Monatshefte für Chemie 136, 1349–1363 (2005)
DOI 10.1007/s00706-005-0336-5

Monatshefte für Chemie
Chemical Monthly
Printed in Austria

L-Carnitine Regulates mRNA Expression Levels of the Carnitine Acyltransferases – CPT1A, CPT2, and CRAT

Alzbeta Godárová[1], **Elke Litzlbauer**[1], **Sylvia Brunner**[1,#], **Alison C. Agu**[1,**], **Alfred Lohninger**[2], and **Reinhold Hofbauer**[1,*]

[1] Department of Medical Biochemistry, Division of Molecular Biology, Max F. Perutz Laboratories Vienna Biocenter, Medical University of Vienna, A-1030 Vienna, Austria
[2] Center of Physiology and Pathophysiology, Institute of Medical Chemistry, Medical University of Vienna, A-1030 Vienna, Austria

Received January 21, 2005; accepted February 3, 2005
Published online July 18, 2005 © Springer-Verlag 2005

Summary. In order to analyze the biological influence of carnitine on the transcriptional regulation of mitochondrial carnitine acetyltransferase (CRAT) and carnitine palmitoyltransferases (CPT1A and CPT2) in conditions simulating carnitine deficiency *in vivo*, we cultured mouse (rat) fibroblasts and human liver cells with dialysed serum (carnitine deficient). The transcript levels of all three genes were 2-3-fold down-regulated after cultivation with dialysed serum compared to mRNA values with non-dialysed serum. The down-regulation of transcripts was fully reverted by carnitine supplementation. CPT1A and CRAT mRNA levels reached peak values 8 and 24 h after carnitine addition, respectively, and declined thereafter. The mRNA levels of CPT2 were induced more than five-fold after 48 h. Carnitine-induced accumulation of the carnitine acyltransferases was not caused by the alteration of mRNAs' stability by carnitine. The levels of CPT1A and CRAT steady state transcripts decreased also during cultivation in fetal calf serum, having a lower carnitine content than calf serum. During the cell cycle, carnitine acyltransferases seem to be regulated in a similar manner. On the other hand, the patterns of regulation after serum stimulus are different and appeared to be dependent on the presence or absence of carnitine in non-dialysed or dialysed serum, respectively.

Keywords. Carnitine acyltransferases; *L*-Carnitine supplementation; Dialysis; Gene expression analysis; Carnitine palmitoyltransferase 1 (CPT1); Carnitine palmitoyltransferase 2 (CPT2); Carnitine acetyltransferase (CRAT); Steady state transcript levels; Real-time PCR; mRNA stability measurements.

* Corresponding author. E-mail: reinhold.hofbauer@univie.ac.at
# Present address: Affiris GmbH, Vienna Biocenter, A-1030 Vienna, Austria
** Present address: Research Institute of Virology and Biomedicine, Vienna University of Veterinary Sciences, A-1210 Vienna, Austria

Introduction

In mammalian cells, the carnitine shuttle is utilized among others for entry of long-chain fatty acids into the mitochondrial matrix, where they undergo β-oxidation or are introduced for anabolic purposes, for excretion of excess acyl moieties from mitochondria, and for detoxification [1]. Since, in healthy tissues, carnitine concentrations are not found in excess, but rather at set levels appropriate for optimal substrate metabolism [2], the impaired fatty acid transport into mitochondria caused by carnitine deficiency may lead to the cytosolic accumulation of lipid intermediates. These intermediates have been shown to inhibit a number of key enzymes [3]. In addition, inhibition of the mitochondrial oxidation of long-chain fatty acids during fasting may cause heart or liver failure [4]. Prolonged inhibition of fatty acid oxidation has also been reported to impair insulin-mediated glucose disposal in rats [5]. Carnitine deficiency contributing to these effects may arise from a genetic disorder or can be acquired as the result of liver disease, renal disease including chronic haemodialysis, premature birth, dietary insufficiency, pregnancy, and certain types of tumours. Tissue and plasma carnitine levels are also affected by *Reye*'s syndrome, *diabetes mellitus*, infections, and heart failure [3]. Secondary carnitine deficiency may also arise as the result of drug therapy such as pivaloyl antibiotics, valproate [6], or zidovudine [7].

Supplemental administration of carnitine has been shown to be effective in some cases of both systemic carnitine deficiency [8] and secondary carnitine deficiency of different origin [9–11]. Also the alterations of carnitine system caused by several xenobiotics can be reverted or minimized by carnitine administration [6, 7, 12].

The acylation state of the mobile carnitine pool is linked to the limited and compartmentalized CoA pools by the action of the carnitine acyltransferase family. In mitochondria, there are three different carnitine acyltransferases – carnitine palmitoyltransferase 1 (CPT1), carnitine palmitoyltransferase 2 (CPT2), and carnitine acetyltransferase (CRAT). CPT1 (palmitoyl-CoA: *L*-carnitine *O*-palmitoyltransferase, EC 2.3.1.21) located within the outer membrane catalyses the initial reaction in the mitochondrial import of long-chain fatty acids. Carrier-mediated translocation of acylcarnitines across the inner membrane is followed by their reesterification to acyl-CoA, by inner-membrane CPT2. The rate of mitochondrial β-oxidation appears to be regulated mostly at the level of CPT1 [13]. However, experiments with administration of 3-thia fatty acids to rats indicated that the regulation of ketogenesis may under some conditions be shifted to step(s) beyond CPT1 [14, 15]. CPT2 might in addition be involved in the export of toxic long-chain acyl-CoA esters from the mitochondria by converting them into the corresponding carnitine esters [16]. The role of the third enzyme – CRAT (acetyl-CoA: *L*-carnitine *O*-acetyltransferase, EC 2.3.1.7) – consists in the regulation of glucose oxidation by removal of excessive acyl groups from mitochondria. By buffering fluctuations in the acetyl-CoA to free CoA ratio, carnitine may thus indirectly regulate the activity of pyruvate dehydrogenase *via* CRAT [3]. In addition, the mitochondrial CoA pool is limited and the depletion of free CoA would also inhibit CPT2 [13].

Low intracellular carnitine concentration may also lead to inhibition of CRAT through increased long-chain acyl-CoA [4]. In estrogen-deficient rats, CRAT

activity in liver and heart was positively correlated with total and free carnitine concentration [17]. Similarly, CRAT activity was reduced in the ischemic muscles where also the free carnitine content was reduced [18].

CPT1 and CPT2 are distinct entities encoded by different genes. CPT1 exists in three isoforms, the liver (CPT1A), muscle (CPT1B), and brain (CPT1C) variants, which also arise from separate genes [19, 20]. CPT1A is expressed in liver, kidney, lung, brain, spleen, intestine, ovary, and pancreas [20, 21]. The CPT1A is also the major isoform in human fibroblasts [22]. Regulation of CPT1 takes place on both protein and mRNA levels. Despite the exhaustive evidence about the regulation of activity of CPT1 (mostly *via* malonyl-CoA), the studies about the regulation of CPT genes on expression level have increased recently [23–25]. The mRNA level of CPT1 and the corresponding enzyme activity are decreased by insulin [26], and increased *e.g.* by fasting [27], thyroid hormone [28], fatty acids, and cyclic *AMP* [29]. CPT2 mRNA is mostly expressed in heart, skeletal muscle, and gonadal tissues [21]. Its activity and mRNA abundance are moderately modified during the fed-starved transition in the rat liver [30]. CRAT mRNA has its highest expression levels in heart, testis, and kidney [31]. CRAT activity was decreased in hypothyroid suckling rats [32]. Treatment of rats with peroxisomal/mitochondrial proliferating agents (*e.g.* clofibrate) caused an increase in the mRNA and activity levels of both CPT1 and CPT2 [20], as well as the activity level and the rate of synthesis of CRAT [33].

Evidence has accumulated that carnitine itself might influence gene transcription [34, 35]. Our aim was to investigate the carnitine effect on transcription of mitochondrial carnitine acyltransferases – CRAT, CPT1, and CPT2. In particular, we wanted to determine the effect of carnitine supplementation in the cell culture under conditions of low and high carnitine levels on the transcription rate and/or mRNA turnover of these carnitine acyltransferases.

Results

CPT1, CRAT, and CPT2 are enzymes belonging to the carnitine system, which is indispensable for the acyl delivery for mitochondrial β-oxidation [38]. To establish the biological influence of carnitine on transcriptional regulation of these genes *in vitro*, mouse or rat fibroblasts were cultured with dialysed (carnitine deficient) serum with or without carnitine supplementation (Fig. 1).

All three transcripts were 2-3-fold down regulated after cultivation of mouse and rat fibroblasts in a medium containing dialysed serum in comparison to mRNA levels in fibroblasts grown with non-dialysed serum. The down-regulation of transcripts was fully reverted by carnitine at its peak effective concentration (80 μM). The highest concentration of carnitine (160 μM) used in this experiment was already out of the range of physiological relevance and did not cause any further increase of the transcription levels. FACS analysis did not reveal any differences in growth states between the cells cultivated with non-dialysed and with dialysed serum (data not shown). Since the action of carnitine reached a maximum between 40 and 80 μM, all subsequent mRNA measurements were carried out with 40 μM carnitine unless stated differently. Taking into account a lower carnitine content in FCS than in CS, it is a strong indication that the observed effect with dialysed

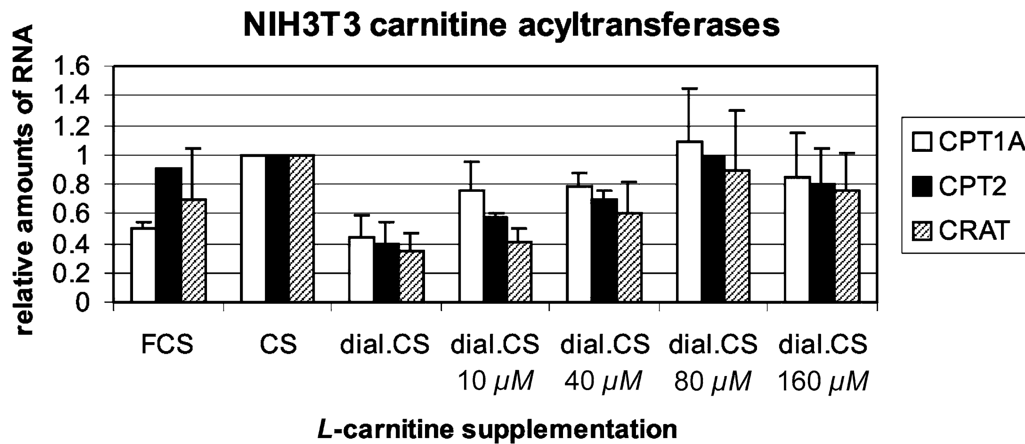

Fig. 1. Response of CPT1A, CRAT, and CPT2 mRNA levels to carnitine supplementation after cultivation of cells in dialysed calf serum (CS); NIH3T3 (or Rat-1) cells were cultivated in medium with non-dialysed 10% (v/v) CS, FCS, or dialysed 10% (v/v) CS for 48 h and subsequently incubated without or with *L*-carnitine for 4 h; the final concentrations of *L*-carnitine were 10, 40, 80, and 160 μM, respectively; northern blot analysis and quantification were performed as described in the experimental section; data shown are mean values of triplicate independent experiments (80 μM concentration was missing in one experiment); to allow direct comparison between different transcripts, data are expressed relative to the mRNA levels detected in cells cultivated in CS (1.0); error bars are standard deviations

serum is rather caused by dialysed out carnitine than by dialysed out low M_w growth factors. The transcript levels of CPT1A and CRAT were also decreased during cultivation in FCS (Fig. 1).

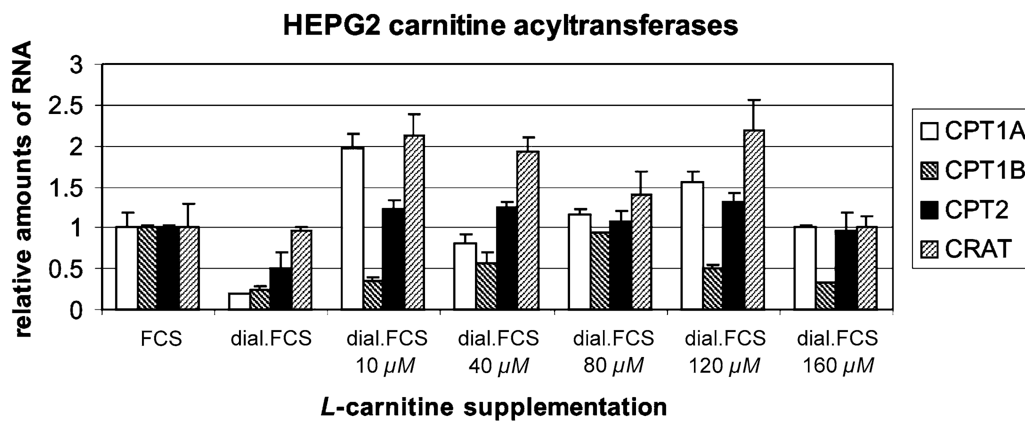

Fig. 2. Response of CPT1A, CPT1B, CPT2, and CRAT mRNA levels to carnitine supplementation after cultivation of cells in dialysed foetal calf serum (FCS); HEPG2 cells were cultivated in medium with non-dialysed 10% (v/v) FCS or dialysed 10% (v/v) FCS for 48 h and subsequently incubated without or with *L*-carnitine for 4 h; the final concentrations of *L*-carnitine were 10 μM, 40, 80, 120, and 160 μM, respectively; steady state level mRNA analysis and quantification by real-time RT-PCR were performed as described in the experimental section; data shown are mean values of triplicate independent experiments; to allow direct comparison between different transcripts, data are expressed relative to the mRNA levels detected in cells cultivated in FCS (1.0); error bars are standard deviations

In analogy to the experimental procedure administrated for Fig. 1, we determined the steady state mRNA levels of CRAT, CPT1A, CPT1B, and CPT2 in human liver cells (HEPG2) treated with non-dialysed foetal calf serum (FCS) and dialysed FCS with or without *L*-carnitine supplementation by real time RT-PCR (Fig. 2). The influence of the dialysis and supplementation on transcript levels of the carnitine acyltransferases in HEPG2 cells is very similar to the patterns observed in NIH3T3 and rat-1 fibroblasts shown in Fig. 1.

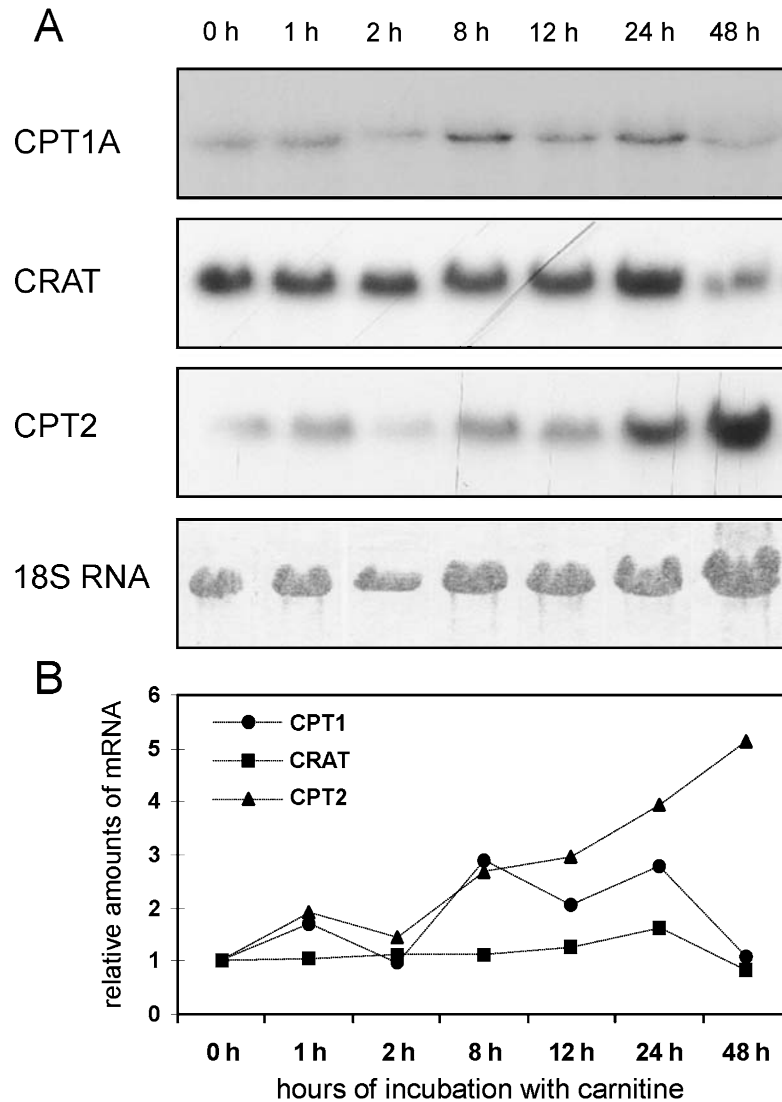

Fig. 3. Time dependent response of CPT1A, CRAT, and CPT2 mRNA levels to carnitine supplementation in NIH3T3 cells grown in dialysed serum; (A) Cytoplasmic RNA (10 μg/lane) was fractionated on a denaturing gel, transferred, and hybridized with random primed cDNAs; the cells were kept in culture medium containing dialysed 10% (v/v) CS and after 48 h *L*-carnitine was added (final concentration 40 μM); the cytoplasmic RNA was prepared from the cells maintained in the presence of *L*-carnitine for 0, 1, 2, 8, 12, 24, and 48 h; (B) The diagram shows the amounts of CPT1A, CRAT, and CPT2 mRNA normalized to 18S rRNA and relative to mRNA values at time point 0 h (1.0)

The time dependence of the effect of carnitine on CPT1A, CRAT, and CPT2 mRNA levels was delineated by incubation with carnitine for 0, 1, 2, 8, 12, 24, and 48 h. As shown in Fig. 3, the CPT1A mRNA amount increased threefold after 8 h, declined after 24 h, and after 48 h reached the level in carnitine-untreated cells. A similar decrease of induced CPT1A transcript levels has been reported for stimulation by palmitate [39] or 3-thiadicarboxylic acid [40].

CRAT mRNA levels reached its peak value after 24 h, although in the previous experiments (Figs. 1 and 2) the mRNA levels were enhanced with twice the concentration of carnitine (80 μM) already after 4 h incubation. The mRNA levels of CPT2 exhibited an interesting pattern with more than a five-fold induction after 48 h, at the time point when the other two carnitine acyltransferases transcripts already declined.

Carnitine induced accumulation of CRAT, CPT1, and CPT2 might result from alterations in transcription rate and/or mRNA turnover. To assess whether carnitine modifies mRNA stability of the transcripts, an experiment with transcriptional inhibitor DRB was carried out.

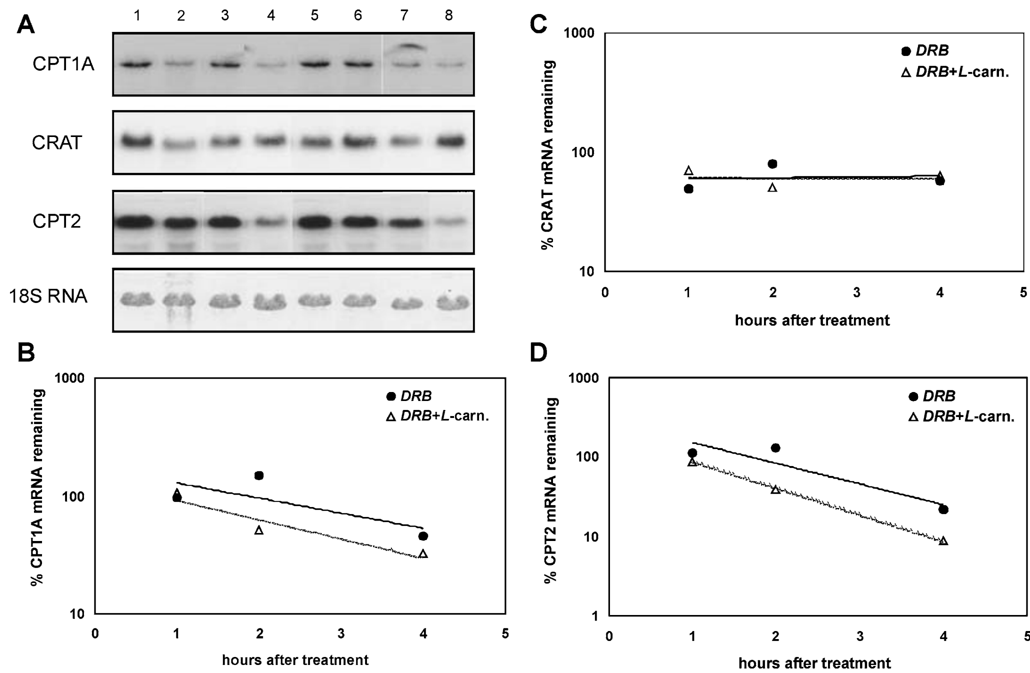

Fig. 4. Effect of carnitine on CPT1A, CRAT, and CPT2 mRNA stability; (A) Cytoplasmic RNA (10 μg/lane) was isolated from NIH3T3 mouse fibroblasts; the cells were grown in medium with dialysed 10% (v/v) CS for 48 h followed by 4 h with (lanes 5–8) or without (lanes 1–4) addition of *L*-carnitine (final concentration 40 μM); afterwards, *DRB* was added (final concentration 30 $\mu g/cm^3$) and cells were harvested after incubation for 0 h (lanes 1, 5), 1 h (lanes 2, 6), 2 h (lanes 3, 7), and 4 h (lanes 4, 8); isolated cytoplasmic RNAs were probed with random-primed cDNAs; hybridization signals for mRNAs relative to 18S rRNA contents were converted to a percentage of the initial (0 h) values and logarithmically transformed; semilogarithmic plots show the decay of CPT1A mRNA (B), CRAT mRNA (C), and CPT2 mRNA (D) in the presence (△) or absence (●) of *L*-carnitine

The results in Fig. 4 clearly show that mRNA stability of any transcript was not increased by carnitine; rather it was decreased (CPT1A, CPT2). Thus, transcriptional activation of the genes appears to account rather for mRNA induction after *L*-carnitine addition and not for a stabilization of the transcripts.

The measured half-life of the CPT1A (2.8 h and 3.2 h with or without carnitine, respectively) and CPT2 (2.2 h and 3.2 h with or without carnitine, respectively)

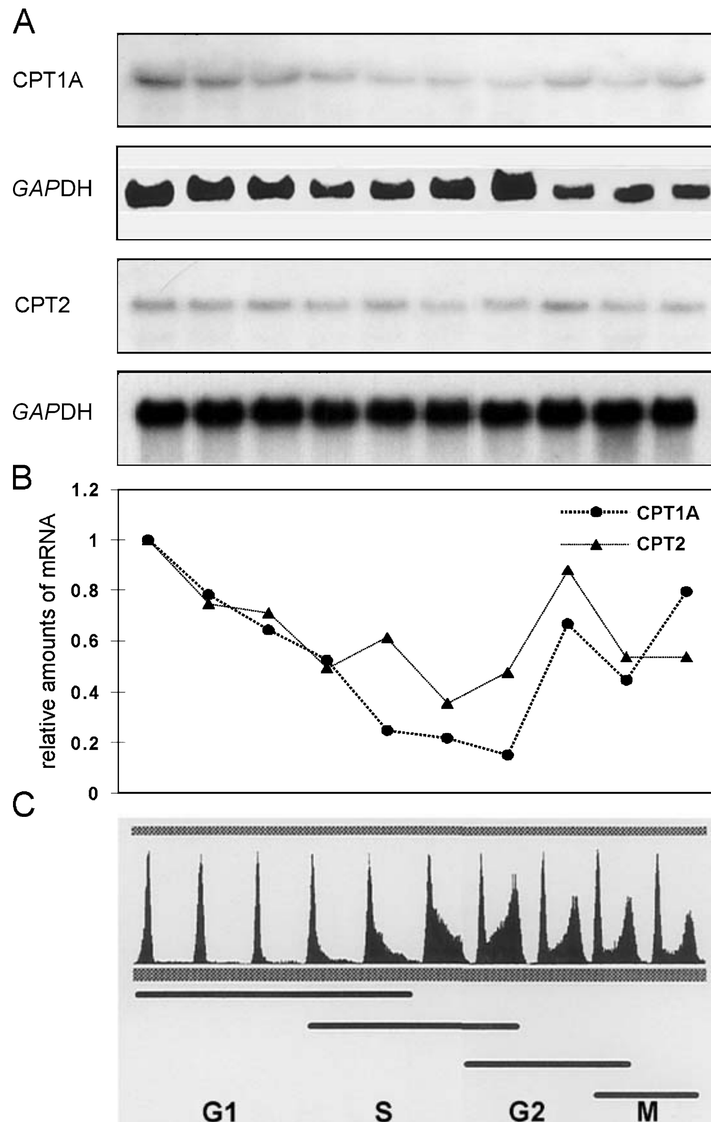

Fig. 5. Northern blot analysis of CPT1A and CPT2 mRNA levels as a function of cell cycle; (A) Cytoplasmic RNA isolated from NIH3T3 cells at different stages of the cell cycle separated by centrifugal elutriation, was fractionated on a denaturing gel, transferred, and hybridised with random-primed cDNAs; *GAP*DH was used as a reference; (B) The different amounts of CPT1A and CPT2 mRNA induction were determined by a densitometric scan and were compared with the first elutriation fraction whose mRNA contents were given relative values of 1.0; (C) A fluorescence-activated cell sorting analysis of the elutriated fractions is shown underneath each lane

transcripts were short for genes encoding metabolic enzymes. In agreement with these results, the half-life of the CPT1A transcript has been shown to be about 3 h [39] and the half-life of CPT2 mRNA 139 ± 17 min [29]. In contrast to the rapid decay of CPT1A and CPT2 mRNA, CRAT mRNA apparently remained at a constant level and therefore seems to belong to 'long-lived mRNAs'.

We have previously demonstrated, that CRAT mRNA is regulated through the cell cycle showing a biphasic pattern with a more than 2-fold induction in G1 and G2 over the level in S-phase [31]. CPT1A and CPT2 transcripts determined from cells sorted into cell-cycle fractions by centrifugal elutriation demonstrated similar behaviour (Fig. 5), although the decrease from G1 to S-phase is, especially in the

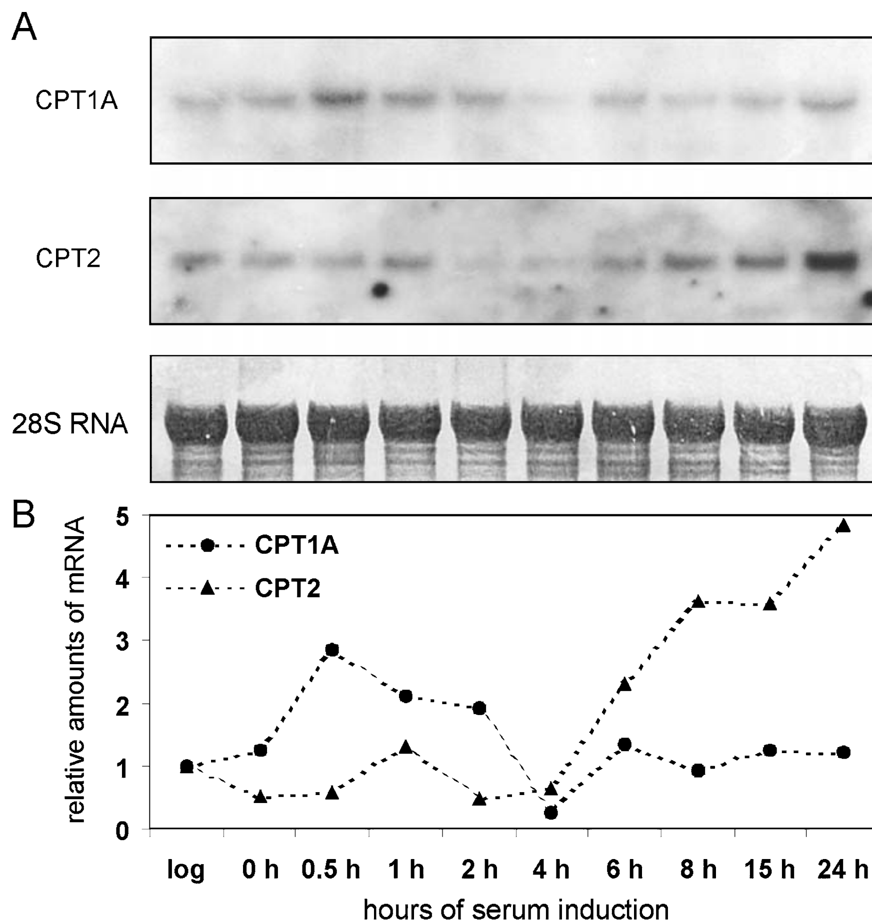

Fig. 6. Northern blot analysis of CPT1A and CPT2 mRNA levels in exponentially growing *versus* starved and serum induced cells; cytoplasmic RNA (10 μg/lane) was fractionated on a denaturing gel, transferred, and hybridised with random-primed cDNAs; the cells were synchronized by serum deprivation for 72 h in a medium containing 0.2% (v/v) FCS; cytoplasmic RNA was prepared from cells that were stimulated with medium containing 20% (v/v) FCS for 0, 0.5, 1, 2, 4, 6, 8, 15, and 24 h; on the lane labelled 'log' cytoplasmic RNA from exponentially growing cells was loaded; the induction behaviour of the CPT1A and CPT2 mRNA in NIH3T3 cells with 28S rRNA control is presented in panel (A); panel (B) shows the amount of mRNA relative to 28S rRNA and after comparison with mRNA values detected in exponentially growing cells (1.0)

case of CPT1A, more pronounced (3–4-fold) and G2-phase levels do not reach the levels in G1-phase. There is also a tendency of CPT2 mRNA to decline after a peak in G2-phase.

In Fig. 6 a complete growth cycle in NIH3T3 cells after mitogenic stimulus is presented. In case of CPT1A, the mRNA increase reaches a maximum already half an hour after growth induction and cells with lowest transcript levels can be measured at the time-point of 4 h. After this minimum, CPT1A mRNA started to rise and remained at constant levels detectable in exponentially growing cells. CPT2 mRNA content shows a tendency to decrease during the first 4 h followed by a steady increase reaching 5-fold transcript peak value 24 h after serum induction.

To determine whether lower carnitine levels caused by dialysis influence serum induction of CPT1A, CRAT, and CPT2 genes, we tested the effect of dialysed serum used for both starvation and serum induction (Fig. 7).

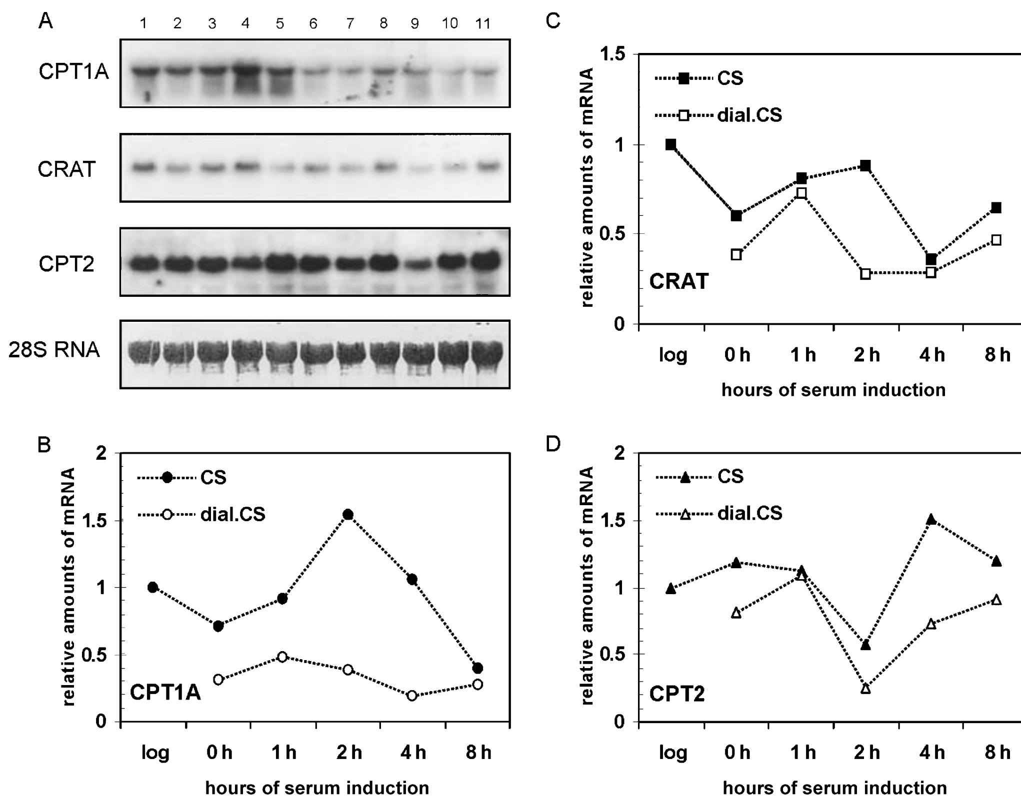

Fig. 7. Effect of dialysed serum on CPT1A, CRAT, and CPT2 mRNA levels during starvation and serum induction; (A) The cells (NIH3T3) for analysis were synchronized by serum deprivation for 48 h in a medium containing 0.2% (v/v) dialysed or non-dialysed CS; the cytoplasmic RNA was prepared from cells that were stimulated with medium containing 10% (v/v) dialysed (lanes 7–11) or non-dialysed (lanes 2–6) CS for 0, 1, 2, 4, and 8 h, respectively; the cells for control were grown in 10% CS (lane 1); RNA was fractionated on a denaturing gel, transferred, and hybridized with random-primed cDNAs; the different amounts of CPT1A mRNA (B), CRAT mRNA (C), and CPT2 mRNA (D) induction determined by a densitometric scan and normalized to 28S rRNA are shown relative to mRNA levels in exponentially growing cells (lane labeled 'log')

For CRAT and CPT2, the stimulation is approximately the same with both sera used. The difference between dialysed and non-dialysed serum is apparent in case of CPT1A. This coincides with the generally admitted fact, that the oxidation of long chain fatty acids is regulated at the level of CPT1A [41], being thus more sensitive to the lack of carnitine. The small differences in induction pattern between the experiments presented in Figs. 6 and 7 could be caused by slightly different conditions for starvation and serum induction. For the investigation shown in Fig. 6, cells were starved for 72 h with 0.2% (v/v) FCS and stimulated with 20% (v/v) FCS whereas 48 h starvation with 0.2% (v/v) dialysed or non-dialysed CS and stimulation with 10% (v/v) dialysed or non-dialysed CS were performed for Fig. 7. The different serum content used for the stimulation could influence the induction patterns since lower serum concentration (5% FCS) was mentioned to maintain endogenous CPT1A activity at low level in COS-M6 cells [42].

Discussion

The aim of the present work was to gain a better understanding of the effect of carnitine on the transcriptional regulation of CRAT, CPT1, and CPT2 expression. The following questions were addressed. (i) What are the conditions for carnitine effect on the gene expression of the three carnitine acyltransferases? (ii) Are the carnitine acyltransferases regulated during the cell cycle, respectively growth cycle after mitogenic stimulus? (iii) Does carnitine have any influence on the growth cycle regulation?

To answer the first question, we developed a cell culture model system, which allowed the exposure to very low carnitine concentrations followed by carnitine supplementation. Mouse NIH3T3 and Rat-1 fibroblasts as well as human HEPG2 liver cells were cultivated in a medium with dialysed serum. Subsequently, carnitine was added at concentrations of 10–80 μM (physiological range). Under these conditions, carnitine showed a quantitatively important (3-and 5-fold induction of CRAT and CPT2 mRNA, respectively) effect on gene transcription.

As expected from the fact that CPT1 is the control step of the channelling of fatty acyl-CoA toward β-oxidation, its response to carnitine supplementation was to some extent different to the response of the other two acyltransferases. Also from the recent gene phylogenic studies, CRAT and CPT2 genes are closer related to each other than to CPT1 [1]. Nevertheless, the results of carnitine supplementation experiment in newborn cardiac myocytes [43] indicated, that carnitine might both increase mitochondrial uptake by CPT1 and decrease acetyl-CoA by CRAT. This outcome clearly means, that carnitine is able to stimulate both CPT1 and CRAT, as we observed in our experiments. Similarly, there is a major increase in the concentration of acetylcarnitine (caused by CRAT) in the liver under conditions that are characterized by high rates of fatty acid oxidation [44]. Under those conditions very likely carnitine palmitoyltransferase(s) are increased as well.

The absence of the induction effect of carnitine supplementation during cultivation of the cells in non-dialysed CS (data not shown), where the basic carnitine content is physiologically normal ($\leq 80\,\mu M$), suggests that the positive effect of carnitine supplementation refers to the range of its physiological concentration. Indeed, it has been reported that increasing carnitine concentration above the

cytosolic carnitine content had no further effect on fatty acid oxidation in isolated rat skeletal muscle mitochondria [3]. Also most of the anticipated metabolic effects of carnitine supplementation have not been observed in healthy persons [45].

The five-fold increase of CPT2 at the time-point of 48 h after addition of carnitine, where CPT1A and CRAT mRNA already decreased, is surprising. However, the mRNA levels for CPT2 were reported to increase up to two-fold by an acute treatment with etomoxir, which competitively inhibits CPT1A [46]. Also administration of 3-thia fatty acids to rats was followed by significantly increased level of CPT2 mRNA while CPT1A mRNA level remained unchanged [14, 15]. Thus, CPT1A and CPT2 mRNAs could probably be induced by different mechanisms.

Transcriptional activation of CRAT, CPT1, and CPT2 genes upon carnitine administration appears to fully account for their mRNA accumulation, since carnitine has not significantly changed their mRNA stability. The half-life of CPT1A mRNA was described to be increased by linoleate approximately 50% [29]. However, palmitate has been shown to have no effect on the half-life of the CPT1A transcript [39]. In the case of carnitine, we have not observed any prolongation of the CPT1A mRNA half-life, although it must be admitted that we only used 4 h incubation with carnitine compared to 48 h incubation in the experiment with linoleate [29]. The half-life of CPT2 mRNA was neither affected by linoleate [29] nor by carnitine in our experiment.

After cultivation with foetal calf serum, mRNAs encoding CPT1A and CRAT were lower than by cultivation with calf serum. CPT1A mRNA values were not decreased after cultivation with foetal calf serum. Interestingly, a similar situation was described *in vivo*, where mRNA levels of CPT1A were low in foetal liver and intestine and rose dramatically during the first day of extra-uterine life, whereas CPT2 mRNA levels were already high prior to birth and remained constant after birth [30, 47]. Also the activity of CRAT was reported to increase during development of chicken embryo [48]. The fact that CPT1A mRNA can be maintained at a high levels in rats weaned on to a high-fat diet, whereas it decreases when the rats are weaned on to a high-carbohydrate diet, suggests that postnatal changes in CPT1A level are dependent on hormonal and/or nutritional factors rather than on the precise stage of development [30]. Our results indicate that beside FFA and glucagon also carnitine is one of those factors, since glucagon, its synthesis and secretion is increased besides FFA [49], has been shown to increase the carnitine content of liver [50]. Carnitine very likely is also one of the milk components that in newborn rats apparently induce the CPT1 expression sooner and with a more pronounced effect [47].

It has been shown that groups of genes whose products act at different steps in a common process are co-ordinately regulated after serum stimulus [51]. This led us to the second goal of our work – to test, if the regulation of the group of carnitine acyltransferases is coordinately expressed during cell cycle or after serum stimulus. Indeed, CPT1, CPT2, as well as previously published CRAT [31] show a resembling pattern of the cell cycle regulation. On the other hand, the patterns of regulation after serum stimulus are different. CPT1 gene expression gaining a maximum half an hour after growth induction seems to belong to the early response genes as has been suggested elsewhere [39]. The observed delay of the

expression of CPT2 compared to CPT1A was also reported during the administration of 3-thia fatty acids to rats [40]. Interestingly, the time courses of the induction by carnitine are very similar to that brought about by transcriptional activation of both CPT1A and CPT2 gene expression in response to mitogenic stimulus. This is in accordance with a published suggestion, that distinct signals can elicit a rather generic transcriptional output [52]. Moreover, carnitine has been shown to act synergistically with hepatocyte growth factor [53]. This led us to the third question. Can the presence or absence of carnitine influence the natural response to CPT1 and CPT2 gene expression after serum stimulation? It was shown that addition of carnitine to low-serum incubation medium of murine hepatocytic cells enhanced cell growth [53]. Using non-dialysed (carnitine containing) and dialysed (lacking carnitine) serum for starvation and serum stimulation, we provided an evidence for the role of carnitine in response of gene expression of carnitine acyltransferases to serum stimulation. All three enzymes are involved in the uptake of activated long-chain fatty acids into mitochondria and their levels were lower during starvation in dialysed serum. In addition, the mRNA levels of CPT1, which represents the main control step in the access of fatty acyl-CoA for β-oxidation, showed nearly no induction by dialysed serum.

The stimulation of both CPT1 and CPT2 gene expression is in case of peroxisome proliferators mediated through peroxisome-proliferator-activated receptor (PPAR) [54, 55]. Although fatty acids have been reported to regulate production of CPT1B transcript also through activation of PPARα (δ) [56–58], the contribution of PPARα in mediating the effects of long-chain fatty acids on CPT1A and CPT2 levels has never been established [1, 59]. A direct modulatory effect of carnitine on the glucocorticoid receptor activity has been demonstrated [60]. Our recently performed promoter studies of the CRAT gene indicate a direct influence on the transcriptional activity of the CRAT promoter. But it is still difficult to hypothesize which mechanism and factors are specifically involved in gene regulation by carnitine. Nevertheless, the influence of carnitine on gene transcription proven also by this study is of interest for the better understanding and management of pathological cases associated with carnitine deficiency.

Experimental

Cell Culture

NIH3T3 mouse fibroblasts (ATCC CRL-1658), Rat-1 rat fibroblasts, and HEPG2 human liver cells (ATCC HB 8065) were grown in *Dulbecco*'s modified *Eagle*'s medium (DMEM) supplemented with 10% (v/v) calf serum (CS) or foetal calf serum (FCS) and antibiotics (30 mg/dm^3 penicillin, 50 mg/dm^3 streptomycin sulphate). For the experiments, cells were kept in DMEM containing 10% FCS, 10% CS, or dialysed 10% CS (dialysis was performed against PBS for 48 h with five buffer changes). Prior any experiment, cells were adjusted to culture conditions for 48 h and subsequently *L*-carnitine (LONZA) was added to obtain a final concentration of 40 μM (or cells were exposed to different concentrations of *L*-carnitine, as indicated in the experiment) in the culture medium. Cells were incubated with or without *L*-carnitine for additional 4 h (in a time course experiment for different periods of time) before lysis and RNA isolation was performed.

For the RNA stability measurement, cells were incubated for 4 h with *L*-carnitine and then the RNA polymerase II inhibitor 5,6-dichloro-1-β-*D*-ribofuranosyl benzimidazol (*DRB*) was added at a

concentration of 30 μg/cm^3 (time indicated as 0 h). After incubation for different periods of time, cytoplasmic RNA was isolated.

For starvation, cells were kept in DMEM containing 0.2% FCS (0.2% CS or dialysed 0.2% CS) for 72 h (48 h in the experiment with dialysed serum). Growth induction was performed by adding fresh DMEM containing 20% FCS (in the experiment with dialysed serum – 10% CS or 10% dialysed CS) for different periods of time as indicated by the time course protocols. The proliferative growth state of cell populations was assessed by a Partec PAS-2 cytofluorimeter measuring the DNA content. Centrifugal elutriation of exponentially growing NIH3T3 cells (3×10^8 cells) was performed using a Beckman elutriation equipment.

RNA Preparation, Northern Blot Analysis and Real Time PCR

To isolate fibroblasts' cytoplasmic RNA, 3×10^7 cells were washed twice with PBS, scraped off with a 'rubber policeman', resuspended in 200 mm^3 of ice-cold buffer A (10 m*M* Hepes, *pH* 7.9, 10 m*M* KCl, 0.1 m*M EDTA*, 0.1 m*M EGTA*, 2.5 m*M* dithiothreitol), and combined with 10 mm^3 of 12.5% Nonidet P-40. After gently mixing for 10 s, the suspension was centrifuged at 15 000 *g* at room temperature for 2 min. The supernatant was transferred into a tube containing 400 mm^3 of buffer B (7 *M* urea, 1% (*w*/*v*) SDS, 0.35 *M* NaCl, 10 m*M EDTA*) and 400 mm^3 of *PCI* (phenol/chloroform/isoamyl alcohol, 25:24:1, by vol.). The samples were mixed and stored at −20°C. After 30 min centrifugation at 4°C (15 000 *g*), the aqueous phase was precipitated with two volumes of ethanol. Following a washing step with 70% ethanol, the RNA was dissolved in diethyl pyrocarbonate (*DEPC*)-treated water.

The RNA (10 μg) was fractionated on denaturing 1% (*w*/*v*) agarose gels containing 1.5% (*v*/*v*) formaldehyde, transferred to GeneScreen nylon membranes (Du Pont-New England Nuclear) and immobilized to filters by UV-irradiation (UV Stratalinker, Stratagene). Afterwards, the membranes were stained with 0.04% methylene blue in 0.5 *M* sodium acetate, *pH* 5.2, for 10 min and subsequently partially destained with distilled water.

Rat CPT1A cDNA [34], murine CRAT cDNA [31], and rat CPT2 cDNA [35] were radiolabeled by random priming [36] and used as probes. The filters were hybridized overnight at 42°C in 50% (*v*/*v*) formamide, 5× SSPE, 0.1 *M* sodiumphosphate, *pH* 6.5, 5× *Denhardt*'s, 10 m*M EDTA*, *pH* 8.0, 1% (*v*/*v*) sarkosyl, 100 μg/cm^3 yeast-tRNA. The final wash was performed in 2× SSC/0.1% SDS at 65°C for 30 min.

Only the HEPG2 RNA was isolated by standard *GTC*-method [37]. For the isolation cells were washed with PBS and lysed directly in the culture dish (*GTC* lysis buffer including *GTC*-stock solution (Guanidium thiocyanate, H_2O, sodium citrate, *pH* 7, 10% *N*-lauryl sarcosinate) and β-mercaptoethanol), 1/10 volume of sodium acetate *pH* 4.5 was added and mixed, finally 1/3 volume phenol and 1/10 volume chloroform were added and vigorously mixed for 15 seconds. Subsequently the RNA was purified and isolated by a standard phenol chloroform extraction. Finally RNA was collected by centrifugation at 4°C/14000 rpm for 10 min. The RNA-pellet was washed with 70% ethanol and again centrifuged for 5 min at 10000 rpm, dried for 5 min in a vacuum dryer, dissolved in 100 mm^3 *DEPC* treated distilled water, incubated for 10 min at 65°C. Quantification was performed by exploiting the absorption properties of the RNA aromatic bases which absorb light at a $\lambda_{max} = 260$ and 280 nm as well as by a Molecular Dynamics Image Quant equipment. Signal and loading corrections were based either on 18S (or 28S) rRNA or on glyceraldehyde phosphate dehydrogenase (*GAP*DH) band intensities.

RNA (3 μg) was used for cDNA synthesis (based on the Revert Aid M-MuLV Reverse Transcriptase Protocol of Fermentas). These cDNAs were taken as a template for Light Cycler measurements (based on Light Cycler Fast Start DNA Master SyBr Green I protocol by Roche Diagnostics). Following human PCR primers and reaction conditions were used: β-Actin sense 5′-TGCCATCCTAAAAGCCAC-3′, antisense 5′-TCAACTGGTCTCAAGTCAGTG-3′, annealing temp. RT-PCR: 62°C, block-PCR: 58°C. CPT1A sense 5′-GAGAGGAGACAGACACCATC-3′, antisense 5′-ACTTGTCAAACCACCTGTC-3′, annealing temp. RT-PCR: 62°C, block PCR: 52°C. CPT1B

sense 5′-GGTGAACAGCAACTATTATGTC-3, antisense 5′-GGATCCTCTGGAACTGCATC-3′, annealing temp. RT-PCR: 67°C, block PCR: 61.8°C. CPT2 sense 5′-GTCACGGTGCAGAAAC-3′, antisense 5′-CTCTTTGTATAGACGGAGGC-3′, annealing temp. RT-PCR: 65°C, block PCR: 57°C. CRAT sense 5′-CCATCCGCTCGGCTTCCATGG-3′, antisense 5′-CTCCGCGCAGCTGTTGTAGGC-3′, annealing temp. RT-PCR: 65°C, block PCR: 59.5°C.

Acknowledgements

This work was supported by the OeNB of Austria (Jubiläumsfonds grant 9642) and the *Herzfelder*'sche Familienstiftung. We are very grateful to *Thomas Sauer* for expert technical assistance during FACS analysis and Lonza AG (Basel, Ch) for providing us with *L*-carnitine.

References

[1] Ramsay RR, Gandour RD, van der Leij FR (2001) Biochim Biophys Acta **1546**: 21
[2] Long CS, Haller RG, Foster DW, McGarry JD (1982) Neurology **32**: 663
[3] Paulson DJ (1998) Mol Cell Biochem **180**: 33
[4] Scholte HR, Rodrigues Pereira R, de Jonge PC, Luyt-Houwen IE, Hedwig M, Verduin M, Ross JD (1990) J Clin Chem Clin Biochem **28**: 351
[5] Dobbins RL, Szczepaniak LS, Bentley B, Esser V, Myhill J, McGarry JD (2001) Diabetes **50**: 123
[6] Arrigoni-Martelli E, Caso V (2001) Drugs Exp Clin Res **27**: 279
[7] De Simone C, Famularo G, Tzantzoglou S, Trinchieri V, Moretti S, Sorice F (1994) Aids **8**: 655
[8] Pons R, De Vivo DC (1995) J Child Neurol **2**: S8–24
[9] Scholte HR, Luyt-Houwen IE, Vaandrager-Verduin MH (1987) Basic Res Cardiol **1**: 63
[10] Touma EH, Rashed MS, Vianey-Saban C, Sakr A, Divry P, Gregersen N, Andresen BS (2001) Arch Dis Child **84**: 58
[11] Matsumoto Y, Amano I, Hirose S, Tsuruta Y, Hara S, Murata M, Imai T (2001) Blood Purif **19**: 24
[12] Melegh B, Pap M, Morava E, Molnar D, Dani M, Kurucz J (1994) J Pediatr **125**: 317
[13] Eaton S, Bartlett K, Pourfarzam M (1996) Biochem J **320**: 345
[14] Raspe E, Madsen L, Lefebvre AM, Leitersdorf I, Gelman L, Peinado-Onsurbe J, Dallongeville J, Fruchart JC, Berge R, Staels B (1999) J Lipid Res **40**: 2099
[15] Madsen L, Garras A, Asins G, Serra D, Hegardt FG, Berge RK (1999) Biochem Pharmacol **57**: 1011
[16] Ventura FV, Ijlst L, Ruiter J, Ofman R, Costa CG, Jakobs C, Duran M, Tavares de Almeida I, Bieber LL, Wanders RJ (1998) Eur J Biochem **253**: 614
[17] Chiu KM, Schmidt MJ, Shug AL, Binkley N, Gravenstein S (1997) Biochim Biophys Acta **1344**: 201
[18] Brevetti G, Angelini C, Rosa M, Carrozzo R, Perna S, Corsi M, Matarazzo A, Marcialis A (1991) Circulation **84**: 1490
[19] Zammit VA (1996) Biochem J **314**: 1
[20] McGarry JD, Brown NF (1997) Eur J Biochem **244**: 1
[21] Brown NF, Hill JK, Esser V, Kirkland JL, Corkey BE, Foster DW, McGarry JD (1997) Biochem J **327**: 225
[22] Britton CH, Schultz RA, Zhang B, Esser V, Foster DW, McGarry JD (1995) Proc Natl Acad Sci USA **92**: 1984
[23] Brady PS, Brady LJ (1989) Biochem J **258**(3): 677
[24] Karlic H, Lohninger S, Koeck T, Lohninger A (2002) J Histochem Cytochem **50**(2): 205
[25] Karlic H, Lohninger A, Laschan C, Lapin A, Bohmer F, Huemer M, Guthann E, Rappold E, Pfeilstocker M (2003) J Mol Med **81**(7): 435
[26] Park EA, Mynatt RL, Cook GA, Kashfi K (1995) Biochem J **310**: 853

[27] Kolodziej MP, Crilly PJ, Corstorphine CG, Zammit VA (1992) Biochem J **282**: 415
[28] Mynatt RL, Park EA, Thorngate FE, Das HK, Cook GA (1994) Biochem Biophys Res Commun **201**: 932
[29] Chatelain F, Kohl C, Esser V, McGarry JD, Girard J, Pegorier JP (1996) Eur J Biochem **235**: 789
[30] Thumelin S, Esser V, Charvy D, Kolodziej M, Zammit VA, McGarry D, Girard J, Pegorier JP (1994) Biochem J **300**: 583
[31] Brunner S, Kramar K, Denhardt DT, Hofbauer R (1997) Biochem J **322**: 403
[32] Wijkhuisen A, Djouadi F, Vilar J, Merlet-Benichou C, Bastin J (1995) Am J Physiol **268**: F634
[33] Mittal B, Kurup CK (1981) Biochem J **194**: 249
[34] Esser V, Britton CH, Weis BC, Foster DW, McGarry JD (1993) J Biol Chem **268**: 5817
[35] Woeltje KF, Esser V, Weis BC, Sen A, Cox WF, McPhaul MJ, Slaughter CA, Foster DW, McGarry JD (1990) J Biol Chem **265**: 10720
[36] Feinberg AP, Vogelstein B (1984) Anal Biochem **137**: 266
[37] Chomczynski P, Sacchi N (1987) Anal Biochem **162**(1): 156
[38] Fritz IB (1955) Am J Physiol **197**: 297
[39] Assimacopoulos-Jeannet F, Thumelin S, Roche E, Esser V, McGarry JD, Prentki M (1997) J Biol Chem **272**: 1659
[40] Vaagenes H, Madsen L, Asiedu DK, Lillehaug JR, Berge RK (1998) Biochem Pharmacol **56**: 1571
[41] McGarry JD, Woeltje KF, Kuwajima M, Foster DW (1989) Diabetes Metab Rev **5**: 271
[42] Swanson ST, Foster DW, McGarry JD, Brown NF (1998) Biochem J **335**: 513
[43] Abdel-aleem S, St. Louis J, Hendrickson SC, El-Shewy HM, El-Dawy K, Taylor DA, Lowe JE (1998) Mol Cell Biochem **180**: 95
[44] Zammit VA (1999) Prog Lipid Res **38**: 199
[45] Brass EP, Hiatt WR (1998) J Am Coll Nutr **17**: 207
[46] Hegardt FG, Serra D, Asins G (1995) Gen Pharmacol **26**: 897
[47] Asins G, Serra D, Arias G, Hegardt FG (1995) Biochem J **306**: 379
[48] Chiodi P, Ciani B, Kentroti S, Maccari F, Vernadakis A, Angelucci L, Ramacci MT (1994) Int J Biochem **26**: 711
[49] Gremlich S, Bonny C, Waeber G, Thorens B (1997) J Biol Chem **272**: 30261
[50] McGarry JD, Robles-Valdes C, Foster DW (1975) Proc Natl Acad Sci USA **72**: 4385
[51] Iyer VR, Eisen MB, Ross DT, Schuler G, Moore T, Lee JCF, Trent JM, Staudt LM, Hudson J Jr, Boguski MS, Lashkari D, Shalon D, Botstein D, Brown PO (1999) Science **283**: 83
[52] Valius M, Kazlauskas A (1993) Cell **73**: 321
[53] Revoltella RP, Dal CB, Caracciolo L, D'Urso CM (1994) Biochim Biophys Acta **1224**: 333
[54] Hashimoto T, Fujita T, Usuda N, Cook W, Qi C, Peters JM, Gonzalez FJ, Yeldandi AV, Rao MS, Reddy JK (1999) J Biol Chem **274**: 19228
[55] Aoyama T, Peters JM, Iritani N, Nakajima T, Furihata K, Hashimoto T, Gonzalez FJ (1998) J Biol Chem **273**: 5678
[56] Brandt JM, Djouadi F, Kelly DP (1998) J Biol Chem **273**: 23786
[57] Mascaro C, Acosta E, Ortiz JA, Marrero PF, Hegardt FG, Haro D (1998) J Biol Chem **273**: 8560
[58] Yu GS, Lu YC, Gulick T (1998) J Biol Chem **273**: 32901
[59] Louet JF, Chatelain F, Decaux JF, Park EA, Kohl C, Pineau T, Girard J, Pegorier JP (2001) Biochem J **354**: 189
[60] Manoli I, de Martino MU, Kino T, Alesci S (2004) Ann NY Acad Sci **1033**: 147