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Chronic Hemodialysis and Pregnancy – *L*-Carnitine Supplementation to Human Sera *in Vitro* is Restoring Normal Expression Levels of Carnitine Acyltransferases

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Summary. We analysed the effects of carnitine substitution on carnitine acyltransferases in Rat1-cells, mouse NIH3T3, and human MRC5 fibroblasts serving as reporter cells. For cultivation we used human sera isolated from well-defined conditions of carnitine deficiency: sera from hemodialysis patients (HD) and pregnant women in the third trimester. Besides many differences in pathology both well-known conditions are characterized by very low carnitine levels ($<20\,\mu M$). Their administration in cell culture media resulted in a marked decrease of cellular steady state mRNA levels of CPT1A and CRAT, determined by Northern blot analysis and real time PCR. Supplementation of increasing *L*-carnitine amounts ($>30\,\mu M$) restored CPT1A and CRAT mRNA-levels in a dose dependent manner. Transcript stability measurements indicated that mRNA degradation is not the major modifying mechanism, the more so transcriptional events take place after carnitine supplementation. Our cell culture studies were able to give solid experimental proof, that the sera from HD patients and from pregnant women as well as dialysed calf serum (CS) directly caused a, decreased, in CPT1A and CRAT expression measured. Interestingly fetal calf serum alone also repressed transcript amounts. In all cases carnitine supplementation was able to restore normal CPT1A and CRAT levels. Even short versus long term hemodialysis was reflected by the observed mRNA levels. These results strongly

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suggest, that normal carnitine levels have to be restored either by substitution or dietary measure in all known physio- and pathophysiological cases of carnitine deficiency. In the situation of HD even very high concentration levels up to 160 μM *L*-carnitine seem to exert the best outcome. Insufficient carnitine supplementation otherwise will cause a persistent reduction of steady state mRNA levels of carnitine dependent gene functions.

Keywords. Hemodialysis serum; Third trimester pregnancy serum; *L*-Carnitine supplementation; Carnitine palmitoyltransferase 1 (CPT1A); Carnitine acetyltransferase (CRAT); Steady state transcript level analysis.

Introduction

Carnitine replacement in end stage renal disease and hemodialysis has been shown to positively influence a variety of clinical symptoms, basically all associated with carnitine deficiency [1]. But general acceptance of the need for carnitine substitution in HD patients has been hampered by the lack of knowledge of both clinical symptoms and metabolic disturbances on the cellular level associated with latent carnitine deficiency. The second physiologically well defined model system of very low carnitine serum levels we used, the pregnancy-related changes of carnitine and its esters, have been analysed and documented in numerous papers [2–6]. In order to evaluate the effects of carnitine deficiency on gene expression we decided to compare in cell culture, blood sera from patients on regular HD with sera donated from women in the third trimester of pregnancy. To be able to test the carnitine effect on carnitine acyltransferases we cultivated MRC5, NIH3T3, and Rat1-rat cells in media containing either calf-, or dialysed calf-, or HD patients, as well as serum from women in third trimester pregnancy with or without carnitine addition. Carnitine palmitoyltransferase 1 (CPT1A) and carnitine acetyltransferase (CRAT) mRNA levels as well as the influence on the half-lives of the mRNAs were evaluated.

It is generally accepted that the rapid decrease in plasma free carnitine levels during HD induces the release of carnitine from muscle stores. The muscle carnitine content in HD patients is frequently lower than in controls [7, 8], is inversely correlated to time on HD, and positively correlated to peak exercise performance [9].

In this study we investigated the effects of dialysed calf-, or serum of a HD patient and of women in the third trimester on the steady state mRNA levels of carnitine acyltransferases of mouse, rat, and human fibroblasts in cell culture. CPT1 enzyme activity in red blood and muscle cells has already been reported to be lower in patients on HD [10, 11]. A 6-fold induction of CPT1A activity was observed during the fetal-neonatal transition in the mitochondria of foetal rats [12]. Therefore the CPT1 enzyme activity as well as its transcript levels seem to immediately react on abnormalities in the carnitine system.

Three different isoforms of CPT1 have been described with distinct tissue distributions. Mammals predominantly express hepatic CPT1A that can be found in kidney (both cortex and medulla), intestine, lung, spleen, brain, pancreatic islets, and ovary [13]. Fibroblasts contain the same CPT1A isoform as the liver, and consequently similar effects of low carnitine levels observed in fibroblasts can be expected in hepatocytes. The data reported indicate that carnitine deficiency causes downregulation of the carnitine acyltransferase transcripts, which can be restored by carnitine substitution. Furtheron supplementation levels and its duration

seem to be very important in achieving the wanted positive effects especially in the case of HD.

Results

Comparison of MRC5 Cells (Human Fibroblasts) Treated with Short and Long Term Hemodialysis Patients and Normal Human Serum on CPT1A RNA Levels

Data are depicted in Fig. 1. The Northern blot analysis was performed with CPT1A mRNA from MRC5 cells, which were incubated in cell culture media supplemented with or without *L*-carnitine, in comparison either to 10% healthy human serum or to 10% serum from long and short term HD patients.

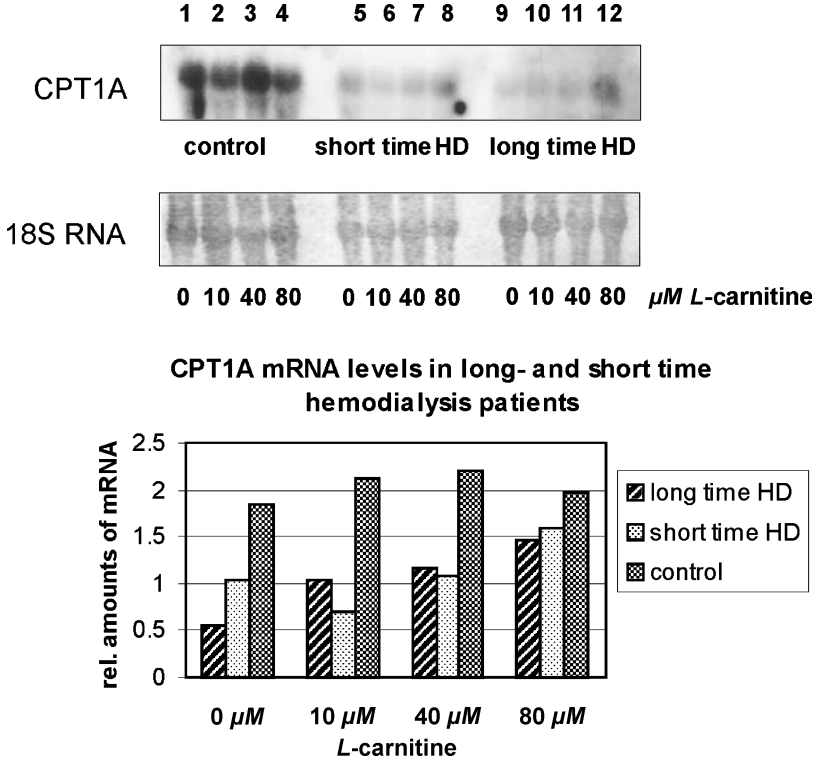

Fig. 1. Northern blot analysis of human CPT1A mRNA levels induced in MRC5 cells after cultivation in a medium containing 10% FCS (lanes 1–4) in comparison to human sera from short HD (lanes 5–8) *versus* long HD patients (lanes 9–12); after precultivation for 48 h the growth media of the MRC5 cells in all three different cases were supplemented with increasing concentrations of *L*-carnitine (control, 10, 40, and 80 μM) and the incubation was continued for additional 4 h; thereafter total cytoplasmic RNAs were isolated and 10 μg/lane were loaded on a 1% denaturing agarose gel; the RNA-gels were transferred and hybridized with a radioactively labeled human CPT1A cDNA fragment; an even loading of the RNA samples was achieved by normalization to constant 18S RNA; Northern blot analysis and quantification were performed as described in the Experimental section; the diagram shows the relative amount of the steady state CPT1A mRNA levels detectable prior and after *L*-carnitine supplementation

The expected increase in CPT1A mRNA level after *L*-carnitine substitution is significantly demonstrated in Fig. 1.

The lanes 1, 5, and 9 contained RNA from unsupplemented cell samples whereas lanes 2–4, 6–8, and 10–12 were loaded with RNA samples isolated from cells supplemented with 10, 40, and 80 μM *L*-carnitine. Control RNA samples (serum from a healthy person) loaded in lanes 1–4 indicate more CPT1A specific mRNA content as that for the short term (lanes 5–8) and the long term (lanes 9–12) dialysed human sera. The incubation with serum of hemodialysis patients resulted in a 2-fold downregulation of CPT1A mRNA steady state levels in comparison to mRNA levels seen in fibroblasts grown in medium supplemented with serum from healthy human donors. The reduction was reverted almost com-

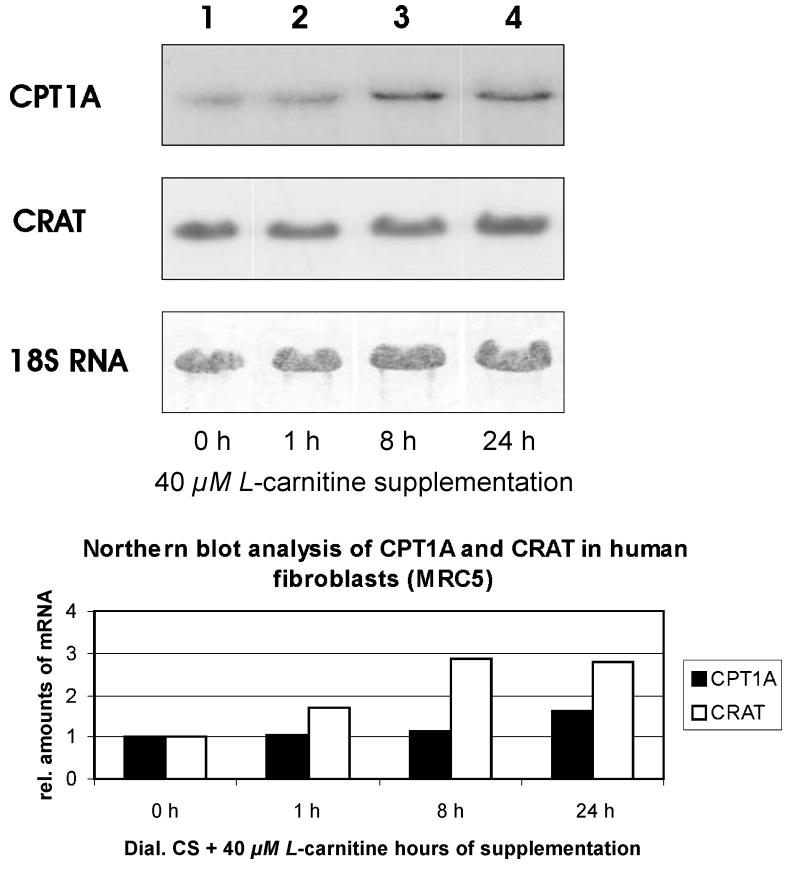

Fig. 2. Northern blot analysis of CRAT and CPT1A mRNA levels in MRC5 (human fibroblasts) grown in dialysed serum as a function of time of incubation with *L*-carnitine; the cells were kept in culture medium containing dialysed 10% CS and after 48 h *L*-carnitine was added (final concentration 40 μM); the cytoplasmic RNA was prepared from the cells incubated with *L*-carnitine for 0, 1, 8, and 24 h and loaded on a 1% denaturing agarose gel on lanes 1–4; cytoplasmic RNA (10 μg/lane) was fractionated electrophoretically, transferred to nitrocellulose, and hybridised with random primed CRAT and CPT1A cDNAs; the diagram shows the amounts of CRAT and CPT1A mRNA relative to 18S RNA; Northern blot analysis and quantification were performed as described in the Experimental section

pletely by *L*-carnitine at supplementation levels reaching a concentration of 80 μM. In a separate experiment similar results were obtained with mouse NIH3T3 fibroblasts (data not shown). Carnitine uptake in fibroblasts is mediated by a high affinity carrier ($K_m = 5\ \mu M$), which is inhibited by palmitoylcarnitine ($K_i = 0.37\ \mu M$) [15].

Time Dependence of L-Carnitine Effects

Data are illustrated in Fig. 2. The effect of *L*-carnitine (40 μM) on CPT1A and CRAT mRNA levels is different with regard to the time of incubation. CPT1A mRNA amount increased threefold after 8 h and remained constant, whereas CRAT

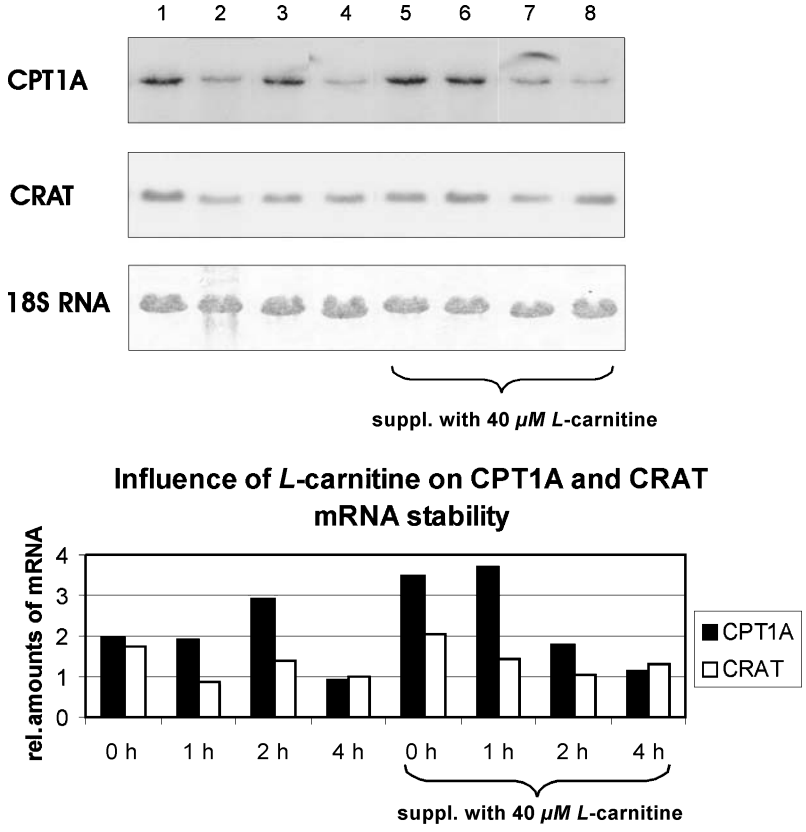

Fig. 3. Effect of *L*-carnitine supplementation on the stability of CRAT and CPT1 mRNAs; cytoplasmic RNA (10 μg/lane) was isolated from MRC5 (human fibroblasts); the cells were grown in medium with dialysed 10% CS (for 48 h) followed by another 4 h without or with addition of *L*-carnitine (final concentration 40 μM) and loaded on a denaturing 1% agarose gel on lanes 1–4 and 5–8, respectively; afterwards, to both cultivation series *DRB* was added (final concentration 30 $\mu g/cm^3$) and the cells were harvested after an additional incubation for 0, 1, 2, and 4 h; the isolated cytoplasmic RNAs were separated electrophoretically, transferred to nitrocellulose and probed with random primed CRAT and CPT1A cDNAs; the different amounts of mRNAs were determined by a densitometric scan with normalization to 18S RNA contents as described in the Experimental section

mRNA induction showed its peak value after 24 h. On the other hand the CRAT mRNA level was enhanced already after 4 h incubation with 80 μM *L*-carnitine.

CPT1A and CRAT mRNAs' Stability at Different Carnitine Levels

Semilogarithmic plots of specific CPT1A and CRAT transcript amounts after *DRB* treatment (see Experimental section) of cells are shown in Fig. 3. It is evident from the decay curves that CPT1A mRNA declined rapidly. In contrast CRAT mRNA very likely belongs to a more stable class of mRNAs (at least under the conditions chosen in this experiment). There is no significant effect of *L*-carnitine treatment on transcript stability. In the case of CPT1A mRNA the transcript even seems to be slightly more stable without *L*-carnitine treatment. Therefore the increase of CPT1A and CRAT mRNAs steady state levels after addition of *L*-carnitine is rather a consequence of transcriptional activation and not due to stabilization of the transcripts.

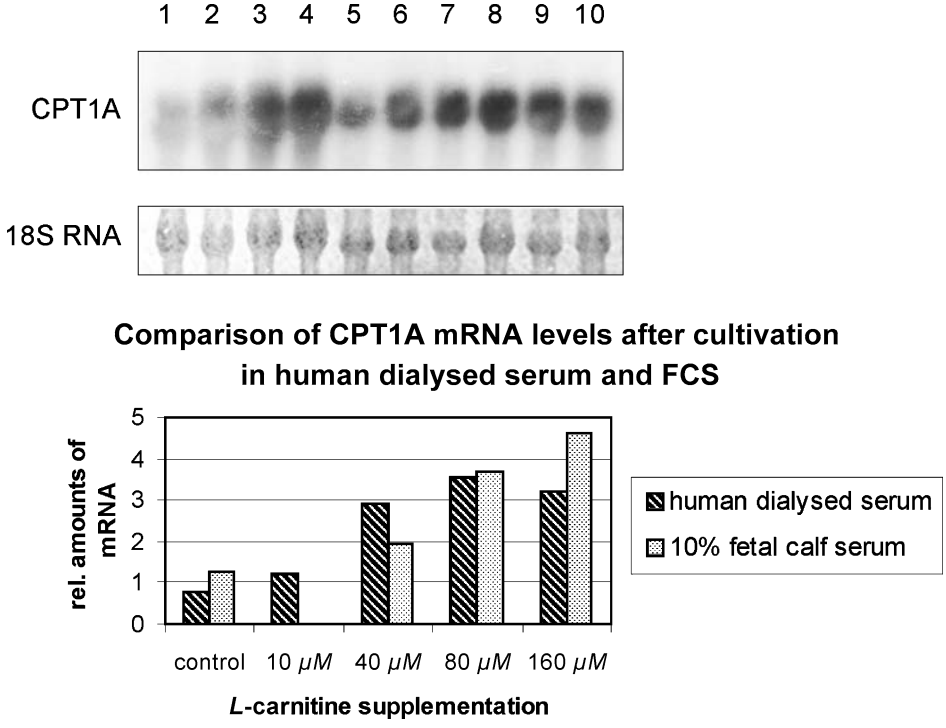

Fig. 4. Northern blot analysis of human CPT1A mRNA levels induced in MRC5 cells after cultivation in a medium containing 10% serum from human HD patients in comparison to 10% FCS; after precultivation for 48 h the growth medium of the MRC5 cells was supplemented with increasing concentrations of *L*-carnitine (up to 160 μM) and the incubation was continued for additional 4 h; lanes 1–4 were loaded with RNAs isolated from cells cultivated with 10% FCS and supplemented with increasing amounts of *L*-carnitine (control, 40, 80, and 160 μM), whereas lanes 5–10 were loaded with samples isolated from cells kept with human HD serum and increasing *L*-carnitine levels (control, 10, 40, 80, and twice 160 μM); thereafter total cytoplasmic RNA was isolated and 10 μg/lane were loaded on a 1% denaturing agarose gel, transferred, and hybridized with a radioactively labelled human CPT1A cDNA fragment; the diagram shows the relative values of the steady state CPT1A mRNAs levels detectable prior to and after *L*-carnitine supplementation

CPT1A Steady State mRNA Levels in MRC5 Human Lung Fibroblasts Cultivated in 10% Serum from Patients under Chronic HD and after Supplementation with L-Carnitine

Figure 4 shows the results of an incubation of human MRC5 cells in 10% serum from HD patients in comparison to 10% FCS. After only 48 h of adaptation to the growth medium the endogenous CPT1A mRNA levels dropped to very low levels in both cell culture systems. In the case of the HD patient serum the transcript levels were even slightly lower than in the FCS counterpart, resembling a model system of very low carnitine content. Only 4 h of *L*-carnitine supplementation were

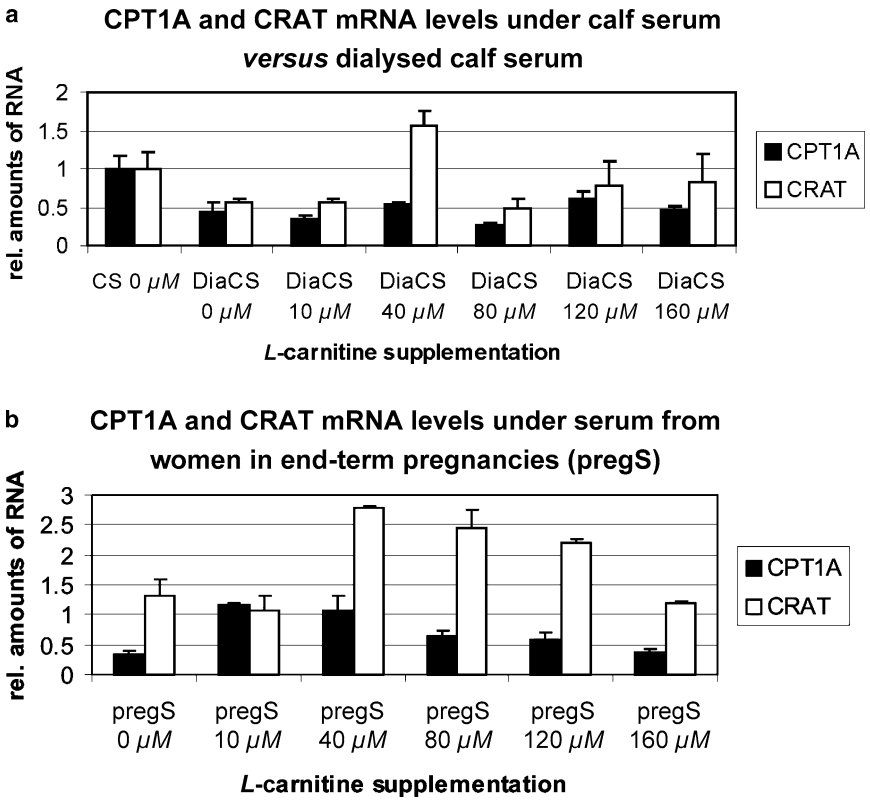

Fig. 5. **a**: MRC5 cells were cultivated in a medium with non-dialysed 10% CS or dialysed 10% CS for 48 h and subsequently incubated with or without *L*-carnitine for additional 4 h, in order to be able to trace the inductional effect on the steady state mRNA levels of CPT1A and CRAT; the final concentrations of *L*-carnitine were 10, 40, 80, 120, and 160 μM; steady state level mRNA analysis and quantification by real-time PCR were performed as described in the Experimental section; **b**: In analogy to the experimental procedure carried out for Fig. 5a, we determined the steady state mRNA levels of CPT1A and CRAT in MRC5-cells treated with 10% serum from women in end-term pregnancy by real time PCR; steady state mRNA level analysis and quantification by real time PCR were performed as described in the Experimental section; data shown in Fig. 5a and b 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 CS alone and pregs. (human serum from end-term pregnancy) respectively without *L*-carnitine substitution (relative expression level set to 1.0); error bars are standard deviations

sufficient to restore the CPT1A mRNA levels in a dose dependent manner, reaching their maximum at 80 μM *L*-carnitine in the cell culture medium. Higher concentrations of *L*-carnitine in the cell culture media even caused a slight reduction of the CPT1A mRNA levels.

Response of CPT1A and CRAT mRNA Levels to Carnitine Supplementation after Cultivation in Dialysed Calf Serum versus Serum from Women in Adolescent Pregnancy

Data are presented in Fig. 5. In analogy to the experimental procedure carried out for Fig. 1, we determined the steady state mRNA levels of CPT1A and CRAT in MRC5 cells treated with nondialysed calf serum (CS), dialysed CS, and serum from women in end-term pregnancy by real time PCR. MRC5 cells were cultivated in medium with non-dialysed 10% CS or dialysed 10% CS in comparison to 10% human (last trimester) serum for 48 h and subsequently incubated with or without *L*-carnitine for additional 4 h. The final concentrations of *L*-carnitines were 10, 40, 80, 120, and 160 μM, respectively. Steady state level mRNA analysis and quantification by real-time PCR were performed as described in the Experimental section. By supplementing cells with *L*-carnitine, the CPT1A and CRAT mRNA levels rose from baseline reaching its maximum at 40 μM *L*-carnitine. The increase of the mRNA levels in MRC5 cells cultivated in pregnancy serum correlates with the results of the experiments of the hemodialysis patients sera.

Discussion

Carnitine metabolism and the effects of *L*-carnitine substitution in hemodialysis patients have been investigated quite thoroughly during the last two decades [1, 15]. Although many beneficial effects of carnitine substitution have been reported, neither the nature of carnitine disturbances nor the mechanisms, by which carnitine substitution relieves uremic symptoms, have been fully understood.

Earlier *in vitro* experiments served as a solid basis for our model system and expression studies of carnitine acyltransferases [16]. In a similar approach we demonstrated now, that fibroblasts growing in a carnitine deficient medium (containing dialysed calf, fetal calf serum, serum of a long term HD patient, or serum from women in end-term pregnancy) showed a reduced steady state expression of carnitine acyltransferase genes. Carnitine substitution induced CPT1A and CRAT transcripts in a dose dependent manner up to normal levels (see Figs. 1, 2, 4, 5). Stability measurements with *DRB* revealed no major changes in mRNA degradation during these treatments. It is well established that CPT1A mRNA concentration is closely related to its immuno-reactive protein [17]. Therefore we conclude that carnitine deficiency causes reduced expression levels of key enzymes of cellular oxidative metabolism like CPT1A and CRAT. The results obtained with fibroblast cell lines kept in serum from HD patients and from pregnant women in the last trimester clearly showed a repression of these genes. Followed by an almost immediate induction of the steady state CPT1A and CRAT transcript levels at 40 μM and more significantly at 80 μM substitution of free *L*-carnitine in the cell culture medium. The effect was observable almost immedi-

ately in our cell culture system, only 4 h after carnitine addition. Moreover 48 h pre-incubation of the cell line in a carnitine depleted cell culture medium, was sufficient to markedly decrease carnitine acyltransferase transcript levels. This is in accordance with clinical trials where the free carnitine level in the so called carnitine nonresponder group was $<30\,\mu M$ and $>40\,\mu M$ in the responders, respectively [18].

Our data also correlate with the observations made in another clinical study, where the significance of carnitine for the energy metabolism in pregnant patients was investigated. It was shown that the antenatal treatment with a low dose betamethasone/*L*-carnitine combination had an advantage over standard betamethasone therapy. The plasma carnitine levels at delivery were decreased to about a half of the concentration found in non-pregnant women. Already in the 12th week of gestation the mean whole blood and plasma carnitine levels were found to be significantly lower than those of controls. Similar low levels were found only in patients with carnitine deficiency. Carnitine substitution (1 g daily) from the 20th gestational week on up to parturition resulted in an increase of free carnitine levels in maternal plasma [3].

In addition these results are supported in part by findings that carnitine administration restored the reduced expression of urea cycle enzymes, of glucokinase, and of the L-type pyruvate kinase genes in carnitine deficient mice by normalizing their transcription [19].

However there are conflicting reports to what extent carnitine deficiency occurs in HD patients and which parameters would characterize patients best, to select them for carnitine substitution therapy. Total plasma carnitine values do not adequately reflect tissue concentrations that may be low due to long term carnitine deficiency [20]. In patients usually a marked reduction of free- and total carnitine levels is observed.

Carnitine has been advocated to improve plasma lipid parameters. Nevertheless the understanding of the regulation of fatty acid oxidation and esterification is incomplete. Partition of long-chain acyl-CoA esters between esterification and oxidation is thought to be regulated by carnitine CPT1A activity, which is sensitive to inhibition by malonyl-CoA [17]. In HD patients a reduced CPT1A activity in erythrocytes and muscle has been reported [10, 11], which is in accordance with the data of our *in vitro* studies and very likely is caused by a reduced transcription rate.

The positive influence of *L*-carnitine supplementation on carnitine acyltransferases expression levels in a dose dependent manner convinced us to try a different approach. In a parallel study we tried to identify additional gene functions influenced by a carnitine treatment. Based on our previous findings we performed chip screen analyses of *L*-carnitine un- *versus* supplemented target cells (*Litzlbauer E et al.*, 2005, in prep). We analyzed the whole human genome and the magnitude of genes, which responded to carnitine supplementation was surprisingly high. Furthermore we were able not only to confirm but also support our results presented above with novel genes filtered out by chip screen evaluation software. The expression levels delineated from the screening data showed an evident increase as well as a decrease, which were in accordance to kidney disease in general and to pregnancy.

One of the most important transporters, in combination with carnitine, is the OCTN2 transporter, member of the SLC22 family [21], which has been correlated to problems occurring during chronic hemodialysis (unpublished results, [22]). The importance of the OCTN2 transporter is underlined by the fact, that mutations in its gene were proven to be the reason for systemic carnitine deficiency [23]. It is evident that other members of the organic cationic transporters (OCTN1, OCTN3) are also involved in the carnitine transport, but with lower specific activity (higher K_m) [24, 25], especially in those situations where OCTN2 is not expressed or not inducible anymore (*e.g.* chronic HD). Therefore higher carnitine levels should be achieved to enable proper import by these transporters. Current clinical therapy protocols recommend normal carnitine concentrations, in a range too low to get it transported in sufficient amounts by less specific membrane transporters.

OCTN2 was described to mediate most of the maternofetal carnitine transport in humans [26] and is remarkably upregulated by carnitine supplementation in pregnant women as well as in normal adult controls (see two other contributions in this issue). In the above mentioned chip screen experiment *L*-carnitine supplementation to deprived cultivation medium affected nearly the whole solute carrier family 16 (about 8 members of family SLC16 were induced by *L*-carnitine). Another frequently used terminus for these genes is monocarboxylate cotransporter (MCT) family, of which only the first four (MCT1-MCT4) have been demonstrated experimentally to catalyse the proton-linked transport of metabolically important monocarboxylates such as lactate, pyruvate, and ketone bodies [27]. In addition to SLC16 genes the solute carrier families 30 (SLC30, Zn-transporters) and 39 (SLC39, Zip-transporters), showed similar dose dependent expression changes, like as the carnitine acyltransferases. There were at least 9 different ZnT- and 15 Zip-transporter genes identified so far in human cells [28]. They are all involved in regulating cellular zinc homeostasis. ZnT-transporters reduce intracellular zinc availability by promoting zinc efflux from cells or into intracellular vesicles, whereas Zip-transporters increase the intracellular Zn availability. Both ZnT and Zip transporter families exhibit unique tissue-specific expression, differential responsiveness to dietary zinc deficiency and excess, and differential responsiveness to physiologic stimuli *via* hormones and cytokines [28]. Therefore these carrier families are responsible for Zn transport and thus supporting the transcription machinery *via* Zn finger proteins [29].

There are obviously a number of aspects how carnitine positively supports the therapy of hemodialysis patients: improved muscle function and reduction of weakness, better wound healing and immune function, increase in ventilatory response and molecular genetic events. One could summarize these points under two different headings: first the improvement of life quality [30, 31] and second the induction of the mRNA levels, as demonstrated by our investigations.

In analogy to HD carnitine treatment did also influence gene expression levels associated with pregnancy. Our screening data of the carnitine supplementation caused a change in transcript levels of distinct plasma- and glycoproteins. It is very interesting and indicative that at least one of these proteins, PAPPA2 (pregnancy associated plasma protein), is also involved in interactions with Zn-ions. Early pregnancy levels of PAPP-A are a risk factor for intrauterine growth restriction, premature birth, preeclampsia, and stillbirth [32]. Maybe the coordinated influence of

Zn transporters and interactors is a possible correlation between the positive effects of carnitine in both pregnancy and dialysis patients [33].

In addition our *in vitro* data strongly argue for a prolonged but also a sufficient carnitine supplementation. Of clinical importance is the administered amount of carnitine supplementation, which should be in the range of 40–80 μM free *L*-carnitine in normal subjects, for hemodialysis patients levels of 80–160 μM are more appropriate in order to saturate low affinity transporters. According to these results studies with a follow up period of three months or less may be misleading, longer administration regimens and observations are indispensable. The combination of molecular biological- and biochemical analytical methods allow an investigation of carnitine effects on the transcriptional level of enzymes involved in carnitine metabolism and might be promising tools for a better understanding of the multitude of clinical carnitine effects.

Experimental

Cell Culture

NIH3T3 mouse fibroblasts (ATCC CRL-1658), Rat-1 rat fibroblasts, or MRC5 human lung fibroblasts (ATCC-CCL 171) were grown in *Dulbecco*'s modified *Eagle*'s medium (DMEM) supplemented with 10% calf serum (CS) and antibiotics (30 mg/dm^3 penicillin, 50 mg/dm^3 streptomycin sulphate). For the experiments, subconfluent cells were kept in DMEM containing either 10% CS, 10% dialysed CS, 10% human serum from HD patients isolated before a dialysis session, or with 10% serum from women in the third trimester of pregnancy. Dialysis of calf serum was performed against PBS for 48 h with five buffer changes. Prior to any experiment, cells were adjusted to culture conditions for 48 h, subsequently *L*-carnitine 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 time course experiment for different periods of time) before the cells were lyzed and RNA was isolated. For RNA stability experiment, 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 $\mu g/cm^3$ (time indicated as 0 h). After incubation for different periods of time, cytoplasmic RNA was isolated.

RNA Preparation, Northern Blot Analysis, and RT-PCR

To isolate cytoplasmic RNA, 3×10^7 cells were washed twice with PBS, scraped off the petri dish 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 gentle agitation for 10 s the suspension was centrifuged at 15000 *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 (15000 *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% agarose gels containing 1.5% formaldehyde, transferred to GeneScreen nylon membranes (Du Pont-New England Nuclear), and cross-linked to filters by ultraviolet light irradiation. Proper transfer was verified by staining the membranes with methylene blue solution (0.04% methylene blue, 0.5 *M* sodium acetate, *pH* 5.2) for 10 min and subsequently partially destained with distilled water. Rat liver carnitine palmitoyltransferase (CPT1A) cDNA and murine carnitine acetyltransferase (CRAT) cDNA were radiolabelled by random priming and used as probes. The filter was hybridized overnight at 42°C in 50% formamide, 5× SSPE, 0.1 *M*

sodiumphosphate, *pH* 6.5, 5× *Denhardt*'s, 10 m*M EDTA*, *pH* 8, 1% Sarkosyl, 100 $\mu g/cm^3$ yeast-tRNA. The final wash was performed in 2× SSC/0.1% SDS at 65°C for 30 min. Steady state transcription levels of the mRNAs were analyzed by the gel analysis software Image Quant (Molecular Dynamics Inc). Signal and loading corrections were based on 18S (or 28S) rRNA contents.

Real Time PCR

RNA was isolated as described above. 2 μg RNA were reversely transcribed based on the Revert Aid M-MuLV Reverse Transcriptase Protocol (by Fermentas) with oligo-(dT) 18-mer primers and used as templates. The mRNA sequences of CPT1A, CRAT, and β2Microglobulin were obtained from the Entrez Nucleotides database. The primers were designed using the Primer 3 software from the Whitehead Institute for Biomedical Research (Cambridge, MA). The following forward (F) and reverse (R) primers and conditions were used: CPT1A F: 5′-GAGAGGAGACAGACACCATC-3′, R: 5′-ACT TGTCAAACCACCTGTC-3′, annealing temp. RT-PCR: 62°C, block PCR: 52°C; CRAT F: 5′-CCATCCGCTCGGCTTCCATGG-3′, R: 5′-CTCCGCGCAGCTGTTGTAGGC-3′, annealing temp. RT-PCR: 65°C, block PCR: 59.5°C; β2Microglobulin F: 5′-GATGAGTATGCCTGCCGTGTG-3′, R: 5′-CAATCCAAA TGCGGCATCT-3′, annealing temp. RT-PCR: 65°C, block PCR: 60°C. Quantitative RT-PCR was performed with the LightCycler Instrument (Roche Diagnostics) using the Fast Start DNA Master SYBR Green I kit for amplification and detection. Relative quantification of target gene expression was performed using a mathematical model developed by *Pfaffl* [14]. The expression of the target molecules was normalized to the expression of β2Microglobulin.

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