (12), who recently measured the phospholipid concentration in the leg muscle of man after exhaustive exercise. These data also corroborate the observations made by Masoro, Rowell, and McDonald (11) that repeated contraction of skeletal muscle induced by electrical stimulation does not alter its phospholipid composition.

However, our results differ from those of Masoro et al. (11) with respect to the effect of exercise on muscle triglycerides. Whereas they concluded that muscle triglycerides are not used as a net source of fuel, results of the present study show that muscle triglyceride levels may increase, decrease, or remain unchanged, depending on the work load. During mild exercise, there is a net deposition of triglyceride in skeletal muscle. This increase in muscle triglyceride levels is similar to observations made by Masoro et al. (11) on fasted rats. Fasting, which stimulates the mobilization of FFA from adipose tissue, resulted in the accumulation of muscle triglyceride. Accumulation of intramuscular lipid has also been observed in other states characterized by enhanced mobilization of endogenous lipid stores. Carlson, Liljedahl, and Wirsén (24) observed that the augmented release of FFA from adipose tissue induced by norepinephrine infusion was reflected in an increased deposition of lipids in various tissue cells including liver and skeletal muscle. In the present study, there was an increased efflux of FFA from plasma to muscle during mild exercise, as evidenced from an increased A-V difference (Fig. 1). The increased supply of FFA may have exceeded the demand; consequently, the excess FFA entering the muscle were deposited as triglyceride. On the other hand, the increased work load imposed by heavy exercise demands a greater turnover of substrate. Our results indicate that the A-V FFA difference markedly narrowed during heavy exercise. It is possible that under these conditions the supply of FFA no longer met the demand. Consequently, the excess triglycerides, deposited during the previous period of mild exercise,

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were depleted. It should be cautioned, however, that the decreased A-V difference might be due to an increased blood flow rather than to a decreased rate of FFA efflux. However, observations made by Issekutz et al. (25) indicate that the decreased A-V difference may be related, at least in part, to a decreased turnover of FFA. They have shown that during heavy exercise blood lactic acid accumulated, and as a consequence the turnover of FFA was significantly decreased. This is further supported by our observation that continuation of heavy exercise is followed by an even greater decrease in A-V difference (Fig. 1). Since there is no experimental evidence that blood flow changes with time at a given work load (26), it is reasonable to assume that the decreased A-V difference reflects a decreased influx of FFA into muscle. Consequently, intracellular triglyceride stores are used, as seen from the significant decrease in intramuscular triglyceride level (Table 3). This is in accord with the recent observations of Carlson et al. (12) that the triglyceride content of human skeletal muscle is decreased after exhaustive exercise.

If we assume that the disappearance of muscle triglyceride and glycogen is a measure of the complete oxidation of these substrates, we see that triglyceride fatty acids yielded 1.7 times as much ATP as did the oxidation of muscle glycogen. Since a significant portion of the glycogen ends up as plasma lactic acid (25), it is not completely oxidized in muscle. Therefore, the ATP yield from glycogen depletion is even less than is indicated in Table 3. It would seem, then, that even at heavy work loads lipid is the major fuel supply for muscle contraction.

This conclusion is at variance with the concept based on RQ determinations during exercise that carbohydrate is the predominant fuel for heavy exercise (27). RQ measurements in an intact organism represent at best an average resultant of all metabolic events occurring in the body. There is no question that muscle glycogen is depleted during severe exercise. However, a great deal of the energy is lost to the muscle, since the resulting lactic acid finds its way to the circulation. The lactic acid is eventually oxidized in extramuscular tissue and thus contributes to the ventilatory RQ, but not to the energy supply for muscle contraction. Even though the total organism is consuming carbohydrate at a rapid rate, it is possible that working muscle still depends primarily on lipid to support muscle activity.

Technical assistance was provided by Donald B. Mellin.

Manuscript received 28 January 1972; accepted 21 August 1972.

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