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Carnitine in Pregnancy

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Summary. By the 12th week of gestation, mean whole blood and plasma carnitine levels are already significantly ($p < 0.01$) lower than those of controls, with a further significant ($p < 0.01$) decrease up to parturition. Diminished carnitine levels may cause a downregulation of carnitine palmitoyltransferase1 (CPT1), both the liver isoform (CPT1A) and muscle isoform (CPT1B), carnitine palmitoyltransferase2 (CPT2), and carnitine acetyltransferase (CRAT) in white blood cells of pregnant women, as determined by real time PCR using the LightCyclerSYBR Green technology.

L-Carnitine-*L*-tartrate supplementation of 2 g/d resulted in an up to 10-fold increase of the relative mRNA abundances of CPT1B, CPT2, and OCTN2 and a 5-fold increase of CPT1A, and CRAT.

There is a relationship between the relative mRNA levels of CPT1A and CPT1B and the FFA plasma levels. The substitution of 2 g *L*-carnitine-*L*-tartrate/d resulted in significant ($p < 0.001$) lower FFA levels compared to untreated controls and the groups substituted with 0.5 and 1 g *L*-carnitine/d although plasma carnitine levels were not significantly increased. The most substantial effect was the reduced portion of acylcarnitines on total carnitine in those women receiving 2 g *L*-carnitine-*L*-tartrate.

Carnitine substitution resulted in an enhanced excretion of both, free carnitine and acylcarnitines, whereas acetylcarnitine accounts for 50–65% of total acylcarnitines.

The results of the present study provide evidence that *L*-carnitine supplementation in pregnancy in sufficient doses avoids a striking increase of plasma FFAs, which are thought to be the main cause of insulin resistance and consequently gestational diabetes mellitus (GDM).

Keywords. White blood cells; *L*-Carnitine-*L*-tartrate-supplementation; Gene transcription; Carnitine deficiency; Free fatty acids.

Introduction

On delivery maternal plasma carnitine levels are decreased to about half of the concentrations seen in non-pregnant women [1–4]. Whole blood carnitine is divided

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between plasma and blood cells, representing two different pools which are influenced by different factors [5–10]. Erythrocyte levels of free carnitine are comparable to those of the plasma, whilst acylcarnitine is more concentrated intracellularly.

In a former study we showed that already in the 12th week of gestation the mean whole blood carnitine level in pregnant women is significantly ($p<0.01$) lower than those of non-pregnant controls. There is a further significant ($p<0.01$) decrease from the 12th gestational week up to parturition. This reduction of total carnitine is mainly caused by a significant decrease of free carnitine levels, since no marked changes of short chain acylcarnitine values are found throughout pregnancy [11].

Similar low levels of free carnitine are only found in patients with carnitine deficiency [5]. Systemic carnitine deficiency is characterized by progressive cardiomyopathy, progressive skeletal weakness, non-ketonic hyperglycaemia and hyperammonaemia and has also been linked to sudden infant death syndrome [12]. *In vitro* studies showed that low carnitine levels (fetal serum or dialyzed serum instead of calve serum) in the cell culture resulted in a diminished expression of carnitine acyltransferases [13]. To address the potential role of carnitine deficiency in the known pregnancy induced metabolic changes, in particular high levels of free fatty acids (FFA), we determined the relative mRNA abundances of carnitine acyltransferases and of the carnitine transporter OCTN2 in the WBC. 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 pregnancies. In athletes we showed that gene expressions were altered in both muscle cells and in WBC, and a positive correlation with one another was shown for CPT1B, CRAT, and OCTN2 [14].

In addition high levels of FFA are an important cause of insulin resistance. The incidence of pregnancies complicated by diabetes has increased, and GDM and type II DM comprised up to 88 and 8%, respectively, of all pregnancies with diabetes [15]. Furthermore maternal hyperglycemia, the most common cause of fetal hyperinsulinemia, has been shown to be associated with adverse fetal outcomes in pregnancies complicated by gestational diabetes mellitus (GDM) [16–21]. Pregnancies in diabetic women are associated with increased risk of spontaneous abortion, congenital malformations, preeclampsia, preterm labor, and macrosomia.

The aim of the present study was to evaluate the effects of carnitine substitution in pregnant women on the expression of carnitine acyltransferases, in particular of CPT1, in order to avoid the striking increase of plasma FFA in pregnancy, the main cause of insulin resistance.

Results

Results from semiquantitative RT-PCR indicate a downregulation of mRNA expression of CPT1A, CPT1B, and CPT-2 in peripheral blood mononuclear cells of pregnant women in relation to β-actin which was used as a standard gene. After *L*-carnitine-*L*-tartrate supplementation the relative mRNA levels rose to levels which were equal to β-actin as confirmed by densitometric evaluation (Fig. 1).

As shown in Fig. 2, this was confirmed by real time quantitative PCR. *L*-Carnitine-*L*-tartrate supplementation of 2 g/d resulted in a 5–12-fold increase of

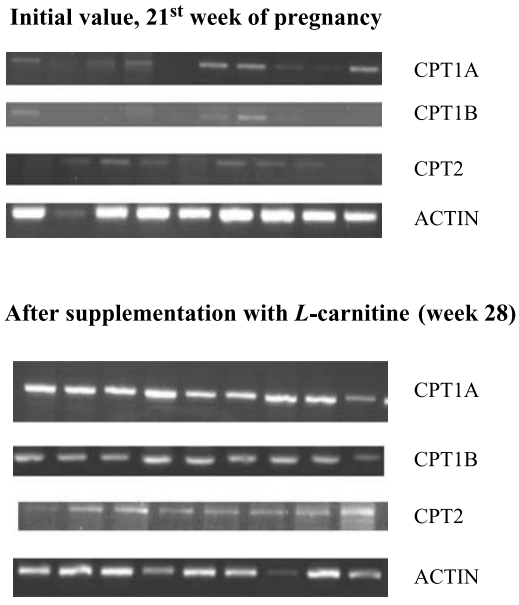


Fig. 1. Semiquantitative RT-PCR analysis of carnitine palmitoyltransferases CPT1A, CPT1B and CPT2; PCR products from block cycler PCR were analyzed on ethidium bromide stained 1.5% agarose gels (Values of 9 pregnant women); primers, PCR conditions, and lengths of amplimers are shown in Table 1

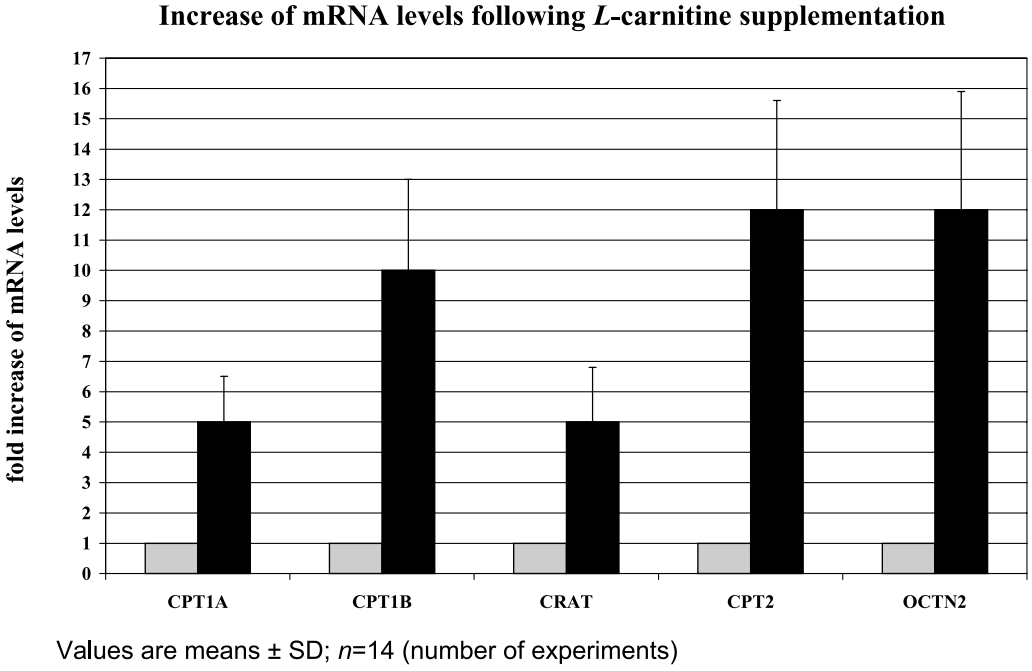


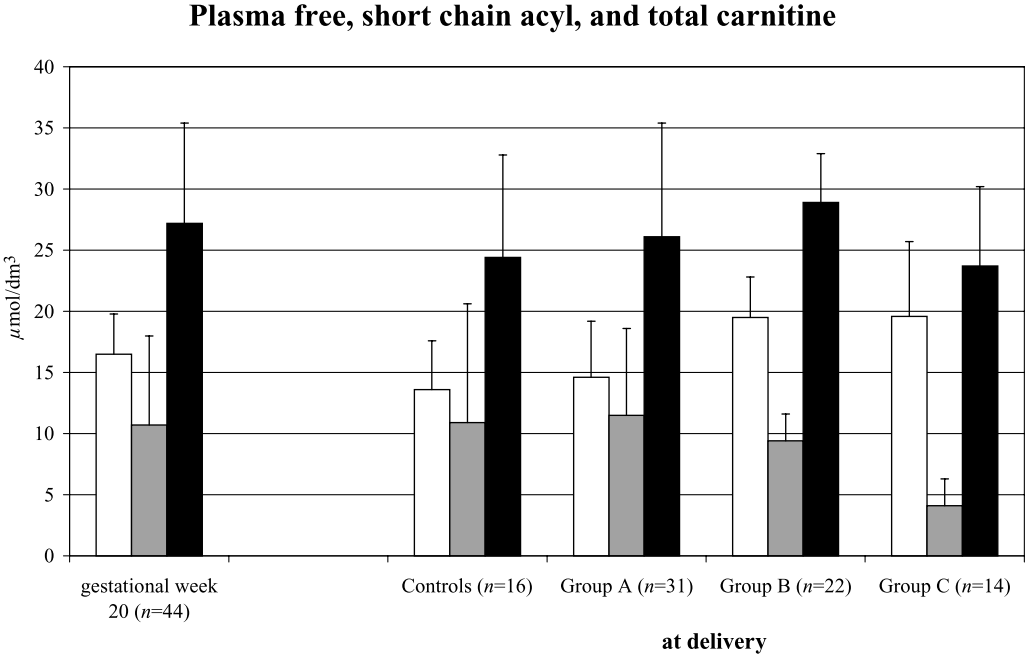
Fig. 2. *L*-Carnitine-*L*-tartrate substitution resulted in a dramatic increase of the relative mRNA abundances of CPT1A and CRAT (5-fold) as well as of CPT1B (10-fold), CPT2, and OCTN2 (12 fold); relative mRNA levels were determined using real time PCR and SYBRgreen detection as described in Materials and Methods; primers, PCR conditions, and lengths of amplimers are shown in Table 1

the relative mRNA levels of carnitine acyltransferases, thus reaching values which were found in non-pregnant healthy adults as previously published [26]. A 10-fold increase of the relative mRNA level for the carnitine transporter OCTN2 led to a relative mRNA level which was 2-fold higher than the mRNA level in non-pregnant healthy adults.

Compared to the 20th gestational week, carnitine substitution resulted in a moderate increase of plasma free carnitine but not of total carnitine. The most substantial effect was the reduced portion of acylcarnitines on total carnitine in those women receiving 2 g *L*-carnitine-*L*-tartrate/d (Fig. 3).

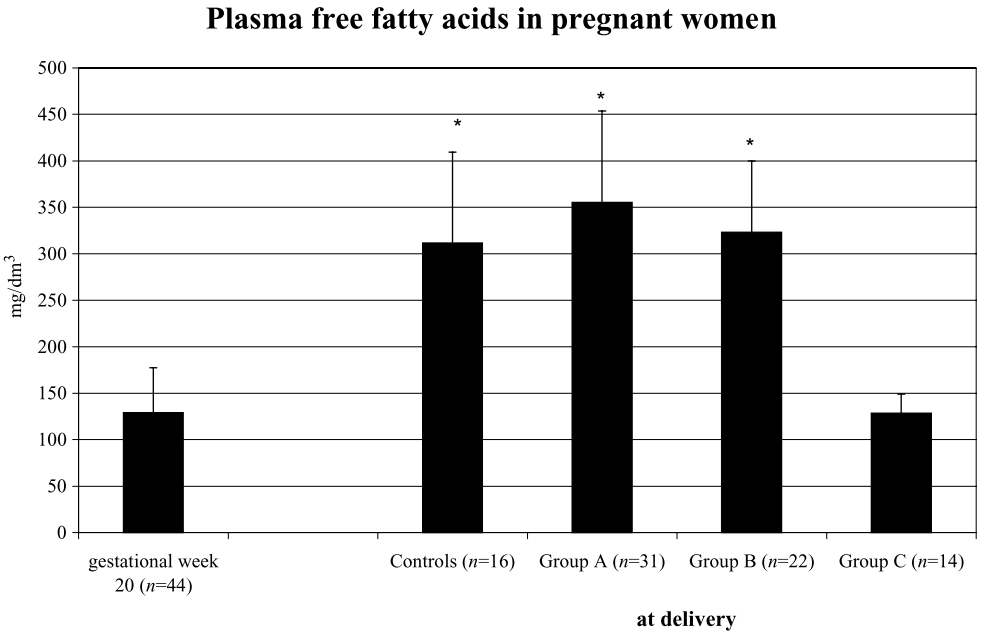
Figure 4 shows that plasma FFA levels increase significantly ($p < 0.001$) during pregnancy up to parturition. But only a dose of at least 2 g *L*-carnitine-*L*-tartrate/d resulted in FFA levels which were significantly ($p < 0.001$) lower than those of pregnant controls and the groups supplemented with 0.5 and 1 g *L*-carnitine/d and comparable to those of the 20th gestational week.

Acetylcarnitine accounts for 50–65% of total acylcarnitines excreted in urine. This portion tended to decrease and the portion of other short chain acylcarnitines tended to increase during the next weeks of gestation (Fig. 5).



Values are means ± SD; n: number of experiments; 20th gestational week initial value; Controls; Group A: supplementation with 0.5 g *L*-carnitine/day; Group B: supplementation with 1 g *L*-carnitine/day; Group C: supplementation with 2 g *L*-carnitine-*L*-tartrate/day; □ plasma free carnitine; ▩ plasma short chain acyl carnitine; ■ plasma total carnitine

Fig. 3. Plasma carnitine and acylcarnitine levels at the 20th gestational week in comparison to those of unsupplemented (controls) and *L*-carnitine supplemented pregnant women at delivery



Values are means ± SD; n: number of experiments; * Significantly higher (p<0.001) compared with the 20th gestational week and group C; 20th gestational week initial value; Controls; Group A: supplementation with 0.5 g *L*-carnitine/day; Group B: supplementation with 1 g *L*-carnitine/day; Group C: supplementation with 2 g *L*-carnitine-*L*-tartrate/day

Fig. 4. Plasma free fatty acids in pregnant women at the 20th gestational week compared to unsupplemented (controls) and supplemented pregnant women at delivery

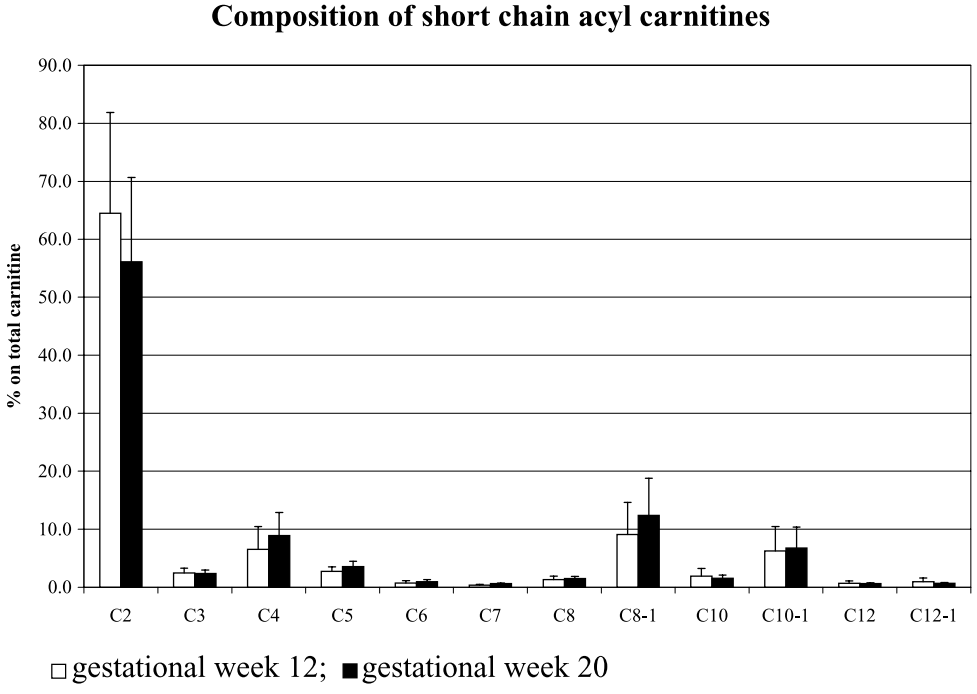


Fig. 5. Composition of excreted acylcarnitines in the 12th and 20th gestational week

Discussion

Pregnancy causes the reduction of plasma total and free carnitine levels, comparable to those found in known carnitine deficiency [11]. The primary purpose of the present study was to test the hypothesis that the diminished levels of free carnitine trigger a coordinate adaptive response in the transcriptional regulation of genes with critical roles in oxidative substrate metabolism. The results of the present study demonstrate that pregnancy induces a reduction in the steady state levels of mRNA of a number of metabolically related genes in WBC particularly those involved in FA metabolism and carnitine substitution restored the relative mRNA abundances of these genes.

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 [27–29]. Furthermore using serum of pregnant women in cell culture media, a reduced expression of CPT1 and CRAT in human-, mouse-, and rat fibroblasts has been observed and addition of carnitine increased the mRNA levels in a dose dependent manner [13, 30].

This is in accordance with animal experiments, where carnitine deficiency changes gene expression and metabolic regulation in liver, heart, and kidney. Juvenile visceral steatosis (JVS) mice were shown to have a marked decrease of carnitine levels in serum, liver, and muscle [31, 32]. The metabolic defect in JVS mice is due to impairment of the renal carnitine transport system due to a mutation of the OCTN2 gene [33]. Severe lipid accumulation, mitochondrial abnormalities in liver and muscle, hypoglycemia, hyperammonemia, and growth retardation have been reported. The lipid accumulation is found within 5 days but the hypoglycemia, hyperammonemia, and growth retardation appears 3 weeks after birth [34, 35].

In JVC mice, the expression of a couple of genes was increased, *i.e.* two glycolytic enzymes, lactate dehydrogenase, and glyceraldehyde-3-phosphatate dehydrogenase and the proto-oncogenes, c-jun and c-fos, coding for nuclear proteins regulate cell growth and differentiation by controlling gene transcription through AP-1 enhancer element. On the other hand urea cycle enzymes, serine dehydratase, tyrosine aminotransferase, and albumin, are suppressed [36, 37]. The pattern of these enzyme expressions resembles rather the one observed in undifferentiated and/or de-differentiated hepatocytes and carnitine substitution restored the expression pattern [38, 39].

Furthermore a close relationship between CPT1 mRNA abundances, the activity of CPT1 and plasma FFA levels has been reported [40, 41] and it is generally accepted that high levels of FFA is the most important cause of insulin resistance [42]. Despite elevated FFA levels insulin resistance is characterized by a reduced ability to oxidize lipids and by accumulation of triglycerides in skeletal muscle. At some point in the genesis of gestational diabetes and also type 2 diabetes, the fat cell appears to become refractory to the antilipolytic effect of insulin such that plasma FFA levels begin to rise [43]. Importantly not only their concentrations become elevated after an overnight fast, but they fail to suppress appropriately after meals, even though postprandial insulin levels may still be in the high range [43]. Despite the link between elevated plasma FFA concentrations and the development of several pathological conditions the significant physiological role of FFA tends to be overlooked.

The hyperlipidemia can influence liver function in a variety of ways, particularly when the FFAs arise from expanded visceral fat stores from which they may reach the liver directly and at high concentration through the portal circulation [44]. In a setting of high insulin, which increases hepatic expression of sterol regulatory binding protein 1c (SREBP-1c) and consequently of ACC and other lipogenic enzymes [45] tissue malonyl-CoA levels will rise. FA esterification will be favoured over oxidation because CPT1 inhibition by malonyl-CoA content of the liver and VLDL secretion increases, contributing to the development of hypertriglyceridaemia [46].

The initial level of maternal hyperglycemia at presentation or diagnosis is associated with the risk of fetal congenital anomalies [16]. Although insulin therapy significantly reduces the risk of spontaneous abortion, drawbacks are associated with its use, *i.e.* several injections per day were often required to achieve euglycemia [47, 48].

L-Carnitine supplementation increases the amount of whole body glucose utilization, and an increase of both glucose uptake and glucose oxidation was reported [49, 50].

Sufficient carnitine levels are also important for oxidative energy production as well as fetal lung maturation and the development of the pulmonary surfactant system. Dietary supplementation 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 *NAD*H-cytochrome C reductase, succinate-cytochrome C reductase, and cytochrome C oxidase [51].

A low activity or inhibition of CPT1 in rat alveolar type II cells diminished the dipalmitoyl-phosphatitidylcholine (DPPC) content [52]. DPPC is functionally and quantitatively the most important constituent of the surfactant complex, and a deficiency on fetal lung surfactant is the primary cause of respiratory distress syndrome, the most severe complication of preterm infants. We have shown, that carnitine-treatment of pregnant rats resulted in a significant increase of the DPPC content of the premature fetal rat lungs [53, 54]. Moreover *Alesci et al.* [55] reported that carnitine mimic the effects of glucocorticoides on glucocorticoid receptor alpha. Glucocorticoides are used to induce fetal lung maturity in cases of preterm delivery [53, 54] but are contraindicated in diabetic pregnancies.

Taken together, pregnancy is characterized by low carnitine levels, resembles to those found in carnitine deficiency. Carnitine substitution in sufficient dosages was shown to prevent dramatic increase of FFA, possibly insulin resistance, and consequently GDM. In addition in cases of imminent premature delivery, *L*-carnitine supplementation stimulates fetal pulmonary surfactant production.

Methods

Abbreviations

ACC acetyl CoA carboxylase; 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.

Study Design

In the first trial 47 pregnant women in the 20th week of normal gestation were split into group A ($n = 31$) and controls ($n = 16$). Group A was supplemented with 0.5 g *L*-carnitine/d up to parturition.

In the second trial 22 pregnant women (group B) in the 20th week of normal gestation were supplemented with 1 g *L*-carnitine/d up to parturition.

The third trial included 14 pregnant women (group C) which were supplemented with 2 g *L*-carnitine-*L*-tartrate/d from the 20th week of normal gestation up to parturition.

Subjects were provided with L-CARNIPURE (Lonza, Basel) for a total of 2 g *L*-carnitine-*L*-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 [22, 23].

Blood Sampling, Isolation of PBMNC mRNA and RT-PCR

Venous blood samples were collected into plain evacuated tubes from a forearm vein with minimal stasis after approximately 10 min of rest in a sitting position.

Table 1. Primers and PCR conditions

PCR-Primer	Sequence	PCR-Product-Size (bp)	Annealing Temp (°C)/ Time (sec)	Extension Temp (°C)/ Time (sec)	Acquisition Temp (°C)/ Time (sec)
β-Actin	S: 5′-TgccATccTAAAAgccAc-3′ A: 5′-TcAAcTggTcTcAAgTcAgTg-3′	289	64/5	72/34	83/1
CPT1A	S: 5′-ccTTccAAcTcATTcAg-3′ A: 5′-ccAggATccTcTgcATcTg-3′	298	62/5	72/34	87/1
CPT1B	S: 5′-ggTgAAcAgcAAcTATTATgTc-3′ A: 5′-ATccTcTggAAgTgcATc-3′	348	62/6	72/34	87/1
CPT2	S: 5′-gggAAgggAAgggAgAcgAg-3′ A: 5′-ccAAgAcAcTgcgTcAggAc-3′	173	63/5	72/34	92/1
CRAT	S: 5′-gAAgcccTTcTccTT-3′ R: 5′-cTccccTAcAccTccTgAg-3′	175	64/5	72/34	91/1
mCPT	S: 5′-cccTcAcATgAcAgAAgAc-3′ A: 5′-cTccTgcATgAcAAAcTTc-3′	270	64/5	72/34	83/1
OCTN2	S: 5′-TccAAgTcAcAcAAggATg-3′ A: 5′-TcccTAgAggAAggTggTg-3′	246	62/5	72/34	86/1

After separation of plasma for *L*-carnitine determination, the mononuclear cells were enriched by density gradient centrifugation using Ficoll – Hypaque separation medium (density according to fraction index: 1.077) and washed several times in phosphate buffered saline (PBS). Aliquots of 1–10 million mononuclear blood cells were frozen in 4 *M GTC* (guanidine isothiocyanate) for preparation of mRNA and cDNA for subsequent RT-PCR.

PCR conditions in block cycler PCR were the same for all assays (1 min 95°C/1 min 55°C/1 min 72°C, 35 cycles) (Table 1). Assays were set up on a block cycler (Perkin-Elmer, Norwalk, CT). PCR products were analyzed on 2% agarose gels as shown in Fig. 1.

Determination of relative mRNA amount using LightCycler technology was carried out as described in detail elsewhere [14].

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 [24].

Radioenzymatic Carnitine Assay

Free and short-chain acylcarnitines were assayed by the radioenzymatic method of *Cederblad* and *Linstedt* [25], with two modifications: HEPES instead of *TRIS* buffer and *N*-ethylmaleimide instead of tetrathionate.

HPLC/tandem-MS assay determinations and the determinations of carnitine and acylcarnitines were carried out as described elsewhere [14].

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