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Endurance Exercise Training and *L*-Carnitine Supplementation Stimulates Gene Expression in the Blood and Muscle Cells in Young Athletes and Middle Aged Subjects

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Summary. Endurance exercise training is known to increase fatty acid (FA) oxidation during exercise and to stimulate mRNA synthesis of mitochondrial carnitine acyltransferases in skeletal muscle. To test the hypothesis that long-term endurance training induces cellular adaptions in different tissues, we determined the relative mRNA abundances of these genes in skeletal muscle and in blood cells from young athletes and middle aged untrained persons. The first trial examined 6 cross-country skiers, at the start of high volume/low intensity exercise training and 6 months later, when training at the same exercise intensity had elicited a significantly slower rate of lactate accumulation. In the second trial of 24 middle aged untrained (12 placebo and 12 carnitine supplemented) probands the mRNA expression was determined at the beginning and after three months of a low intensity endurance training program.

A 5-fold increase of the muscle form of carnitine palmitoyltransferase 1 (CPT1B), a 4-fold increase in carnitine acetyltransferase (CRAT), and a 6-fold increase in the mRNA content of the main carnitine carrier OCTN2 in the muscles of the athletes were determined by reverse transcription quantitative real time polymerase chain reaction (RTPCR). The corresponding values examined in white blood cells were 12-(CPT1B), 4-(CTP1A), 3-(CRAT), and 5-fold (OCTN2).

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In WBC of middle aged untrained subjects, the mRNA content of the liver form of the carnitine palmitoyltransferase 1 (CPT1A) was stimulated 2-fold (placebo group) and 8-fold in the carnitine supplemented probands. The relative abundances of CPT1B mRNA were increased by a factor of 3 (placebo) and 5 (carnitine supplemented), respectively. The mRNA abundances of OCTN2 increased 5-fold (placebo) and 7-fold (carnitine supplemented).

While the plasma carnitine levels also remained low during the study period in athletes, normal levels were determined in untrained subjects and significantly increased levels were found after carnitine supplementation. A marked increase of acylcarnitine excretion resulting in a reduction of the portion of short-chain acylcarnitines, other than acetylcarnitine, due to carnitine supplementation could be interpreted as a detoxifying function.

The results of the present study may offer the opportunity to use blood cells as a target for differential gene expressions and as an indicator for responses of skeletal muscle to exercise and/or nutrient supplementation.

Keywords. Training; Gene transcription; Muscle and blood cells; *L*-Carnitine-*L*-tartrate supplementation; Acylcarnitine ester; GC; HPLC/tandem-MS.

Introduction

Endurance training results in an overall increase in fat oxidation during rest and exercise. The difference in the capacity to oxidize fatty acids (FA) between trained and untrained individuals is due to enhanced cellular FA uptake by the FA-translocase (FAT/CD36) and an increased FA transport into the mitochondria by the carnitine carrier system [1–4]. *Tunstall et al.* reported that 9 days of repeated exercise increased total fat oxidation over the course of 1 h by 24% which equated to 4.5 g of additional fat oxidized [4].

The mitochondrial transport system for long-chain FAs consists of three proteins, the malonyl-CoA sensitive carnitine palmitoyltransferase 1 (CPT1), carnitine palmitoyltransferase 2 (CPT2), and carnitine-acylcarnitine translocase (CACT). CPT1 exists in at least three isoforms, a liver form (CPT1A), a smaller counterpart (CPT1B) found in cardiac and skeletal muscle, and CPT1C recently described in the brain. The isoforms are encoded by genes localized on different chromosomes and exhibit different tissue distribution and different transcriptional regulation. The most prominent kinetic difference between them is the 10–100-fold difference in their sensitivity to malonyl-CoA (CPT1B being more sensitive) [1–4].

It is generally accepted that a bout of sustained muscular activity exerts the capacity to transiently activate the expression of many genes [5]. For example, GLUT4, hexokinases, and uncoupling protein (UCP-3) gene expression are observed to peak from within 30 min to 3 h after exercise [3, 6]. Also adaptive processes to long-term endurance exercise induce long lasting changes of the transcriptional rates of numerous genes in different tissues, and an increase in mRNA expression and activity from CPT1B and CPT2 in skeletal muscle has been described [4, 7, 8]. The mechanisms coupling muscle contractile activity, mitochondrial oxidative capacity, and expression of distinct genes are poorly understood.

We chose to apply reverse transcription quantitative real time polymerase chain reaction (RTPCR) in blood and muscle samples in an effort to identify patterns of gene expression that were possibly altered simultaneously in blood and skeletal muscle cells. It has been reported that white blood cells (WBC) have appreciable

beta-oxidation activity and that patients with FA disorders or with pathogenic mutations in the CPT2 gene have also very low rates of WBC FA oxidation [9–11]. After adriamycin treatment an excellent correlation of the blood cell and heart gene expression has been demonstrated in rats. It has been concluded that gene expression in blood cells particularly in relation to oxidative stress may be useful in monitoring and predicting cardiac muscle damage [12]. The presence of sufficient numbers of nucleated cells (predominantly neutrophils, lymphocytes, and monocytes) may offer the opportunity to use blood cells instead of muscle tissue biopsies for the evaluation of metabolic changes in response to exercise. Consequently, the aim of the study was to determine the relative mRNA abundances of three carnitine acyltransferases (CPT1A, CPT1B, and CRAT) and the organic cation transporter OCTN2, responsible for carnitine uptake by most cells.

Nevertheless professional and non-professional athletes seek nutritional supplements that will enhance exercise performance. These substances theoretically improve exercise capacity by enhancing lipid oxidation and reducing rates of muscle glycogen depletion [13]. During high-intensity exercise muscle carnitine content decreases under various conditions, including a decrease secondary to redistribution into acylcarnitines [14]. On the other hand the carnitine transport system (OCTN2) makes it difficult to increase muscle carnitine content in healthy subjects and a long duration of carnitine supplementation would likely be required to modify skeletal muscle homeostasis.

However dietary supplementation of endurance athletes with *L*-carnitine (2 g/day for 4 weeks) resulted in substantial increases in activities of skeletal muscle pyruvate dehydrogenase and the respiratory chain enzymes *NADH*-cytochrome C reductase, succinate-cytochrome C reductase, and cytochrome C oxidase [15]. This is in line with studies in untrained humans without disturbances in fatty acid metabolism which show that oral *L*-carnitine supplementation results in an increase in long chain fatty acid oxidation [16, 17].

In animal experiments we demonstrated that *L*-carnitine administration significantly increases the relative m-RNA abundances of CPT1A, CPT2, and CRAT in the livers of old but not of young rats, as well as the activity of CPT1A [18]. In trained rats carnitine supplementation increases FA oxidation in skeletal muscle by a mechanism that includes increasing total carnitine content in soleus muscle mitochondria and the total content of acylcarnitine. The most interesting finding was that the effect of supplementation was even greater in trained rats after a three-week carnitine supplementation [19].

A new line of research studying the interplay between *L*-carnitine and exercise has recently evolved. Studies have indicated that an important benefit of *L*-carnitine supplementation may reside in its ability to optimize recovery from hypoxia effects. Exercise induced increases in plasma markers of purine catabolism and circulating cytosolic proteins were significantly attenuated and the increases in plasma malondialdehyde returned to resting value sooner in treated subjects compared to controls [20].

It is well known that acylcarnitine ester concentration and the excretion of acylcarnitine rapidly increase following on oral or intravenous administration of *L*-carnitine, which possibly mirrors the mitochondrial matrix pool, but the quantitative and functional significance of this exchange has not been evaluated. Consequently it was an additional goal to characterize and quantitate the acylcarnitines in

the urine of the probands by HPLC/tandem-MS. In order to distinguish between *n*- and *iso*-isomers we developed a simple GC method.

Results

Subject Characteristics of Young Athletes

Considering a low proportion of cardiopulmonary load in the training protocol, there was a small but not significant increase in $VO_{2\,max}$. Both workloads at fixed lactate levels P2 and P4 increased significantly ($p < 0.05$) during the 6 month training period. The exercise training did not result in significant changes in body weight.

Expression of Carnitine Acyltransferases and OCTN2 in Blood and Muscle

Transcript levels of CPT1B, CPT2, CRAT, and OCTN2 are shown in Fig. 1. After a 6 months training period CPT1B expression showed a 4-fold increase in the expression in muscle and a 8-fold increase in blood cells. CPT2 expression was also higher in peripheral blood and muscle (data not shown).

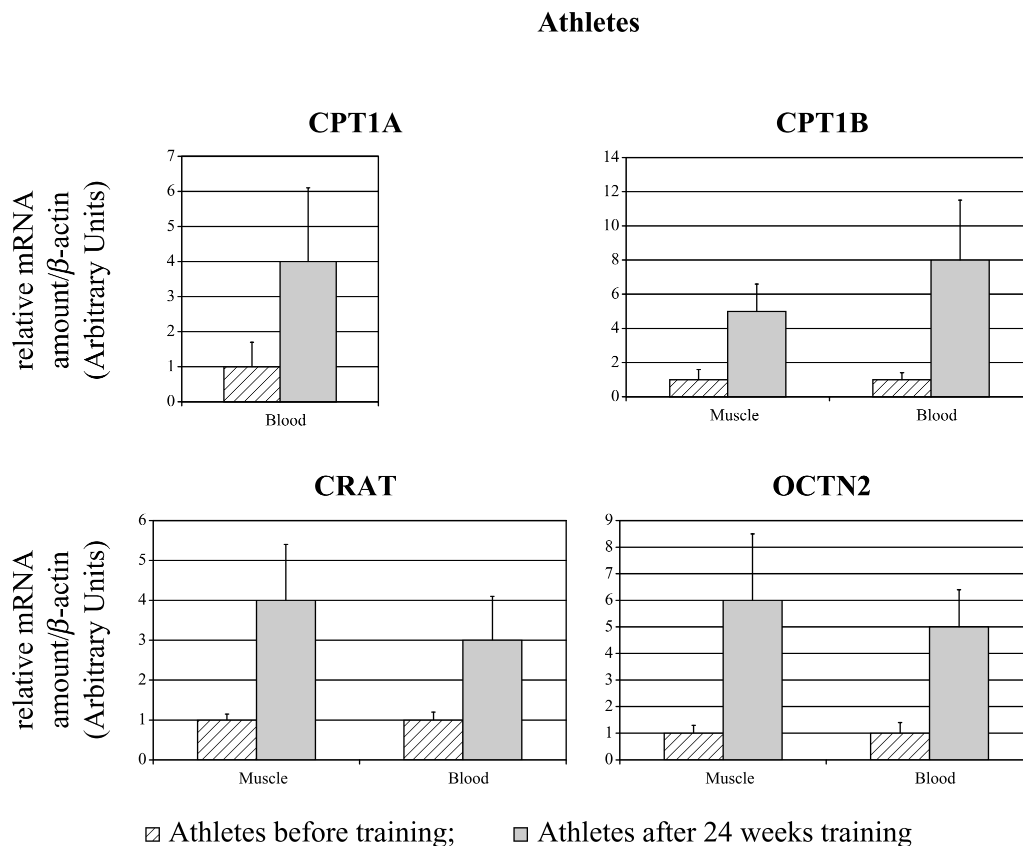


Fig. 1. Comparison of the relative mRNA abundances of genes encoding proteins involved in long chain fatty acid transport in skeletal muscle and white blood cells (WBC) of athletes before (white bars) and after 24 weeks training (black bars); specific mRNA levels were quantified by real time PCR in total RNA preparations from muscle tissue and WBC

Table 1. Plasma carnitine, triacylglyceroles and free fatty acids (FFA) levels and the portion (%) of a single FA on total FFAs of athletes before and after 6 months of endurance training

	Before	After	p
Free Carnitine (μmol/dm^3)	33.0 ± 9.5	33.7 ± 8.9	n.s.
Total Carnitine (μmol/dm^3)	40.1 ± 9.9	42.0 ± 5.8	n.s.
Short Chain Acylcarnitine (μmol/dm^3)	7.1 ± 4.7	8.2 ± 5.5	n.s.
Triacylglyceroles (mg/100 cm^3)	41.3 ± 12.8	54.5 ± 27.2	n.s.
Total Free Fatty Acids (g/dm^3)	88.9 ± 42.3	55.6 ± 18.9	$p < 0.05$
% Palmitic Acid (C16:0)	19.0 ± 2.0	36.0 ± 2.7	$p < 0.001$
% Palmitoleic Acid (C16:1)	8.2 ± 1.4	10.4 ± 1.1	$p < 0.001$
% Stearic Acid (C18:0)	11.6 ± 1.6	10.8 ± 1.1	n.s.
% Oleic Acid (C18:1n9)	48.4 ± 2.3	30.1 ± 3.2	$p < 0.001$
% Linoleic Acid (C18:2n6)	11.2 ± 3.1	10.5 ± 3.0	n.s.
% Linolenic Acid (C18:3n3)	1.6 ± 0.7	2.2 ± 0.9	n.s.

The mean expression of CRAT showed a 4-fold increase in muscle and a 3-fold increase in WBC. After a 6 months training OCTN2 expression showed a 6-fold increase in muscle and an equal 5-fold increase in blood cells. The liver isoform CPT1A, is also expressed in blood cells, but not in skeletal muscle. In the blood cells a 4-fold increase in expression was found.

Plasma Carnitine Levels and Lipid Profile

The levels of free, total, and short chain acylcarnitine and triacylglycerol were generally lower as compared to controls and values of the middle aged untrained, and showed no training induced changes (Table 1).

In contrast, plasma levels of free FA diminished significantly after 6 month training (Table 1). The composition of FFA elicited significant changes, after 6 months of training the proportion of palmitic and palmitoleic acid increased significantly ($p < 0.001$) and the proportions of myristic and oleic acid expressed as a percentage of total FA was significantly reduced ($p < 0.001$) (Table 1).

Subject Characteristics of Middle Aged Untrained Probands

The endurance exercise training resulted in a significant reduction in body weight, a significant increase of $VO_{2\,max}$ and of performance (W/kg) in both, the supplemented and the control group. There were slightly higher values in the carnitine supplemented subjects, but the difference was statistically not significant.

Expression of Carnitine Acyltransferases and OCTN2 in Blood Cells

The low intensity exercise training resulted in an increase in the expression of CPT1A, CPT1B, and OCTN2 by a factor of 2, 3, and 5, respectively (Fig. 2). Exercise and carnitine supplementation together enhanced the effect on gene expression by a factor of 8, 5, and 7, respectively (Fig. 2).

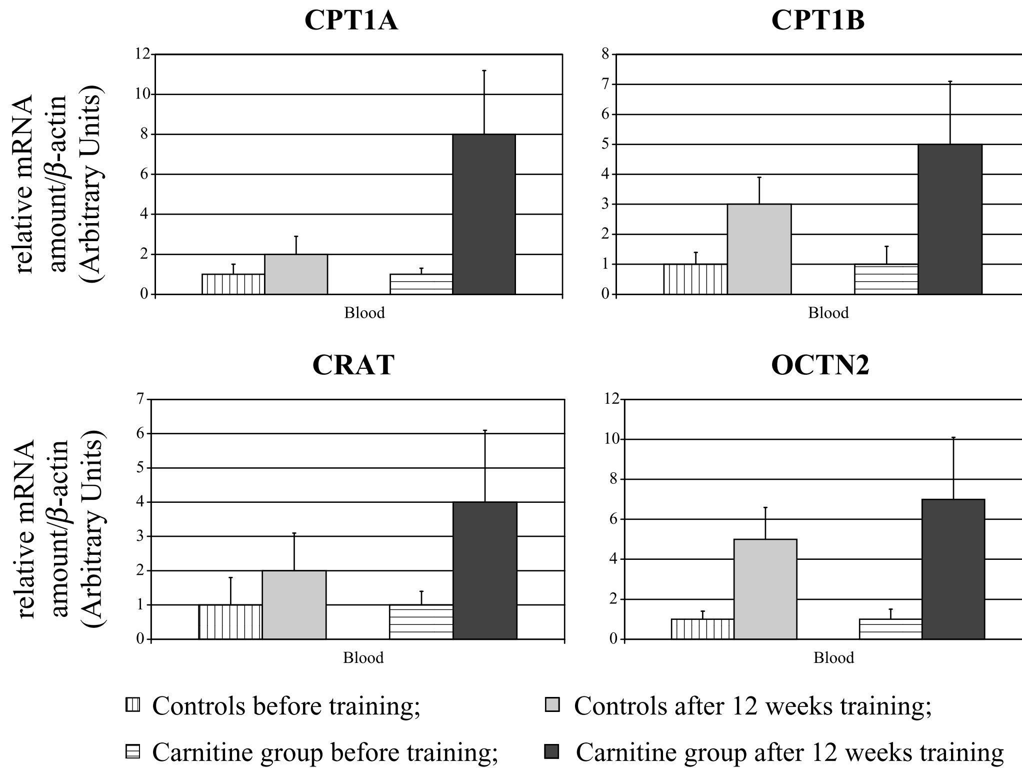


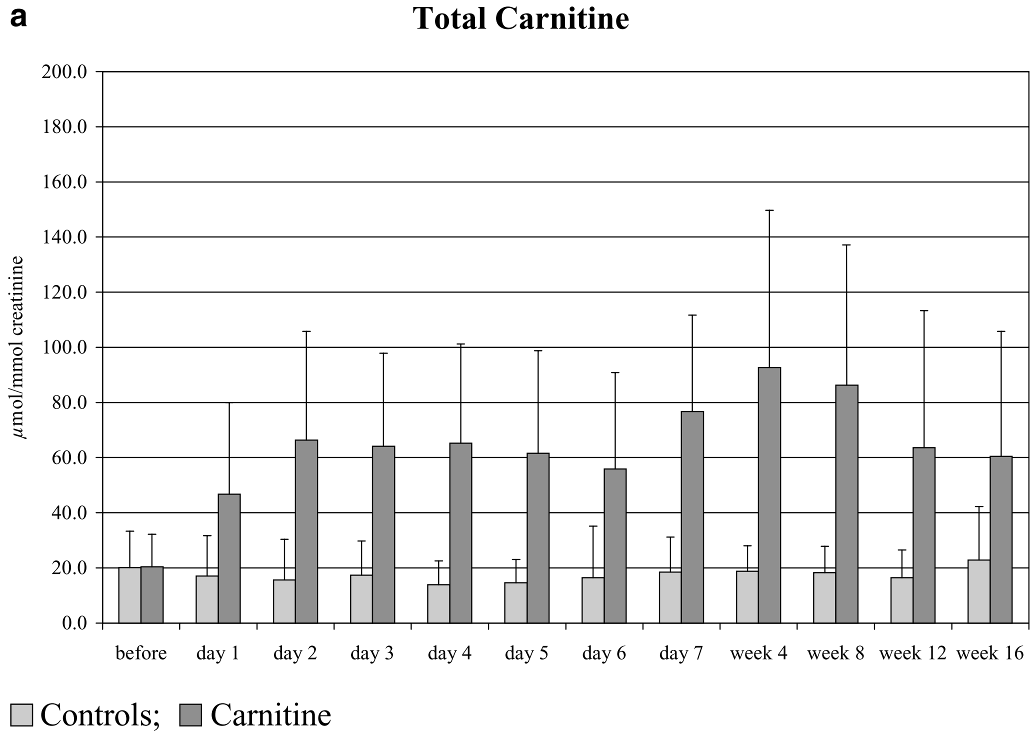
Fig. 2. Comparison of the relative mRNA abundances of genes coding proteins involved in long chain fatty acid transport in white blood cells (WBC) of control and carnitine supplemented middle aged untrained subjects before (white bars) and after 12 weeks training (black bars); specific mRNA levels were quantified by real time PCR in total RNA preparations from WBC

Plasma Carnitine Levels and Lipid Profile

Compared to the placebo group, plasma total, free, and short-chain acylcarnitine concentrations were significantly higher during *L*-carnitine supplementation at all

Table 2. Plasma carnitine, triacylglyceroles, and free fatty acid (FFA) levels of untrained middle aged probands before, after 1, 12, and 16 weeks of endurance training

	Before training		After 4 weeks training		After 12 weeks training	
	Controls ($n=12$)	Carnitine group ($n=12$)	Controls ($n=12$)	Carnitine group ($n=12$)	Controls ($n=12$)	Carnitine group ($n=12$)
Total Carnitine (μmol/dm^3)	49.3 ± 2.3	52.1 ± 10.2	47.5 ± 8.7	56.7 ± 8.0	50.9 ± 9.8	61.2 ± 10.2
Free Carnitine (μmol/dm^3)	38.7 ± 8.7	39.0 ± 6.1	40.1 ± 8.4	46.3 ± 5.4	40.3 ± 8.1	47.9 ± 8.9
Short Chain Acylcarnitine (μmol/dm^3)	10.6 ± 5.6	13.1 ± 8.7	7.4 ± 3.9	10.4 ± 3.7	10.6 ± 3.9	13.3 ± 7.2
Triglyceroles (mg/100 cm^3)	97.5 ± 60.1	78.1 ± 21.7	93.3 ± 65.5	71.2 ± 21.4	83.8 ± 33.1	68.8 ± 16.8
Total Free Fatty Acids (mg/dm^3)	127.0 ± 48.8	132.2 ± 46.9	134.3 ± 31.3	136.2 ± 26.0	145.7 ± 52.6	140.2 ± 46.0



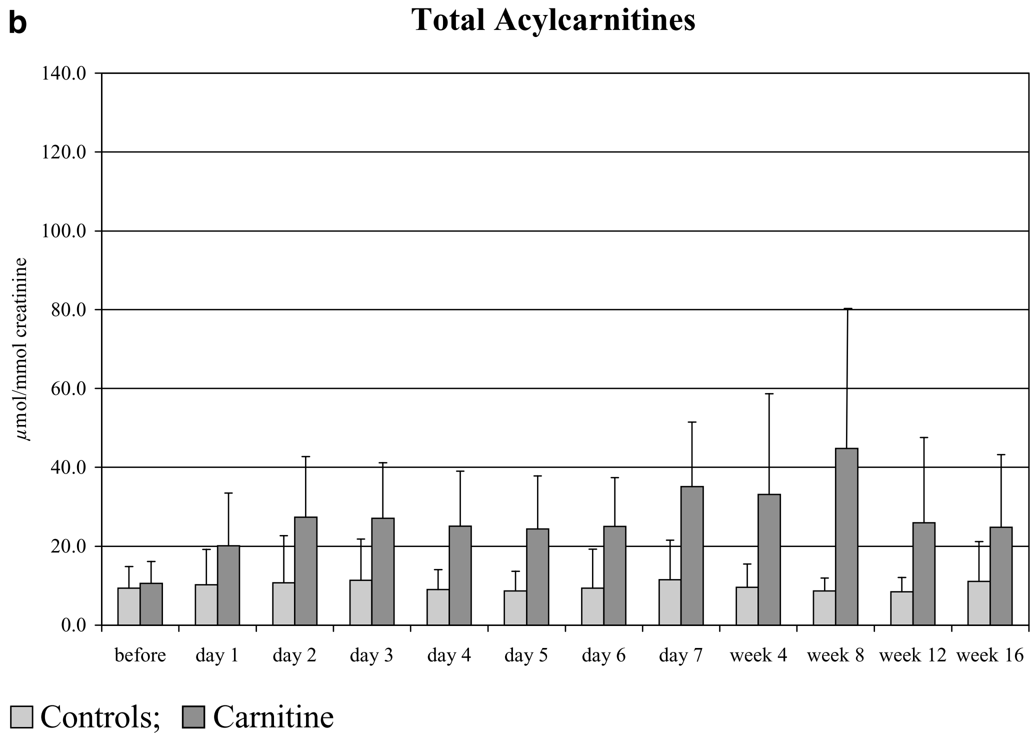


Fig. 3. a. Comparison of the excretion of total carnitine in the urine of middle aged untrained probands before and after day 1 to 7, week 4, 8, 12, and 16 of endurance training; controls (white bars) *versus* carnitine supplemented subjects (black bars); **b**. Comparison of the excretion of acylcarnitines in the urine of middle aged untrained probands before and after day 1 to 7, week 4, 8, 12, and 16 of endurance training; controls (white bars) *versus* carnitine supplemented subjects (black bars)

time points measured. The plasma levels of triacylglycerols and free fatty acids were not influenced in either group by the exercise training or by carnitine supplementation (Table 2).

Excretion of Acylcarnitines in Urine

Figure 3 shows the excretion of total carnitine and the total acylcarnitine esters at the start, the first seven days, and thereafter every four weeks of exercise training and carnitine substitution. In comparison to placebo, carnitine substitution increases the excretion of total carnitine 3- to 4-fold, but very importantly, also those of acylcarnitines in the same order of magnitude.

Before exercise training middle aged untrained probands excrete more free than esterified carnitine. The proportion of carnitine esters increased significantly within 7 days from 46 to 65% and this ratio was sustained up to 3 months (data not shown).

Table 3. Portions of single acylcarnitine on total acylcarnitines in the urine of middle aged untrained probands before, after day 1–7, week 4, 8, 12, and 16 of endurance training

	C2 %	C3 %	*i*C4 %	*i*C5 %	C8 %	C8-1 %	C10-1 %	D3 %	D4 %	D5 %	D6 %	D7 %
Controls												
before	52.6	3.7	8.6	5.2	1.9	8.5	3.09	1.6	4.1	3.7	1.1	2.5
day 1	56.5	3.5	7.6	5.0	1.9	7.1	3.33	1.8	3.8	3.1	3.0	1.5
day 2	64.7	2.3	5.5	4.3	2.3	6.6	3.02	2.2	3.8	3.7	8.5	1.5
day 3	56.0	2.0	5.4	4.1	2.0	7.0	3.04	1.5	3.4	3.0	7.5	1.3
day 4	49.7	2.8	7.2	5.3	2.5	9.0	4.24	1.9	4.4	3.7	4.6	1.7
day 5	51.1	2.6	7.8	5.2	2.1	9.3	3.92	1.7	4.4	3.9	2.1	1.6
day 6	56.1	2.7	6.6	4.4	2.0	8.2	3.57	1.7	3.7	3.3	3.4	1.5
day 7	61.3	2.2	5.6	4.5	1.6	6.5	3.23	1.4	3.0	2.7	1.7	1.2
week 4	54.2	2.7	8.2	5.7	2.0	9.0	2.74	1.5	4.6	4.2	1.0	2.5
week 8	43.7	2.9	9.7	6.7	2.5	11.0	3.34	1.6	4.7	4.1	1.3	2.6
week 12	44.2	3.2	8.9	6.3	2.4	11.3	3.73	1.6	5.2	4.6	1.2	2.8
week 16	57.1	3.2	7.3	5.3	1.8	8.9	2.93	1.4	3.7	3.2	1.0	2.1
Carnitine supplemented Group												
before	54.9	2.6	7.2	5.0	1.9	7.6	3.39	1.1	3.7	3.3	0.9	2.2
day 1	62.0	2.9	6.0	6.9	1.2	6.3	2.11	1.0	1.8	1.4	0.5	0.8
day 2	70.2	3.0	4.2	5.7	1.1	4.7	1.70	1.0	1.3	1.0	0.6	0.7
day 3	70.8	3.3	4.9	5.0	1.1	4.6	1.21	1.2	1.3	1.1	1.6	0.7
day 4	70.8	3.4	4.9	5.0	1.1	3.5	1.55	1.0	1.3	1.0	0.6	0.7
day 5	70.7	2.9	5.0	4.7	1.0	4.1	1.56	0.9	1.3	1.1	0.4	0.7
day 6	71.4	2.4	3.8	3.3	1.1	6.4	1.56	1.1	1.3	1.1	0.7	0.6
day 7	74.0	2.6	4.2	4.8	1.0	3.8	1.35	1.0	1.2	1.0	1.0	0.6
week 4	73.9	3.2	3.5	3.4	1.1	5.0	1.25	1.1	1.4	1.3	0.3	1.2
week 8	84.7	1.7	2.2	1.9	0.7	2.7	1.12	0.7	0.8	0.7	0.2	0.7
week 12	76.5	3.0	4.3	3.4	1.0	4.3	1.35	0.8	1.3	1.3	0.3	1.2
week 16	76.2	2.8	3.6	3.0	1.0	4.1	1.26	0.8	1.5	1.4	0.3	1.1

Interestingly, acetylcarnitine accounts only for about 50 to 60% of the carnitine esters with a tendency to increase during the study period. The other carnitine esters determined in significant amounts were identified as isobutyryl-, isovaleryl-, octenoyl-, and decenoylcarnitine using either HPLC/tandem-MS or the GC method. Also carnitine esters of dicarboxylic acids were found in significant amounts.

For each fraction, values are expressed as percent of the total amount of short chain acylcarnitine. The composition of short chain acylcarnitines of urine specimens is comparable to those reported by others [30]. Carnitine supplementation resulted in an increase of the proportion of acetylcarnitine expressed as a percentage of total acylcarnitines and in a diminution of the other short- and middle-chain acylcarnitines (Table 3).

Discussion

Endurance exercise training stimulates the oxidative capacity of skeletal muscle and leads to an increase in total fat oxidation whereas high-intensity exercise training did not affect total fat oxidation during exercise [1, 3, 4, 6, 16, 31]. The results of the present study provide further and extended evidence that long term endurance exercise training with a high volume/low intensity protocol is effective in increasing the relative mRNA abundances of enzymes involved in mitochondrial fatty acid transport, CPT1B, CPT2, and CRAT.

The novel findings are that the gene expressions were altered in both muscle cells and in WBC, and a positive correlation with one another was shown for CPT1B, CRAT, and for OCTN2, suggesting a common mechanism of induction.

Carnitine-acyltransferases are important enzymes for energy homeostasis and fat metabolism through their modulation of the pools of acetyl-CoA and long chain acyl-CoA in distinct cellular compartments. These enzymes belong to the family of carnitine choline acyltransferases and have distinct properties with respect to intracellular location, substrate specificity, kinetics, and physiological function. The CPTs and COT (carnitine octanoyltransferase) transesterify medium and long-chain fatty acyl chains whereas CRAT, which is a mitochondrial matrix enzyme, transesterifies short chain acyl groups [32–35].

Recently, it has been shown that even a low amount of physical activity in lean sedentary humans leads to a significant depression of acetyl-CoA carboxylase 2 and an increase in lipoprotein lipase mRNA expression [6]. Then changes may result in an enhanced cellular FA supply and a reduced synthesis of malonyl-CoA, and consequently in an increased activity of CPT1, which may contribute to the observed significant statistical increase in FA oxidation in human skeletal muscle. It is possible that immediate early genes and genes encoding growth factors and signalling intermediates are more responsive to exercise in the untrained state, whereas other genes, such as those encoding metabolic enzymes and transporters may be more strongly influenced in the trained state [23].

Nevertheless, the mechanisms coupling muscle contractile activity to the increase in metabolic gene expression are incompletely understood. In relation to exercise, potential stimuli include stretch and muscle tension, the pattern of

motor nerve activity, the energy charge of the cell, substrate availability, oxygen tension, and circulating hormones [36]. Various cellular signaling mechanisms such as the mitogen-activated protein kinase family, protein kinase B, protein kinase C, Ca^{2+}/calmodulin dependent protein kinase, and the *AMP*-activated protein kinase are of importance [36]. Finally, the various transcription factors involved in the activation of gene transcription must be considered.

The concept that endurance exercise training induces metabolic adaptation mechanisms which are not specific to the skeletal muscle is further supported by the finding that the relative amount of CPT1A mRNA was also increased in mononuclear blood cells in the same order of magnitude as the muscle isoform CPT1B.

CPT1A expression is controlled by thyroid and pancreatic hormones as well as by long chain FA, which have been shown to activate both, CPT1A and CPT1B transcription [37, 38]. It is known that endocrine factors, including thyroid and steroid hormones, are influenced by endurance training [39–41]. The fat-activated fatty acid response element (FARE) is activated through the peroxisome-proliferator-activated receptor alpha (PPARα) [42, 43]. On the other hand several studies suggest that the effects of long chain FA on CPT1A expression are mediated by a pathway different from that of peroxisome-proliferator-activated receptors. *Tunstall et al.* [4] found that training for 9 days increased the cellular uptake of FA by increasing the FAT/CD36 and CPT1 expression, while the expression of PPARα remained constant and that of PPARγ was lowered. It is known that LCFAs induces the gene expression of CPT1 but not CPT2 [37]. In a previous study a stimulation of the transcriptional rates of both, CPT1 and CPT2 was found, which is in accordance with the view that the effects of endurance exercise training on gene transcription may not only be mediated via PPARα.

Regardless of the exercise intensity, in young athletes all plasma triglyceride levels before and after training remained in the lower control range, those of free FA were in the normal range but dropped significantly after the training period (Table 1). From stable isotope studies it is known that about 50% of total fat oxidation is accounted for by plasma FFA oxidation. However, the source of the additional oxidized FAs is not clear. The following fat sources available during exercise for skeletal muscle include: (1) Plasma FFA released from lipolysis of adipose tissue triglycerides into the blood, (2) fatty acids released from circulating VLDL triglycerides by the action of lipoprotein lipase, and (3) release of FA from the intramuscular triglyceride depots. Thus it is possible that in young athletes in the trained state substrate availability may limit overall FA oxidation and consequently the physical performance.

The composition of FFA elicited significant changes. At the start of endurance exercise training, the composition of FFA is rather comparable to that of adipose tissue, whereas after 6 months of training the portion of those FA, known to be the main products of endogenous FA synthesis (palmitic and palmitoleic acids), increased significantly.

Variations in the dietary intake of macronutrients are associated with marked changes in substrate availability, oxidation, and energy metabolism. Alterations in plasma glucose and fatty acid levels can influence gene transcription and the avail-

ability of these substrates, and their effects on gene expression, may be involved in the adaptive response to dietary manipulations [44].

In contrast in middle aged untrained subjects the plasma levels of triacylglycerols and FFAs were not influenced, neither by the exercise training nor by carnitine supplementation (Table 2).

The low plasma levels of total, free, and esterified carnitine found in the present study in young athletes were rather unexpected. Accumulation of acetyl groups was observed during exercise when formation of acetyl-CoA is greater than its utilization by the tricarboxylic acid (TCA) cycle [45]. The excess acetyl-CoA units are transferred to carnitine to regenerate free CoA which was found to be essential for continued PDH activity.

Submaximal exercise reduced muscle free carnitine content, matched by an almost equivalent increase in acetylcarnitine [45]. A decrease in acetylcarnitine during prolonged moderate intensity exercise has been described and coincided with a concomitant decrease in PDH activity and whole body carbohydrate oxidation. These results were interpreted to indicate that in spite of increased fat metabolism, *ATP* derived from acetylcarnitine stores was required to fuel oxidative metabolism late in exercise [45, 46].

It is known that acetylcarnitine contributes only about 50 to 60% of total short chain (acid soluble) acylcarnitine excreted in urine, a portion which is further reduced after exercise [47]. The kidneys play an important role in the homeostasis of carnitine levels in the blood [48]. The organic cation transporters (OCTNs) are also found in the kidney and favor the transport of carnitine [49]. More than 95% of free carnitine is reabsorbed in the ultrafiltrate. Acetylcarnitine is also reabsorbed in significant amounts but the longer and/or branched chain carnitine esters are not [48]. Consequently, reduction in carnitine levels in the body can be induced by the following sequence of reactions: (i) Increased protein turnover causes a rise in free branched chain amino acid level. (ii) By transamination, branched chain fatty acids are then formed and subsequently esterified, yielding carnitine esters. (iii) Substantial amounts of carnitine may then be excreted *via* the kidney, resulting in a reduction of free and total carnitine levels. This interpretation is substantiated by the findings in the untrained subjects, who excreted significant amounts of acylcarnitines other than acetylcarnitine. In addition carnitine substitution resulted in a significant increase of acylcarnitine over the whole study period. It has been shown that carnitine supplementation increased the total amount of carnitine in mitochondria by 50% in sedentary and by 80% in trained rats. This increase was paralleled by a decrease in total content of acetyl-CoA [45].

Interestingly, in supplemented subjects of the present study the proportion of acetylcarnitine on total acylcarnitine increased and that of other acylcarnitines decreased, which may be interpreted as detoxifying function of carnitine in mitochondrial metabolism.

This is in accordance with the expression of CRAT mRNA which was increased 4-fold in muscle and 3-fold in WBC of athletes and 2-fold in untrained subjects. Within the mitochondrial matrix, the capacity of transesterification of short chain FA *via* the carnitine acetylcarnitine equilibrium reaction may also be important, if acetyl-CoA formed from acetylcarnitine is the major rate determinant of the

oxidative phosphorylation at the onset of exercise, as suggested by *Greenhaff* and colleagues [46].

The metabolic adaptations to exercise include the transcriptional activation of target genes like carnitine acyltransferases. The increased mRNA synthesis correlates in muscle and WBC. Notably, the enzymes investigated in this study are involved in fatty acid transport in mitochondria and microsomes as well as in the transfer of short chain fatty acids, *via* the acyl-CoA – acylcarnitine equilibrium reaction. It is suggested that long term endurance exercise training induces simultaneous common metabolic adaptation mechanisms by changing transcriptional rates of numerous genes in various cells and tissues.

Experimental

Abbreviations

CACT carnitine-acylcarnitine translocase; COT carnitine octanoyltransferase; CPT1A carnitine palmitoyltransferase 1 liver form; CPT1B carnitine palmitoyl transferase 1 muscle form; CPT2 carnitine palmitoyl transferase 2; CRAT carnitine acetyl transferase; FA fatty acid; FARE fatty acid response element; FAT fatty acid translocation; GC gaschromatography; GLUT4 glucose transporter 4; GTC Guanosin isothiocyanate; HPLC high performance liquid chromatography; LCFA long chain fatty acid; MS mass spectronometry; OCTN2 organic cation transporter 2; PDH pyruvate dehydrogenase complex; PPAR peroxisome-proliferator activated receptor; PTV programmed temperature vaporizer; RTPCR real time polymerase chain reaction; UCP-3 uncoupling protein 3; UEFA unesterified fatty acids; WBC white blood cells.

Reagents and Standards

Acetyl- and octanoylcarnitine was purchased from Sigma Aldrich (St. Louis, MO). Crotonyl-, isobutyryl-, isovaleryl-, and heptanoylchlorides, as well as diisopropylether (used for precipitation of carnitine-esters), methanol, trifluoroacetic acid, acetone, and hydrochloric and perchloric acid were purchased from E. Merck, Darmstadt, FRG. *L*-Carnitine was a gift from Leopold (Graz). Dowex 50 W × 8 (100–200 mesh, H^+-form) was purchased from Biorad, Dowex 1 × 8 (200–400 mesh; Cl^--form) was purchased from Serva (Heidelberg, FRG).

Kit Histopaque – 1077 (Sigma-Aldrich, MO, USA); RNA/DNA Stabilizing Reagent for Blood/Bone Marrow (Roche Diagnostics, Mannheim, Germany); mRNA Isolation Kit for Blood/Bone Marrow Kit (Roche Diagnostics, Mannheim, Germany); Protector RNAse Inhibitor (Promega, WI, USA); 1st strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany); Ladder Marker, dNTPs, Taq Polymerase (MBI Fermentas, USA); Gradient Thermocycler (Eppendorf, Hamburg, Germany); GenElute PCR Clean-up Kit (Sigma-Aldrich, MO, USA); BioPhotometer (Eppendorf, Hamburg, Germany); LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany); LightCycler Instrument (Roche Diagnostics, Mannheim, Germany).

Synthesis of Acylcarnitines

Crotonyl-, isobutyryl-, isovaleryl-, and heptanoylcarnitine were prepared by the method described by *Bohmer et al.* [21]. Using diispropylether and a crystallisation time of 2 days at -18°C, a yield of 90% was obtained. By means of the radioenzymatic assay all synthesized *L*-carnitine esters were found to have a purity of more than 99.5%. GC analysis revealed a single peak for each species. Distribution coefficients were determined either by the batch equilibrium or the column method as described by *Korkisch* [22].

Subject Characteristics

a) Young Athletes

Six male endurance trained junior cross-country skiers volunteered to participate in the study. All subjects had finished their competitive season without major health problems. The subjects entered the study at the end of a 3 weeks period with no training after the end of the competitive season. The observation period was 6 months.

Training status and level of condition of the subjects was low compared to the level of condition at the beginning of the past competitive season. All subjects had a regular endurance training experience of a minimum of 2 years and competed on local or national level. Mean $VO_{2\,max}$ was 58.7 $cm^3/kg/min$ at the beginning. Our test design consisted of two treadmill-tests for each participant, muscle and blood samples were taken after informed consent during routine health- and performance tests. All subjects were familiar with the examined test procedure.

Training Protocol

Over the study period of six months the subjects followed a low-intensity training programme with a minimum of seven 90–120 min sessions per week. The intensity of the exercise was individually set according to the results of the treadmill test. More than 85% of the total training load was performed below the ventilatory threshold or below HR at fixed lactate levels of 2 $mmol/dm^3$, respectively. To monitor and adjust the training intensity according to training improvements, heart rate monitors were used for all training sessions. Blood lactate concentrations were measured (Eppendorf ESAT 6661, Hamburg, Germany) during training.

b) Middle Aged Untrained Probands

Twenty four male subjects volunteered to participate in the study. Subjects were matched for pretesting clinical values, nutritional patterns, and body size and then randomly assigned to either an *L*-carnitine or placebo group in a double-blind fashion. Subjects were provided with L-CARNIPURE (Lonza, Basel) for a total of 2 g carnitine tartrate (corresponds to 1.45 g *L*-carnitine)/day, or an identically-looking placebo. It has been shown that when used as a dietary supplement (3 g/day) *L*-carnitine-*L*-tatrate (L-CARNIPURE) has no adverse effects on metabolic and hematological variables in normal healthy men [23].

The test design consisted of 4 tests, one at the beginning, after 4, 8, and 12 weeks. At each test anthropometric data were measured, blood samples were taken, and cycle ergometer tests were carried out.

Training Protocol

On the basis of the results of the bicycle tests the subjects followed a low-intensity training program with a minimum of 3 sessions per week (60 min) during the 12 weeks. The intensity of the exercise was individually set according to the results of the bicycle test. Individual training workload was examined before the first training by determination of the individual HR at fixed lactate levels and at the ventilatory threshold. At least once in a week subjects had to train at the training-center, using ergometers (Bikerrace, Technogymm Gambettola, Italy).

Treadmill Testing (TT)

Each subject's $VO_{2\,max}$ was measured during maximal cycle ergometer exercise and each participant performed four bicycle tests at the beginning, after four and eight weeks, and at the end of the training period (12 weeks). The cycle ergometer test was performed as an incremental load exercise test to exhaustion on an electromagnetically braked bicycle ergometer. The incremental test was started with

40 W with an increment of 40 W every 4 minutes and a constant rotation about 75 rpm until the respiratory exchange ratio was equaled 1.1 and there was a plateau in oxygen consumption despite increasing work load.

Capillary blood samples from the earlobe were taken at the beginning, after completion of each stage, and after 1.5, 2.5, 4, 5.5, 7, and 9 minutes during the recovery period for analysis of blood lactate (LA). Heart rate was determined by means of a chest-belt telemetry monitor (Polar, Kempele, Finland) which transmitted and recorded data at the beginning and at the end of each stage. Blood lactate was determined with the fully enzymatic method utilizing Eppendorf (ESAT 6661, Eppendorf, Hamburg, Germany). The plasma lactate response (PLR) was fitted to an exponential function according to $LA = a * e^{bx}$ (where x = workload, a and b are fitting parameters).

Muscle Biopsies

Muscle biopsies from athletes were taken from the right *musculus vastus lateralis* at the midtigh level at baseline and 6 months later at the end of the training period. Sampling was performed as described previously [24]. Samples were immediately frozen in liquid nitrogen; aliquots of homogenized muscle tissue were frozen in 4 *M GTC* (guanosin isothiocyanate) for mRNA preparation.

Blood Samples

Venous blood samples were collected into plain evacuated tubes from a forearm vein after an overnight fast and at least 24 h from the last workout. Blood was immediately centrifuged and then stored at −20°C before analysis.

Analysis of mRNA Expression

Isolation of Lymphocytes

Viable mononuclear blood cells were isolated from venous blood with Kit Histopaque – 1077 supplier. Enriched aliquots with calculated equal numbers of cells were used for mRNA extraction. The same aliquot volume was taken for the mRNA extraction procedure (500 mm^3), thereby standardizing the samples internally. The samples were pre-treated with RNA/DNA Stabilizing Reagent for Blood/Bone Marrow.

Isolation of Polyadenylated mRNA

The mRNA fraction was isolated and purified by virtue of hybridization to biotin-labeled oligo (dT) and capturing by streptavidin-coated magnetic particles, followed by magnetic separation. 40 U/mm^3 of RNAse Inhibitor were added to each extraction. Samples were stored at −80°C.

Due to the low levels of mRNA present in cells and tissues (only 1–5% of total RNA) most or all of the mRNA yielded in an extraction would have to be used for this purpose. Using 1st strand cDNA Synthesis Kit and the provided oligo (dT) primer, the corresponding single-stranded (ss) cDNAs were synthesized by AMV Reverse Transcriptase supplier.

Determination of Relative DNA Amount

Real time PCR technique and SYBR Green I for detection of double-stranded (ds) DNA amplified in the PCR were used as described elsewhere [25].

The primers were tested in a Gradient Thermocycler for the required annealing temperature. The accuracy of real time technique is confirmed by recording the melting curves and melting point of the PCR products of each sample after completion of the reaction.

The standard curve of each gene was obtained using the LightCycler. The sequence of interest was initially amplified in Block PCR, then cleaned (GenElute PCR Clean-up Kit), quantified (Biophotometer), and finally diluted in a 1:10-step serial dilution (1 ng-1 ag).

At least three runs were made for each sample. The mean value obtained was then normalized to a chosen housekeeping gene (β-actin). The housekeeping gene mRNA triplicates were prepared accordingly and internally standardized to measured value corresponding to 1 fg β-actin.

Analysis of Plasma Lipids

Unesterified fatty acids (UEFA), free cholesterol, cholesteryl esters, and triacylglycerols were determined directly from total lipid extract by capillary gas chromatography in two separate runs using a programmed temperature vaporizer (PTV) injector as described previously [26].

Carnitine Measurements

Radioenzymatic Carnitine Assay

Free and short-chain acylcarnitines were assayed by the radioenzymatic method of *Cederblad* and *Linstedt* [27], with two modifications: *HEPES* instead of *TRIS* buffer [28] and *N*-ethylmaleimide instead of tetrathionate [26].

HPLC/Tandem-MS Assay

An API-3000 Tandem MS system (Sciex, Canada) with an Agilent HPLC-system 1100 (Agilent, Germany) was used.

100 mm^3 of urine sample were diluted with 100 mm^3 of internal standard working solution (d3-carnitine, d3-propylcarnitine, d3-palmitoylcarnitine about 5 $\mu g/cm^3$ in 20% methanol) and 800 mm^3 of 50% methanol. Afterwards samples were vortexed for about 20 s and 5 mm^3 were injected onto a silica column. A total of 44 MRMs was determined.

Carnitine, propionyl-, butyryl-, heptanoyl-, lauroyl-, myristoyl-, and palmitoylcarnitine were determined with calibration standards in 20% methanol from 0.25–25 $\mu g/cm^3$. Std2 was injected within analytical sequences to prove system stability. All other substances were evaluated with the calibration curve of the most similar substance (with regard to hydrocarbon chain length). Such were ethyl-, butenyl-, pentanoyl-, hexanoyl-, octanoyl-, octenoyl-, decanoyl-, decenoyl-, dodecenoyl-, dicarb11 = hydroxy-dodecenoyl-, C14-1, dicarb137 = hydroxy-myristyl-, C16-1, dicarb15 = hydroxy-palmitoyl-, C18, C18-1, C18-2, dicarb17 = C18-OH, dicarb2 = C3-OH, dicarb3 = C4-OH, dicarb4 = C5-OH, dicarb5 = C6-OH, dicarb6 = C7-OH, dicarb7 = C8-OH, dicarb8 = C9-OH, dicarb9 = C10-OH, dicarb10 = C11-OH, dicarb12 = C13-OH, dicarb14 = C15-OH, dicarb16 = C17-OH, dicarb18 = C19-OH, glutaric-O-methyl. The daughter ion used for all 44 transitions was m/z 85.1. Dwell time was 30 ms each.

Chromatographic Conditions

Mobile phase A: 20 m*M TFA* in water; mobile phase B: 20 m*M TFA* in methanol; 0.0–2.3 min isocratic at 75% B; flow: 0.5 cm^3/min.

Detection Parameters

Detection mode: MRM; operational mode: ESI in positive ion mode; ionisation voltage: +5.0 kV; temperature: 540°C; nebulizer gas: flow = 13 device units; curtain gas: flow = 13 device units; collision gas: flow = 4 device units; mass spec. resolution: unit → unit.

GC Method

Dowex 1 (1 g) was mixed with 5 cm^3 of dist. water. After 15 min the resin was quantitatively transferred to a commercial 1 g plastic column. Three different specimens were divided in two portions. Total carnitine and unesterified carnitine were determined before the chromatographic step and from the combined eluents. In a first trial 3 cm^3 portions were passed through a column of 1 g Dowex 1×8 (chloride form) and washed with 3 cm^3 of dist. water. In a second trial 3 cm^3 portions of urine were first acidified with 150 mm^3 1 *M* $HClO_4$ to give a final concentration of 50 m*M* $HClO_4$ and passed through 1 g of Dowex 1×8 (chloride form, equilibrated with 50 m*M* $HClO_4$), and washed with 3 cm^3 of 50 m*M* $HClO_4$.

Separation and Accumulation of Short Chain Acylcarnitine Esters

Dowex 50 (1 g in the pyridinium-form) was mixed with 5 cm^3 of water and after 15 min the resin was transferred to a commercial 1 g plastic column. Urine (1–3 cm^3) were acidified with 1 *M* $HClO_4$ to a final concentration of 50 m*M* $HClO_4$ and precipitates, if any, were removed by centrifugation.

To the acidified urine, 200 nmol of crotonyl- and heptanoylcarnitine were added as internal standards. The resulting solution was passed through a column with 1 g of Dowex 50 W $\times$ 8 (pyridinium-form). After adsorption of acylcarnitines on the resin, anions, mostly amino acids and urea, were eluted from the column by consecutive washings with 6 cm^3 of 0.012 *M* HCl and 6 cm^3 of water. Elution of the acylcarnitines was achieved with 6 cm^3 of 2 *M* NH_3; this eluate was collected in a screw cap tube. To the eluate 100 mm^3 of 0.6 *M* KOH was added to ensure complete hydrolysis of acylcarnitines, to avoid formation of amides, and to obtain the corresponding short chain fatty acids in the ionic form. Saponification was found to be complete after 45 min at 70°C.

The saponified eluate was vacuum dried in the screw cap tube. The dry residue can be stored for up to several weeks at −18°C without deterioration. To liberate the corresponding free fatty acids from the acylcarnitines, 100 mm^3 of ice-cool bidistilled water and 120 mg of dry Dowex 50 W $\times$ 8 (hydrogen-form) were added to the residue. This step also provides a further purification. After addition of the resin, 150 mm^3 of bidistilled water were added and the slurry mixed vigorously for 150 s on a Vortex mixer and cooled for 60 s in ice-water. Then the slurry was mixed for 150 s and the supernatant transferred to a capped sample vial and 2–4 mm^3 were used for gas chromatographic analysis.

Retention Behaviour of L-Carnitine and Acid Soluble L-Carnitine Esters on Ion-Exchangers

The question arose, if short-chain acylcarnitines in human urine were unspecifically adsorbed to the resin matrix. Dowex 1×8 in the chloride form has the same matrix as Dowex 50 W $\times$ 8. Dowex 1×8 does not exchange chloride-ions against weakly dissociated carboxylic functions of organic acids. Thus any adsorption would be based on unpolar interactions. The sensitivity is in accordance with the data obtainable *via* radioenzymatic assays. Consequently interaction of short chain acylcarnitines and resin-matrix seems to be negligible.

To determine the behaviour of medium-chain acylcarnitines, distribution coefficients were determined on Dowex 1×8 (chloride form) for acetyl- and octanoylcarnitine using the column method and acidic, neutral, and alkaline eluents. Only with octanoylcarnitine a slight hydrophobic adsorption was determined to the resin matrix in acidic media. This interaction was diminished by addition of methanol (35% v/v) to the eluent (data not shown.)

In screwcap-tubes 100 mm^3 of 0.6 *M* KOH and 100 mm^3 of crotonic acid standard were added and vacuum dried. The residue was dissolved in 100 mm^3 of chilled water and 120 mg of dry Dowex 50 W $\times$ 8 in the hydrogen-form were added. 2–4 mm^3 were used for the gas chromatographic determination.

Gas–Liquid Chromatography

A 30 m (0.25 mm i.d.) fused silica capillary column with chemically bonded FFAP (0.25 μm coating thickness) was used for all analyses. Supplier hydrogen was used as the carrier gas at 0.4 bar (6–8 cm^3/min flow rate) and nitrogen as the make-up gas. The injector temperature was kept at 250°C. The oven temperature was programmed to increase from 100 to 240°C, at a rate of 8°C/min. The quantitation was based on the relative peak area to internal standard area. The amount of metabolites was calculated as the weight equivalent to that of internal standard on the basis of area ratio.

Statistics

All data are presented as means ± SE. A nonparametric ranking sum test was used to detect significant differences between unpaired (*Mann-Whitney*) and paired (*Wilcoxon*) followed by *Dunnets t*-test for multiple comparison [29].

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