Original Article

Exercise-trained but not untrained rats maintain free carnitine reserves during acute exercise

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Exercise training is known to induce physiological adaptations that improve exercise performance and alter patterns of energy substrate utilization to favour fatty acid oxidation. L-Carnitine is an essential cofactor for the oxidation of fatty acids under all physiological conditions, including exercise. This study evaluated the effect of acute exercise on carnitine concentrations in tissue and serum, liver carnitine palmitoyltransferase-I activity and expression, and serum lipids in both trained and untrained rats as compared to non-exercised rats. Serum acyl- and total carnitine was significantly higher in the trained animals, whether exercised or not, suggesting an exercise-induced increase in a renal threshold for carnitine. Untrained rats had significantly higher acylcarnitine in skeletal muscle and an acyl/free carnitine ratio of 0.63 ± 0.06 compared with 0.31 ± 0.16 in trained animals receiving an identical acute bout of exercise, demonstrating that untrained animals utilized a significantly higher percentage of free carnitine reserves during exercise. This study suggests that free carnitine reserves may be reduced during exercise in untrained rats, an effect that has the potential to impair both carbohydrate and fat metabolism during exercise.

Key Words: carnitine, carnitine palmitoyltransferase-I, exercise, rat.

Introduction

Carnitine β-hydroxy-γ-trimethylammonium butyrate) is an essential cofactor for facilitating fatty acid transport into the mitochondrial matrix where β-oxidation occurs.[1] Carnitine is synthesised from the essential amino acid lysine after post-translational methylation by S-adenosylmethionine to tri-methyllysine. Ascorbate, vitamin B_6 and iron are also required for the synthesis of carnitine. It is believed that the source of substrates for carnitine biosynthesis is mainly food derived.[2] Because muscle tissues lack carnitine biosynthesis capability and long-term exercise results in a loss of muscle carnitine, exogenous carnitine may be necessary to maintain muscle carnitine concentrations,[3,4] and exogenous carnitine may be required to maintain adequate carnitine stores for fat metabolism during active exercise in athletes.[5,6]

Many attempts have been made to increase exercise endurance time and exercise performance by carnitine supplementation.[5,7–10] These studies have yielded inconsistent results, and the role of supplemental carnitine for the enhancement of exercise performance remains controversial. The inconsistent results may be due to differences in carnitine dosages, exercise type and intensity, or differences in exercise training by the subjects prior to the study. These studies have also evaluated performance enhancement by looking at very different outcomes. Some studies have measured actual performance[5,11,12] while others have employed indirect calorimetry to determine energy substrate usage.[5,6,9] A few studies have measured tissue carnitine concentrations,[9,13] but even that has not been consistent, with one study showing an increase in tissue carnitine from supplemental carnitine[13] and another showing no change.[9] The effects of supplemental carnitine, during exercise, on carnitine concentrations in tissue is complicated by the effects of exercise itself on muscle carnitine. Although many studies have evaluated the effect of carnitine supplementation on exercise performance, few studies have evaluated the effects of exercise on carnitine status. This research evaluated the effect of long-term training, acute-exercise, and the combined action of both on blood and tissue concentrations of lipids, carnitine fractions and liver carnitine palmitoyltransferase-I (CPT-1) activity. In so doing, we have attempted to separate the short-term effects of exercise from the effects of exercise training on carnitine status, employing exercising rats as a model.

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Materials and methods

Diets and exercise protocol

The animal-use protocol for this study was approved by the Korea Science and Engineering Foudation (KOSEF). Twenty male Sprague–Dawley rats, aged 7 weeks, were fed an AIN-76 diet and divided into two groups, one of which was exercised daily (long-term trained, LT) and one not exercised (non-trained, NT). The trained rats were run on a treadmill for 60 min per day (10°incline, 25 m/min). Each rat was individually housed in a stainless steel wire mesh cage and allowed free access to feed and water. Feed consumption and weight-gain records were maintained throughout the 60 day study. At the end day of study, the two groups were each divided into two subgroups, one of which was exercised (single exercise, SE) and one that was not exercised (NE) before decapitation. All of the trained rats were before decapitation. All of the trained rats were able to maintain the exercise for 60 min, and all but two of the untrained rats were able to do so. The two rats that did not run for 60 consecutive minutes were allowed a short break and then run for the balance of the 60 min. Blood was collected in tubes, centrifuged, and the serum frozen at –20°C until assayed. Liver, kidney and skeletal muscle (gastrocnemius) tissues were surgically removed, quickly frozen in liquid nitrogen, and stored at –80°C until assayed.

Analytical procedures

Total cholesterol and triacylglycerol in liver and serum were assayed enzymatically with a commercial kit (Asan Pharmaceutical, Seoul, Korea). Total lipids were assayed with a commercial kit based on the sulfo-phospho-vanillin method[14] (Kokusai Pharmaceutical, Kobe, Japan). Carnitine was assayed using a modified version of the radioisotopic method of Cederbland and Lindstedt.[15,16] In this assay, acid-insoluble acylcarnitine (AIAC) is precipitated with perchloric acid and centrifugation, leaving the acid-soluble acylcarnitine (ASAC) and the non-esterified carnitine (NEC) in the supernatant. An aliquot of the supernatant is assayed to determine the NEC content and another aliquot hydrolysed with 0.5 mol/L KOH to assay all acid-soluble carnitine (ASAC + NEC). ASAC was calculated as the difference between the NEC and the total acid-soluble carnitine. The pellets containing the AIAC were drained, washed and hydrolysed in 0.5 mol/L KOH for 60 min in a hot water bath at 60°C. In each case, carnitine was assayed by using carnitine acetyltransferase (Sigma Chemical Company, St Louis, MO, USA) to esterify the carnitine to a [^{14}C]acetylcarnitine from [1–^{14}C]acetyl-CoA (Amersham, Little Chalfont, Buckinghamshire, England). Radioactivity of samples was determined in a Beckman LS-3801 liquid scintillation counter (Beckman Instruments, Palo Alto, CA, USA).

Liver mitochondria were prepared for the determination of CPT-I activity by the procedure of Johnson and Lardy.[17] The liver was homogenised in cold, Tris-buffered 0.25 mol/L sucrose and the mitochondria were isolated from the homogenate by differential centrifugation, washed and resuspended three times. After the final wash, the mitochondria were resuspended in the buffer. The mitochondrial protein content was determined by the method of Bradford using γ-globulin as the standard. A modified procedure of Guzman *et al.*[18] was used to measure CPT-I activity. Final concentrations of the reaction mixture in a total volume of 1 mL at 37°C were: 80 mmol/L sucrose, 70 mmol/L imidazole (pH 7.0), 1 mmol/L ethyleneglyco-ltetraaceticacid (EGTA), 1 μg antimycin A, 2 mg bovine serum albumin. A 5 min preincubation period was initiated by the addition of myristoyl-CoA. The reaction was started with L-carnitine (0.4 mCi/mmol l-[methyl-^{3}H]carnitine) and stopped after 5 min by adding 4 mL of 1.0 mol/L perchloric acid.

CPT-I mRNA levels

Total RNA from fresh rat liver was isolated by the guanidine hiocyanate/phenol/chloroform extraction procedure,[19] as described by Park *et al.*[20] CPT-I mRNA abundance was measured by Northern blot analysis, as described by Mynatt *et al.*[21] The RNA was resolved on an agarose gel and transferred to a nylon membrane (Ambion, Austin, TX, USA). The CPT-I DNA fragment obtained from the CPT-I cDNA[21] was labelled with biotin using the Psoralen–Biotin labelling kit (Ambion). Hybridisation of the probe to the membrane-bound mRNA was conducted at 42°C for 20 h. The membrane was washed to remove non-specifically bound probe and was incubated successively in the blocking, conjugation, blocking and CDP-star solutions of the BrightStar™ BioDetect™ kit (Ambion). The membrane was then exposed to X-ray film (Fuji, Tokyo, Japan) for 45 min.

Statistical analysis

All values are expressed as group means ± SD. Significance of differences were determined using 2-way analysis of variance (ANOVA) using SAS version 6 (SAS Institute, Cary, NC, USA). When the *F*-test indicated differences between groups, the differences were separated using Tukey's test.

Results

There was no significant difference in dietary intake between the two primary groups; LT and NT animals. However, feed efficiency and weight gain were lower in the LT animals (Table 1). LT animals had lower serum triacylglycerols and total cholesterol, but there was no difference in serum total lipids between the groups. Acute-exercised animals in the NT group had higher liver triacylglycerol levels, but total lipids were higher in both groups following acute exercise (Table 2). LT animals had higher concentrations of serum acylcarnitine fractions and total carnitines than did NT animals (Table 3). There was no difference in any of the liver carnitine fraction concentrations following either acute exercise or training (Table 4). Kidney carnitine concentrations were not affected by acute exercise and, at most, only marginally by exercise training. Acute exercise in untrained animals, however, resulted in a lower ASAC and total carnitine concentration in the kidney.

Table 1. Effects of exercise on food consumption and body weight gain in rats

	Non-trained group	Long-term trained group
Food consumption (g/day)	28.8 ± 2.9[a]	30.2 ± 4.4[a]
Initial body weight (g)	313.1 ± 14.7[a]	310.8 ± 13.9[a]
Weight gain (g)	188.7 ± 56.3[a]	134.5 ± 40.2[b]
Food efficiency ratio†	0.12 ± 0.03[a]	0.08 ± 0.03[b]

All values are mean ± SD (n = 10). Values with different superscripts ([a,b]) are significantly different ($P < 0.005$). †Food efficiency ratio was calculated as weight gain (day)/dietary intake (day) during the experimental period.

Table 2. Lipid concentrations in serum and liver

	Group				ANOVA†		
	NTNE	NTSE	LTNE	LTSE	LT	SE	LT x SE
Serum							
Triglyceride (mmol/L)	0.87± 0.23[a]	0.99 ± 0.19[a]	0.43 ± 0.10[b]	0.50 ± 0.05[b]	0.005	NS	NS
Total cholesterol (mmol/L)	2.35 ± 0.62[a]	2.17 ± 0.31[a,b]	1.53 ± 0.41[b,c]	1.03 ± 0.67[c]	0.005	NS	NS
Total lipid (g/L)	0.026 ± 0.006	0.018 ± 0.000	0.020 ± 0.003	0.024 ± 0.007	NS	NS	0.005
Liver							
Triglyceride (µmol/g)	15.7± 0.9[b]	58.9 ± 31.6[a]	14.6 ± 4.0[b]	26.5 ± 9.3[b]	NS	0.0050	0.05
Total lipid (mg/g)	0.031 ± 0.016[b]	0.081 ± 0.036[a]	0.046 ± 0.009[b]	0.080± 0.004[b]	NS	0.0001	0.05

All values are mean ± SD (n = 5). Values with different superscripts ([a–c]) are significantly different ($P < 0.05$). †The degrees of significance resulting from the 2-way ANOVA are shown with effects of long-term trained, single-exercise, and the interaction of long-term trained and single-exercise (LT x SE) being expressed as the numerical value or as not significant (NS) when $P < 0.05$. LT, long-term trained; LTNE, long-term trained, non-exercise; LTSE, long-term trained, single-exercise; NTNE, non-trained, non-exercise; NTSE, non-trained, single-exercise; SE, single exercise.

Table 3. Carnitine concentrations and ratio of acyl/free carnitine in serum

Carnitine	Group				ANOVA†		
(µmol/L)	NTNE	NTSE	LTNE	LTSE	LT	SE	LT x SE
Non-esterified	30.1 ± 7.3	28.7 ± 2.3	36.1 ± 1.1	36.1 ± 1.1	0.05	NS	NS
Acid-soluble acyl	20.9 ± 16.3[c]	31.8 ± 4.2[c]	72.7 ± 14.7[b]	101.3 ± 8.9[a]	0.001	NS	NS
Acid-insoluble acyl	2.1 ± 0.5[b]	2.9 ± 1.3[a,b]	4.7 ± 2.1[a]	4.7 ± 0.6[a]	0.001	NS	NS
Total	64.4 ± 12.9[b]	65.25 ± 7.1[b]	111.4 ± 19.4[a]	142.1 ± 8.3[a]	0.01	NS	NS
Acyl/free	1.23 ± 0.29[c]	1.21 ± 0.07[c]	2.14 ± 0.1[b]	2.94 ± 0.03[a]	NS	NS	0.01

All values are mean ± SD (n = 5). Values with different superscripts ([a–c]) are significantly different ($P < 0.05$). †The degrees of significance resulting from the 2-way ANOVA are shown with effects of long-term trained, single-exercise, and the interaction of long-term trained and single-exercise (LT x SE) being expressed as the numerical value or as not significant (NS) when $P < 0.05$. LT, long-term trained; LTNE, long-term trained, non-exercise; LTSE, long-term trained, single-exercise; NTNE, non-trained, non-exercise; NTSE, non-trained, single-exercise; SE, single exercise.

In skeletal muscle, acute exercise increased all acylcarnitine concentrations in both trained and untrained animals. The acyl/free carnitine ratio was twice as high in untrained animals following acute exercise as that of any other group.

The average maximum inhibition (I_{max}) of CPT by malonyl-CoA was 82%, indicating that the majority of the CPT activity was from CPT-I. Acute exercise significantly increased liver CPT-I activity in trained rats, but not in untrained rats (Table 5). The expression of liver mRNA for CPT-1 was also higher in both trained groups and appeared highest in the trained animals following acute exercise (Fig. 1).

Discussion

This study evaluated the effect of acute exercise on carnitine status and lipid parameters in the blood and tissues of trained and untrained rats. This design allowed us to evaluate differential effects of acute exercise that may result from adaptation in trained animals, compared with untrained animals. At the end of the study, both exercised subgroups of trained and untrained animals were treadmill exercised for the same duration, speed and incline as was used during training. Exercise intensity was fixed for all animals and not adjusted to a percentage of VO_{2max}. Presumably, the final exercise in

Table 4. Carnitine concentrations and ratio of acyl/free carnitine in tissues

Carnitine	Group				ANOVA†		
	NTNE	NTSE	LTNE	LTSE	LT	SE	LT x SE
Liver (nmol/g dry weight)							
Non-esterified	911 ± 254	1156 ± 564	1262 ± 295	1436 ± 365	NS	NS	NS
Acid-soluble acyl	90 ± 139	87 ± 64	98 ± 106	134 ± 107	NS	NS	NS
Acid-insoluble acyl	20 ± 17	25 ± 25	35 ± 12	20 ± 18	NS	NS	NS
Total	1021 ± 371	1267 ± 639	1396 ± 368	1591 ± 420	NS	NS	NS
Acyl/free	0.12 ± 0.12	0.09 ± 0.06	0.10 ± 0.07	0.11 ± 0.05	NS	NS	NS
Kidney (nmol/g dry weight)							
Non-esterified	2634 ± 474^{b}	2000 ± 389^{c}	2986 ± 342^{a}	3675 ± 176^{b}	NS	NS	NS
Acid-soluble acyl	645 ± 599	221 ± 171	254 ± 205	226 ± 194	0.05	NS	NS
Acid-insoluble acyl	17 ± 24^{b}	9 ± 4^{b}	$27 \pm 14^{a,b}$	51 ± 18^{a}	NS	NS	NS
Total	3286 ± 617^{a}	2330 ± 383^{b}	3267 ± 409^{a}	3952 ± 152^{a}	0.05	NS	0.01
Acyl/free	0.28 ± 0.31	0.12 ± 0.09	0.09 ± 0.07	0.08 ± 0.06	NS	NS	NS
Skeletal muscle (nmol/g)							
Non-esterified	$3124 \pm 692^{a,b}$	2260 ± 378^{b}	$3120 \pm 744^{a,b}$	4116 ± 1235^{a}	NS	NS	NS
Acid-soluble acyl	775 ± 410^{b}	1563 ± 134^{a}	941 ± 260^{b}	1121 ± 419^{b}	NS	0.01	NS
Acid-insoluble acyl	25 ± 12^{b}	105 ± 63^{a}	21 ± 19^{b}	24 ± 25^{b}	0.05	0.01	0.05
Total	3924 ± 867^{b}	$4329 \pm 447^{a,b}$	$4082 \pm 883^{a,b}$	5261 ± 915^{a}	NS	NS	NS
Acyl/free	0.26 ± 0.13^{b}	0.63 ± 0.06^{a}	0.32 ± 0.12^{b}	0.31 ± 0.16^{b}	NS	0.01	0.01

All values are mean ± SD (n = 5). Values with different superscripts ($^{a–c}$) are significantly different ($P < 0.05$). †The degrees of significance resulting from the 2-way ANOVA are shown with effects of long-term trained, single-exercise, and the interaction of long-term trained and single-exercise (LT x SE) being expressed as the numerical value or as not significant (NS) when $P < 0.05$. LT, long-term trained; LTNE, long-term trained, non-exercise; LTSE, long-term trained, single-exercise; NTNE, non-trained, non-exercise; NTSE, non-trained, single-exercise; SE, single exercise.

Table 5. Activity of carnitine palmitoyl transferase-I activity (nmol/minute/mg protein)

	Group				ANOVA†		
	NTNE	NTSE	LTNE	LTSE	LT	SE	LT x SE
CPT-I							
Mean	2.83	0.41	1.73	8.60	NS	0.05	0.001
± SD	1.92^{b}	0.21^{b}	1.74^{b}	3.96^{a}			

All values are mean ± SD (n = 5). Values with different superscripts (^{a,b}) are significantly different ($P < 0.005$). †The degrees of significance resulting from the 2-way ANOVA are shown with effects of long-term trained, single-exercise, and the interaction of long-term trained and single-exercise (LT x SE) being expressed as the numerical value or as not significant (NS) when $P < 0.05$. CPT-I, carnitine palmitoyl transferase-I; LT, long-term trained; LTNE, long-term trained, non-exercise; LTSE, long-term trained, single-exercise; NTNE, non-trained, non-exercise; NTSE, non-trained, single-exercise; SE, single exercise.

the animals that were untrained was more difficult and strenuous than for the trained animals, and required greater exertion. The design mimics, in human terms, the 'weekend warrior' who engages in occasional strenuous exercise as compared to persons who maintain a consistent exercise program. It is important to note that the effects of exercise seen in this study may not be the same in animals or people exercising at similar percentages of maximal exercise capacity, and further research may be warranted to make that determination.

The important difference in tissue carnitine concentrations between groups was the significantly higher muscle acyl-carnitne of the NTSE group, which also resulted in a two-fold higher acyl/free carnitine ratio. In untrained animals the exercise bout resulted in utilisation of a substantial portion of the free carnitine reserve, an effect that was prevented by long-term training. This may have been due to a difference in exercise intensity between groups. Others have shown that maximal intensity exercise, but not submaximal exercise, causes a depletion of free carnitine accompanied by increased acylcarnitine,[22–24] which would be consistent with our results if the untrained animals were exercising at a much higher percentage of their VO_{2max}. Apparently, the acylcarnitines are a result of increasing carbohydrate metabolism and are not byproducts of fatty acid oxidation because muscle acylcarnitines accumulate during high intensity exercise (>75% VO_{2max}) when measured respiratory exchange ratios (RER) indicate that mostly carbohydrate is utilised for energy,[23] underscoring the importance of carnitine in

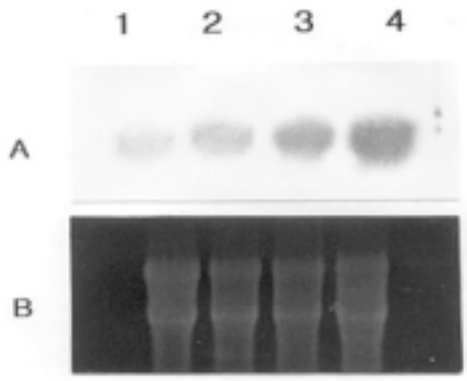

Figure 1. Northern blot analysis showing changes in hepatic carnitine palmitoyltransferase-I mRNA in trained rats. Liver samples were collected from rats in each of the following groups: NTNE (lane 1), NTSE (lane 2), LTNE (lane 3) and LTSE (lane 4). Total RNA (30 g) was separated by 1% agarose gel electrophoresis and transferred to a nylon membrane. Hybridisation and visualisation were conducted as described in Materials and methods. (a) Northern blot of rat mRNA; (b) ethidium bromide stain of RNA from rats. LTNE, long-term trained, non-exercise; LTSE, long-term trained, single exercise; NTNE, non-trained, non-exercise; NTSE, non-trained, single exercise.

carbohydrate metabolism as well as fat metabolism. The muscle acyl/free carnitine ratio in acutely exercised, untrained rats (0.63 ± 0.06) is in very close agreement with results seen in humans engaging in high-intensity exercise above the lactate threshold (0.67 ± 0.07).[20] The CPT-1 data support the assertion that the acylcarnitines formed during acute exercise in untrained animals are carbohydrate derived as the only group with increased CPT-1 level was the acutely exercised trained rats, which did not have higher acylcarnitine levels.

Unlike CPT-1 activity, CPT-1 mRNA expression was high in both of the exercised groups, demonstrating that the lack of activity in the NTSE group was a result of short-term inhibition of CPT activity. How this might occur is not clear as the primary inhibitor of CPT-1 activity is malonyl-CoA. It is unlikely that high levels of malonyl-CoA would be present during intense exercise, therefore there is presumably another inhibitor of CPT-1 activity during high-intensity exercise.

It is interesting that exercise training, but not acute exercise, affected changes in serum carnitine concentrations. Both groups of trained rats had two-fold or higher serum concentrations of acyl- and total carnitine. A possible explanation is that long-term exercise training results in an increase in urinary conservation of carnitine by increasing a renal threshold for carnitine, acylcarnitine in particular. Several studies have shown that free carnitine is more efficiently reabsorbed in the kidney than are acylcarnitines.[25–27] If there is an increased renal threshold in trained animals, it could be a protective mechanism that prevents carnitine loss as a result of exercise.

It is generally recognised that exercise plays an important role in weight maintenance. We previously demonstrated that 30 days of exercise training resulted in less weight gain in young rats,[28] and others have shown, in humans, that total energy expenditure, regardless of exercise type or intensity, is the critical factor in exercise-mediated weight loss.[29] In this study, long-term (60 days) exercise training also resulted in reduced weight gain and food efficiency ratio in adult rats.

Plasma triglyceride is a potential source of energy for muscle and is important for replenishing intramuscular triglyceride stores during long periods between exercise.[30] Previous studies by ourselves and others have found that exercise training reduces serum triacylglycerol and total cholesterol in humans, as was seen in rats in this study.[31,32] However, acute exercise did not lower either serum triacylglycerol or total cholesterol in this study, but both were lower in rats in the LT training groups. Triacylglycerol and total lipids in liver were unchanged by long-term exercise training, but acute exercise resulted in higher concentrations of both. Furthermore, untrained animals had higher concentrations of liver total lipids following exercise than trained animals, possibly reflecting a reduced capacity for mobilising fat from liver as compared to trained animals. It has been generally observed that triacylglycerol accumulates in liver during exercise, as was the case in this study.[33,34] When rats are injected with nicotinic acid, thereby blocking lipolysis in adipose tissue, triacylglycerols do not accumulate in the liver during exercise.[35] This would suggest that synthesis of triacylglycerol from fatty acids released from adipose stores exceeds the capacity of the liver to export triacylglycerol. When hypoglycaemia develops during exercise, the accumulation of triacylglycerol in the liver is even greater.[34] In the present study, liver triacylglycerol accumulated more in the untrained than in the trained animals during exercise. The additional accumulation might be due to an increased capacity of the trained animals to export triacylglycerol, or might have been due to a hypoglycaemic effect of exercise in untrained animals due to lower glycogen stores, which is known to occur in untrained muscles.[36, 37]

The observation that untrained rats utilised a much greater portion of their free carnitine pool and accumulated acylcarnitine is important. Other investigators have demonstrated the effects of exercise on muscle carnitine concentrations. Lennon *et al.*[3] found that acylcarnitines were increased and free carnitine decreased after 40 min of exercise on a cycle ergometer at 55% of their maximal aerobic capacities. They also reported that total muscle carnitine was reduced after the exercise, a result that was disputed by Carlin *et al.*[38] who found that after 90 min cycle ergometry, acylcarnitine was increased at the expense of free carnitine in muscle, but the total carnitine was unchanged. Neither of these studies, however, made a comparison between untrained and highly trained individuals after acute exercise.

It is often assumed that carnitine is primarily important as a facilitator of fatty acid oxidation during exercise. However, it is increasingly evident that carnitine also facilitates carbohydrate metabolism by maintaining pyruvate dehydrogenase activity by acting as a sink for acetate as acetylcarnitine and maintaining free coenzyme A.[39] This is known

to maintain active pyruvate dehydrogenase, thereby assuring a continuous flow of glucose-derived acetate toward oxidative metabolism. Furthermore, when the acetyl-CoA/ CoA ratio is high citrate concentrations are increased, which inhibits phosphofructokinase, a rate-limiting enzyme in glycolysis. It has been observed that humans given a bolus dose of carnitine with glucose during a glucose tolerance test had greater glucose disposal and oxidation when compared with subjects given only glucose.[40] Carnitine therefore appears to play an important role in maintaining active energy metabolism by storing labile acetate in an activated state. Therefore, reduction of free carnitine stores would be expected to have the potential for limiting both carbohydrate and fat metabolism during exercise. Trained rats were able to maintain their free carnitine stores during exercise, a training effect that would be expected to facilitate energy metabolism from both carbohydrate and lipid substrates.

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