universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„L-Carnitine and its transcriptional modulation“

Verfasser

Klemens Kienesberger

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag.rer.nat.)

Wien, 2012

Studienkennzahl lt. Studienblatt:	A 490
Studienrichtung lt. Studienblatt:	Molekulare Biologie
Betreuer:	Ass.-Prof. Dr. Reinhold Hofbauer

Acknowledgements

A diploma thesis is always a good opportunity to say thanks to all those people who participated in the genesis of such an „epic“ work (from a very subjective point of view). And that is exactly what I want to do right here.

At first I want to address my thanks to my supervisor Ass.-Prof. Dr.Reinhold Hofbauer, who gave me the opportunity to do my diploma thesis in his lab. Thank you for teaching me science and for the introduction into the fascinating world of L-carnitine and nutrigenomics. Especially I want to say thank you for being more than a teacher and for the freedom I have enjoyed planning and executing my experiments throughout the last year.

Of course I also want to thank my parents, Ulrike and Rudolf. Thanks for the financial support, the patience, the understanding and support for the last 27 years. I am very grateful that you continued supporting me when I „surprised“ you with my „medical masterplan“.

A big thank you I want to address to my grandparents Rudolf and Franziska for supporting me my whole life long.

I also want to say thanks to my brothers, Klaus and Michael. Since my early childhood you pushed me forward, encouraged me to explore my own boarders.

Special thanks I want to address to my cousin Hubert. You have been an awesome roommate and I think of you as a great and true friend since the time I was born.

At this point I also want to address a very special thanks to my late grandmother Theresia. She has been such an inspirational person in my life and she has been and still is a huge motivation for my studies.

I also want to thank my closest friends Jakob, Matl and Michi. Thanks for the great moments we shared in the past years in Vienna and also back home. It was always a pleasure!

Thanks to my friend and colleague Muatl for your great scientific and organisational support through the past years. Thanks Fips for making the daily lab routine more fun and all your technical support. Also a big thank

you to my lab partners Marion and Thomas who both contributed in their ways to the genesis of this thesis.
Finally I want to thank my terrific girlfriend Romina. Without you the last few years wouldn’t have worked out that well. Thanks for pushing me forward and guiding me through hard times. You have always been there for me when I was desperate and doubting myself and I was close to giving up. This thesis would not have been possible without you. Thanks for your understanding when I spent evenings and also weekends in the lab. Thank you for everything you did for me!

INDEX

1 Introduction

1.1 L-Carnitine

L-Carnitine (L-3-hydroxy-4-N, N, N – trimethylaminobutyrate) is a naturally occurring amino acid derivate with essential functions in intermediary metabolism.[1] Chemically it can be described as a water-soluble zwitterion.[2] Carnitine is synthesized from the two essential amino acids lysine and methionine. Lysine provides the carbon backbone whereas methionine supplies with 4-N-methyl groups.[3]

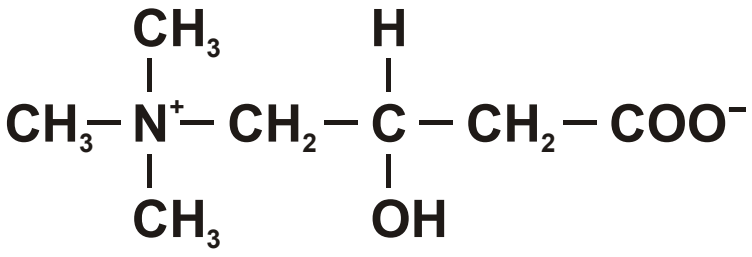


Figure 1: L-Carnitine

1.1.1 Sources and metabolism of L-Carnitine

L-Carnitine can be considered as a “semi-essential” biological compound. Mammals are basically able to synthesize it, but most important source is nutritional uptake.[2] About 75% of total body L-carnitine derives from diet, whereas about 25% are produced endogenously. Mainly the kidneys are responsible for synthesis of L-carnitine, but also liver, heart, bone muscle and brain tissues are able to produce this compound.[4]

Dietary L-carnitine uptake ranges from 1-15 µmol/kg bodyweight per day. Regular consumption of red meat leads to uptake of 6-15 µmol/kg whereas vegans and vegetarians acquire a maximum of 8 µM carnitine/kg bodyweight per day.[5] People strictly following a red meat lacking diet like vegetarians might use carnitine dietary supplements. In this case it has to be considered that supplementary L-carnitine shows a decreased bioavailabilty (14-18%). Dietary L-carnitine on the other hand has a

bioavailabilty of 54-87% depending on the carnitine-concentration in the meal [6]. This dietary part of the carnitine-pool is absorbed actively and passively across the membrane of enterocytes. Carnitine homeostasis is kept constant through this dietary uptake, minor endogenously synthesis and very effective renal absorption. [7] The whole physiological L-carnitine pool is made up of free L-carnitine and many different acylcarnitine-esters like acetyl-L-carnitine, propionyl-L-carnitine or long chain fatty acid carnitine-esters.[6]

After entering the blood stream L-carnitine gets transported across the cell membrane by the transport protein OCTN2 (Organic Cation/Carnitine Transporter 2) or also called solute carrier family 22 member 5 SLC22A5 [8]. This membrane protein, physiologically a uniporter, is expressed in several different tissues like skeletal muscle, kidney, heart and brain. OCTN2, as a sodium/carnitine co-transporter, has a high affinity for carnitine, but can also serve as a polyspecific Na+ independent cation uniporter. [9]

OCTN2 is not only important for L-carnitine uptake into adipocytes and myocytes, it also plays a crucial role in reabsorption of organic cations and carnitine in the proximal tubulus of the kidney.[10]

1.2 Mitochondrial carnitine shuttle system

After entering the cells in muscles, fat tissue or brain, L-carnitine fulfils its manifold roles. One central function of L-carnitine is its key role as a cofactor for the transport of long-chain fatty acids prone for β-oxidation across the mitochondrial membrane. The network executing this process is called carnitine acyltransferase system or “carnitine shuttle system”. Within this network several transferases and translocases enable important steps of energy metabolism and homeostasis.

Long-chain fatty acids are the main energy source for many tissues. These fatty acids are produced by lipolysis in adipocytes and can be transported through the blood system bound to albumin.[11] Special transport proteins mediate the transport across the plasma membrane of the fatty acids. [12]

After entering the cell long-chain fatty acids are first bound to fatty acid binding proteins that are present in the cytosol.[13] Before long-chain fatty acids are introduced into metabolism (β-oxidation) or get stored or are used for building of triglyzerides for the plasma membrane - depending on tissue and demand - they have to get activated by conversion to acyl-CoA. This reaction is catalysed by long-chain co-enzymA synthethases (LCAS). [14]

The next step in this cascade is the transport into the intermembrane space of the mitochondria. The mechanism of this step has not yet been defined and characterized enough. The opinion that fatty acids are able to pass the outer mitochondrial membrane by diffusion has not been confirmed.[13]

Two different pathways have been proposed for this process, both depending on the orientation of the Carnitine-Palmitoyltransferases 1 (CPT1) in the outer mitochondrial membrane. This enzyme catalyses the important conversion of acyl-CoA + free L-carnitine to acylcarnitine + free CoA. This step is the rate-controlling step of the whole “carnitine-shuttle system” and also β-oxidation.

Originally it has been proposed that the catalytic domain of CPT1 faces the intermembrane space between outer and inner mitochondrial membrane.[15] This orientation would mean that the activated fatty acids would have to pass the outer mitochondrial membrane.

In the meanwhile a different pathway has been proposed in which the orientation of CPT1 is directed to the cytosol. [16] This topology can be explained by the fact that the effective substrate of CPT1 is the complex of acyl-CoA and acyl-CoA binding protein (ACBP), which would be too large to be transported into the intermembrane space. [17] Further on the voltage dependent pore-forming molecule (VDAC) in the outer mitochondrial membrane is too small to transport long-chain acylcarnitine esters. [18] Recently it has also been shown that CPT1 forms a hexamer in vivo [19]. The diameter of the resulting pore would allow the passing of molecules of the size of palmitoylcarnitine indicating that CPT1 might also fulfil transport duties. [17]

The carnitine-acylcarnitine translocase (CACT), residing in the inner membrane catalyses the transport of long-chain acylcarnitines formed by CPT1 across the inner mitochondrial membrane into the matrix in exchange for L-carnitine. [20]

Carnitine Palmitoyltransferase 2 (CPT2) is associated with the inner mitochondrial membrane facing the mitochondrial matrix. This enzyme reconverts the acyl-carnitineesters to its original conformation of acyl-coA and free L-carnitine. [13] These long-chain fatty acids can now be used for ATP production by β-oxidation and subsequent introduction of the generated acetyl-CoA into the TCA (tricarboxylic acid cycle). Free L-carnitine gets again exported into the cytosol via CACT for another round of fatty-acid transport across the mitochondrial membrane.

In the mitochondrial matrix another transferase is located. The Carnitine-Acetyltransferase transfers acyl groups of different sizes from acyl-coA to L-carnitine resulting in an acyl-carnitine-ester. [21] It defines the equilibrium between acetyl-CoA (+ free L-carnitine) and acetylcarnitine (+ free CoA) and therefore regulates the acetyl-CoA/CoA ratio.[22]

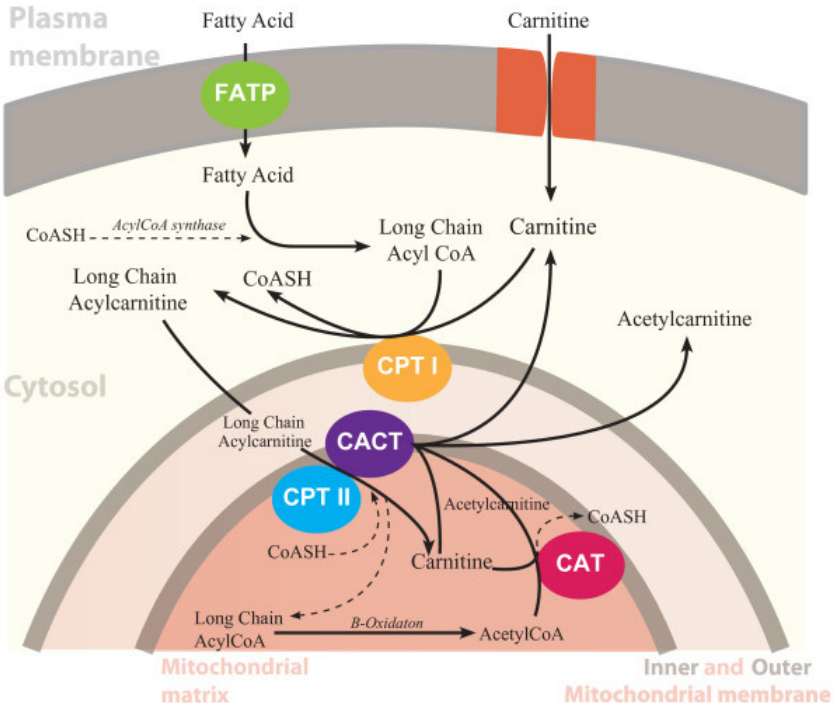


Figure 2: Carnitine-Acyltransferase System (Flanagan et al, 2010)

Another carnitine transferases which itself is not active in mitochondria but in peroxisomes is the Carnitine Octanoyltransferases. It enables the transport of medium-chain fatty acids from peroxisomes to mitochondria through the conversion of acyl-CoA´s, shortened by peroxisomal oxidation, to acylcarnitine. [23]

1.2.1 The members of the "Carnitine-Shuttle-System" – enzymology and genomics

1.2.1.1 Carnitine Palmitoyltransferase 1 (CPT1)

The basic enzymology of CPT1 (EC 2.3.1.21) has been described above. Mammals express three isoforms of CPT1, all localized on different chromosomes.[24, 25] CPT1a represents the liver isoform, which is also expressed in kidneys. CPT1b is called the "heart isoform" and is mainly expressed in cardiac and skeletal muscle. [17] These two isoforms have been known and characterized for many years. In 2002 a third isoform, CPT1c, mainly expressed in brain tissue, has been discovered. [25] This enzyme is specialized for neural tissue fatty acid oxidation. It is mainly localized in the endoplasmatic reticulum of neuronal cells. [26] CPT1c also seems to play an important role in central control of energy metabolism, since CPT1c deficient mice showed abnormalities in hepatic gluconeogenesis, weight gain and insulin resistance. [25] Overexpression of the neural specific CPT1c in the hypothalamus leads to increased food uptake in mice by antagonizing the anorectic effects of leptin. [27]
Also the CPT1a isoform seems to be located not only to the outer mitochondrial membrane. It may also enable the transport of long-chain fatty acids into the endoplasmatic reticulum for the synthesis of triacylglycerol. [28]
As already mentioned above CPT1 catalysis the rate-controlling step of fatty acid degradation. This step can be inhibited by malonyl-coA, a product of the acetyl-carboxylase during fatty acid anabolism. This enables the inhibition of fatty acid oxidation under conditions where synthesis is

needed.[23] The three isoforms of CPT1 show different affinities to malonyl-coA. The liver-isoform (CPT1a) shows higher affinity to L-carnitine and lower affinity to malonyl-coA in contrast to the muscle form (CPT1b).[24] This provides the body the important possibility to control the fatty acid metabolism especially in the heart where both isoforms are expressed at different levels.[22] Affinity of the CPT1 isoforms to malonyl-coA also correlates with the pH enabling decreased fatty acid oxidation during acidosis. [29]

1.2.1.1.1 Transcriptional regulation of *CPT1a*

CPT1a transcription rate in liver is increased with high fat diet, during starvation and streptozotocin induced diabetes.[30] Also multiple hormones influence the expression of CPT1a like glucocorticoids, thyroid hormone (T3) and insulin. Insulin inhibits transcription whereas cAMP increases CPT1a expression.[31] Fatty acids and hyperlipidemic drugs like fenofibrate activate CPT1a expression via Peroxisome Proliferator Activated Receptor alpha (PPARα).[30] PPARα binds to PPRE (PPAR responsive elements) as a heterodimer with RXRα (Retinoid X Receptor). [32]
An important cofactor for activation of PPAR regulated genes is the peroxisome proliferator activated receptor gamma coactivators 1 alpha (PGC-1α).[33,34] Coactivators are transcriptional regulators that bind to already DNA liganded proteins. They act as gene expression stimulating factors, histon modifiers and they mediate interaction to other transcription factors.[35] Thyroid hormone T_3 for instance influences CPT1a gene expression via PGC-1α.[36] T_3 induced different PCG-1α isoforms, which further on activate in cooperation with T_3 CPT1a expression. [37]
The promoter structure in the different model organisms is highly conserved with only small differences. The main difference is that the human *CPT1a* gene contains two alternate first exons (exon 1a and exon 1b) whereas the other organisms like rat and mouse only have one first exon. [38,39]. A TATA-less promoter regulates CPT1a expression. The proximal region of the promoter start is similar to other TATA less promoters with a GC- rich

region. Two SP1 (specifity protein 1) binding sites -21 to -27 and -100 to -107 relative to the start site of transcription and one CCAAT box for binding of nuclear factor Y have been identified. These two factors are responsible for basal transcription levels of CPT1a [40]. cAMP induced transcription is mediated via a “multi responsive element” containing a cAMP responsive element and a DR1 motif. CREB (cAMP responsive element binding – protein) and HNF4α (hepatic nuclear factor 4 alpha) bind to this element and mediate the effect of cAMP on *CPT1a* expression.[31]

Long chain fatty acids also induce *CPT1a* expression.[30] Also PPARα and PPARγ coactivators (PGC-1α) activate *CPT1a* transcription [41]. The exact mechanism how these factors regulate *CPT1a* has been discussed in the last few years quite controversial. It is generally accepted that fatty acids activate gene expression via PPARα. This model has been strengthened after the analysis of PPARα knockout mice, which showed diminished induction of genes regulated by this factor, amongst those *CPT1a*. But publications in the last few years reported different mechanisms for fatty acid mediated *CPT1a* expression. Long chain fatty acids are also able to induce *CPT1a* transcription in a PPARα independent pathway. [42] But a fatty acid responsive element independent from PPARα has been localized in the first intron. This element is mainly induced by PCG-1α during fasting.[38] A PPARα dependent element, which mediates the *CPT1a* expression induced by long chain fatty acids and also during fasting, has been identified in the second intron. This pathway again is independent from PCG-α.[41] This duplex regulatory mechanism indicates the importance of CPT1a expression for energy homeostasis during fasting.

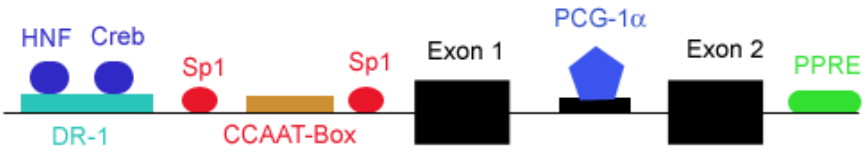


Figure 3: Scheme of CPT1a promoter

1.2.1.1.2 Transcriptional regulation of *CPT1b*

The *CPT1b* promoter structure is highly conserved throughout the different model organisms. At least two different regulative elements control CPT1b expression in humans and sheep as well as in rats and mice .One promoter region is located up to 330nt upstream the first exon, whereas the second one is a complex secondary structure downstream of the transcriptional start site. [43]

CPT1b promoter is very similar to *CPT1a* and *CRAT* a TATA-less promoter. Basal transcription is mediated by SP1 (specificity protein 1) and an enhancer complex. The binding site of this enhancer has been localized to the first intron/exon of the rat *CPT1b* promoter. [44]

A FARE (fatty acid response element) drives the fatty acid induced *CPT1b* expression, which has been mapped in rat and human promoters. Peroxisome Proliferator Factor α (PPARα) activates this element. [45, 46] PPARα forms heterodimers with the nuclear receptor retinoic X receptor α (RXRα). This FARE overlaps with a COUP-TF binding site [45], also an element which is highly conserved in mammalian CPT1b promoters. Further promoter elements found in the upper promoter are E-boxes, more SP1 sites and a SRF (serum response factor) site, which is known for regulation of CPT1b in rats in combination with GATA. [47] Further it has been shown that the deletion of the CA-box sequence CCACCC leads to drastic decrease of reporter gene expression. [47]

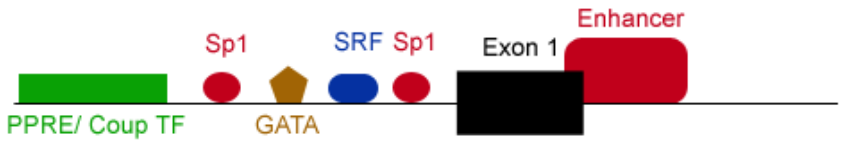


Figure 4: Scheme of CPT1b promoter

1.2.1.2 Carnitine/Acylcarnitine Translocase (CACT)

As described above the carnitine-acylcarnitine translocase is an integral inner mitochondrial membrane protein. It catalyses the mole-to-mole exchange of carnitine and acylcarnitine to transport long chain fatty moieties can be transported into the mitochondrial matrix. [13, 48] CACT is also located in peroxisomes. [13] The enzyme shows a very high affinity for carnitine and acylcarnitine, but very low affinity for other compounds of the fatty acid metabolism. This substrate specificity makes CACT an important regulator of carnitine levels in mitochondria and cytoplasm and explains also the severity of CACT deficiencies. [13] CACT can be inhibited by acyl-D-carnitine, which therefore regulates fatty acid oxidation. [49]

1.2.1.2.1 Transcriptional regulation of *CACT*

CACT promoter is also lacking a TATA box. A DPE element at -18/-13bp proximal the first exon mediates binding of TFIID enabling basal transcription levels. [50] DNA microarray analysis indicated that *CACT* expression is regulated by PPAR alpha [51, 52] Various analysis of the human *CACT* promoter showed that a PPRE is present –105bp to -75bp proximal of the first exon. This PPRE shows response to fibrates, statines and 9-cis retinoic acids, which results in elevated *CACT* mRNA levels. [53]
Further on Sp1 and FOXA2 sites, located at -237/-227bp respectively -300/-290bp have been identified in the *CACT* promoter. Sp1 contributes to *CACT* transcription in various cell types and tissues, whereas FOXA2 only promotes *CACT* expression in hepatic cells. [54]
In the murine *CACT* promoter sequence four putative PPRE have been identified at following positions: -808/-796bp, -580/-568bp, -332/-324bp and +45/+57bp relative to promoter start. PPARα and PPARδ are able to bind this specific PPRE (AGGTCAAAGGTCA) located in the first exon +45/+57bp in the 5' UTR of the murine *Cact* gene up-regulating the transcription. [48]

Figure 5: Scheme of CACT promoter

1.2.1.3 Carnitine Palmitoyltransferase 2 (CPT2)

Carnitine Palmitoyltransferase 2 is a protein of the mitochondrial matrix, which is anchored to the inner mitochondrial membrane. It reverts the carnitine esters to their original compounds namely acyl-coA and free carnitine.[13] CPT2 is expressed as the same protein in all tissues of the body with no different splice variants. It also shows no sensitivity to malonyl-coA, meaning that this compound does not inhibit CPT2 and further no important regulative function in β-oxidation can be described for CPT2.[23]

1.2.1.3.1 Transcriptional regulation of *CPT2*

The human CPT2 promoter showed putative binding elements for the following factors after theoretical promoter analysis: a TATA box, a PPRE, a Sp1 binding site and also a binding site for the insulin upstream factor 1 (IUF-1).[55] The PPRE element is inducible by PPARα/RXRα heterodimers and is further on modulated by the orphan receptors COUP-TFI and ERRα.[56] The murine CPT2 receptor in the contrary shows no TATA box but several Sp1 binding sites and a putative AP-1 binding cassette. [56, 57]
PPARα also plays an important role in the transcription of this member of the "carnitine-shuttle system", as data from DNA microarray with PPARα deficient mice show.[51] PPARα deficient mice lack the ability to up-regulate CPT2 expression after administration of fibrates or during starvation in contrary to wild type mice. [58]
A notable characteristic of the human CPT2 promoter is the sequence of the PPRE. This PPRE only contains one half proportion with a perfect consensus sequence (TGACCT). The second part of it is not matching the

previous accepted consensus sequence (GAGCAC). By chromatin immunoprecipitation PPARα binding to this sequence and functionality of this site has been shown.[51]

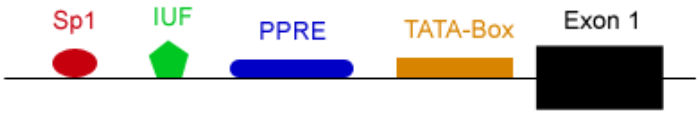


Figure 6: Scheme of human CPT2 promoter

1.2.1.4 Organic Cation/ Carnitine Transporter 2 (OCTN2)

OCTN2 is a sodium ion-dependent cation transporter and the most important carnitine transporter in the plasma membrane. [8] Most adult tissues highly express OCTN2, including skeletal muscle, kidney, placenta, and heart.[59] This transporter regulates the carnitine pools within the cell. In the plasma a carnitine concentration of 50 µM is physiological. OCTN2 allows the cell to reach millimolare carnitine concentrations in the cytoplasm. [60]

1.2.1.4.1 Transcriptional regulation of the Organic Cation/ Carnitine Transporter 2 (OCTN2 or SLC22A5)

PPARα is also involved in regulation of OCTN2 mRNA levels. Administration of fibrates leads to upregulation of OCTN2 gene expression. Additionally carnitine biosynthesis is also upregulated through PPARα agonists. This synergetic effect results in carnitine accumulation within the cell. [61, 62]

The *Octn2* promoter in rat hepatocytes shows sensitivity to PPARα-agonists ciprofibrate and fenofibrate. Several imperfect PPRE were identified in the rat promoter, but the sequence mediating the PPARα induced upregulation of transcription has not been defined yet. [63]

The murine *Octn2* promoter is also regulated by PPARα via a PPRE in the first intron in mouse hepatocytes. It has been shown that PPARα binds as a

heterodimer with RXRα this element, but it can not be excluded that other PPREs are also contributing to the *Octn2* expression after administration of a synthetic PPARα agonist like WY14,643.[64]

OCTN2 in the tissue of the large intestine is regulated in a different way. Recent data suggest that *OCTN2* in colon of mice and humans is rather modulated by PPARγ than by PPARα.[65]

The administration of cisplatin leads to massive urinary loss of L-carnitine. This effect of cytostatic therapy is traceable to regulative deactivation of the transcription factor PPARα and subsequent down regulation of OCTN2 in the kidney. [66]

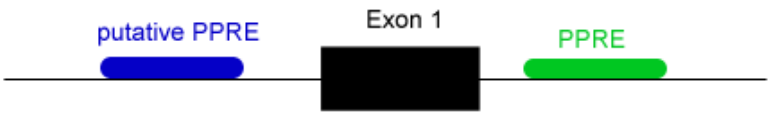


Figure 7: Scheme of OCTN2 promoter

1.2.1.5 Carnitine Acetyltransferase (CRAT)

The Carnitine Acetyltransferase is mainly localized in the mitochondrial matrix, but can also be found in peroxisomes and the endoplasmatic reticulum. [67] CRAT regulates the equilibrium between short-chain acyl-coA (mainly acetyl-coA) and CoA, and acyl-L-carnitine and free L-carnitine within the mitochondrial matrix. [68] The nomenclature suggests that this enzyme mainly catalyses the conversion of acetyl-coA and free L-carnitine to acetylcarnitine and free Co-A but the optimum acyl-chain length for CRAT is C4. [69] The fact that CRAT is not localized in the cytosol shows the importance of this protein for the equilibrium of Acetyl-CoA/Co-A within organells and cellular compartments. [22]

CRAT is also important in cell cycle regulation and is necessary for the progression from G1 into S-phase. [21] Decreased CRAT activity has been connected with disorders of the nervous system like Alzheimer's disease. [70] But regarding this hypothesis opposing data has also been published,

saying that carnitine acyltransferases like CRAT and CPT1 are not changed in Alzheimer's disease. [71]

Very low expression of CRAT has been connected with skeletal muscle and Purkinje cell degradation. [72] Also a connection between the pathophysiology of peripher vascular disease and low CRAT activity has been proposed. [73]

1.2.1.5.1 Transcriptional regulation of *Crat*

As DNA microarray analysis indicated that PPARα also plays a key role for the regulation of *CRAT* expression [51, 52] The central role of this nuclear receptor in fatty acid metabolism is depicted in the figure below, where genes which are induces by PPARα are listed.

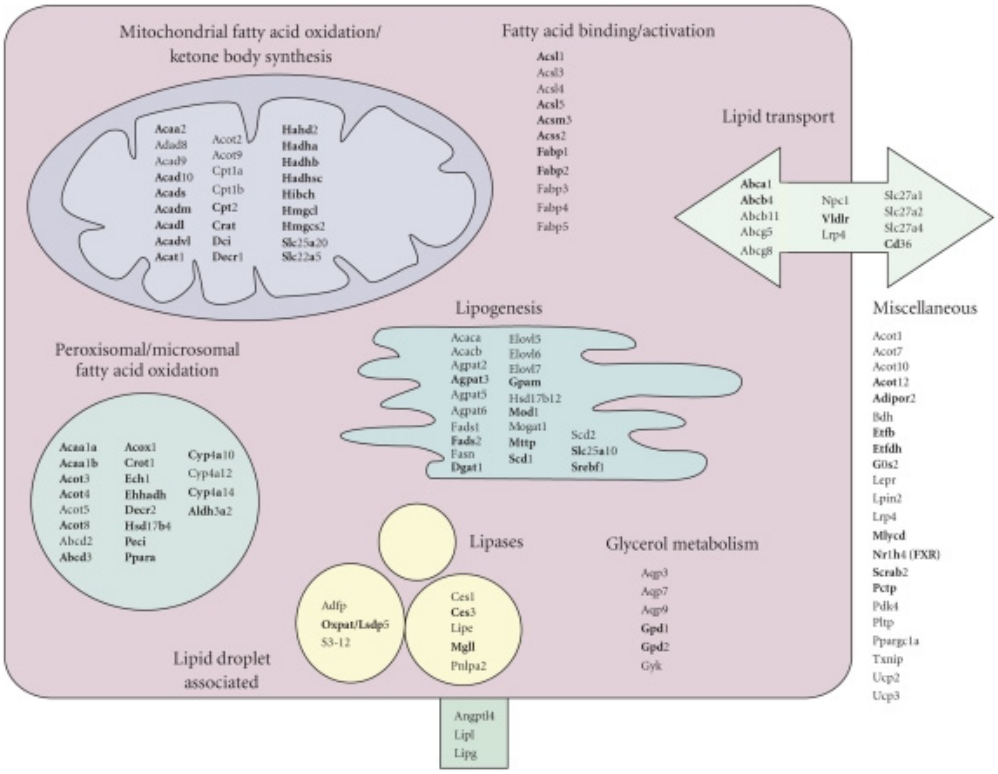


Figure 8: Central role of PPARα in fatty acid metabolism: DNA microarray defined PPARα responsive genes (from Rakhshandehroo M et al, 2007).

Most knowledge about the murine *Crat* promoter is known from data produced in our lab. Aniko Ginta Pordes published in her diploma thesis that gene expression of the murine carnitine acetyltransferase (*Crat*) gets

upregulated in case of L-carnitine supplementation after initial carnitine deficiency. The responsible promoter region for this effect could be detected. Reportergene-assays located this region within (-342/+15) in relation to the transcription start. [74]

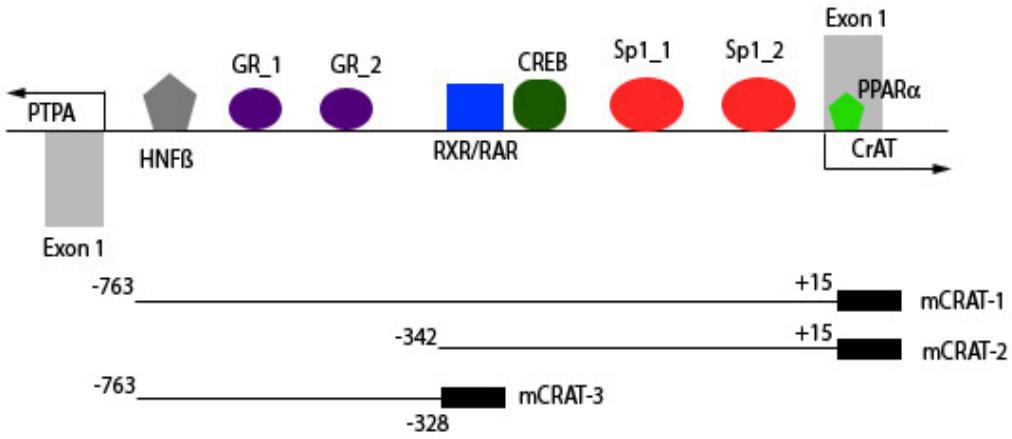


Figure 9: Reportergene Assay constructs:

The theoretical promoter analysis with bioinformatical tools like TESS and Patch several binding sites for regulating transcription factors could be identified. Analysing these elements with band shift assays revealed that a RXRα/RARγ element showed the strongest signals correlating with the "L-Carnitine effect".

The figure below shows the results of the theoretical promoter analysis.

HNF-beta (Foxa2)
-435 TTTGTTTGTT TGTTTTGGTG AGGGGTGATG GAGGGAATAG GTATGGCAGC AGTATTCTTG CGGAGCAAGG
GR GR
-365 CCACCTGTCA TCTTCTTTGT GCATGCCTCC ACCCAAGCAA TCTGAGACAG TCCCCAGTCA ACAGTTGTGT
TREB USF (CREB TGACCG)
-295 TCTCCTGCCA TTCCTAGAGT GACGTTCAAG GACACCAGTC AGACTTGAAC GCACGTGACC GCCTCGACAG
HES-1 RXR-alpha/RAR-beta CREB
-225 CTAAGGTGGC TTTCGGAGGC GGAGAGCACG AGCGGGAC**AG CGCCTACCGT TGTGACCC*CG* *TGACGGGAGC***
SP1
-155 *CCAGCGCCGG* GCTGCTAACA GCTGAGCTAG AGGTCCGTCT CCGCAAGCCC CGCCCTCTGC GGCCACTCCC
SP1
-85 CGGCTTGGGC CCACCCCGAG ATCGGGGCGA GCGGCCGGGG ACTGCGAGCC ACCAAAGCTC CGCCCCGTGA

-15 GTCCTGCCCC ACCGGCAGCC CGCCGGGCCC GGTGGCCGCG GGGCCGCCTC TACGGCTAGC GGCAGTGACT

+1 CGGCGACCTT GAACC

Figure 10: Theoretical promoter analysis performed with TESS (adapted from Ginta Pordes[74])

1.3 The importance of Acetyl-CoA pools

Mammalian cells contain sequestered acetyl-coA pools, which are functionally separated by intracellular membranes. The most important acetyl-coA pool is located in the mitochondrial matrix. There acetyl-coA depicts an important crossroad of metabolic pathways and fulfils important regulative functions between catabolic and anabolic processes.

A very important metabolic control function of acetyl-coA is the change from catabolism to anabolism.[17] During longer periods of fasting neuronal cells and red blood cells are still depending on glucose as energy source. In liver gluconeogenesis allows the synthesis of glucose out of non-carbohydrate precursors like lactate, alanine, glutamine and glycerol. [75] The rate-controlling step as depicted in Figure 11, is catalysed by the pyruvate carboxylase, which mediates the conversion of pyruvate to oxaloacetate. This step is allosterically induced by acetyl-coA and ATP.[76] So in liver cells, when high amounts of other energy sources are available, like fatty acids, the acetyl-coA pool and ATP levels are high and it is more likely that the conversion of pyruvate to oxaloacetate is catalysed and gluconeogenesis is induced than acetyl-coA gets introduced into the Krebs cycle.[77] Acetyl-coA also activates pyruvate dehydrogenase kinases, which phosphorylate the pyruvate dehydrogenase. Phosphorylated pyruvatedehydrogenase is inactive and further on the utilization of acetyl-coA in TCA circle is blocked. Also during fasting the pyruvate dehydrogenase gets blocked by phosphorylation sparing the free carbon compounds for gluconeogenesis. [78]

One aspect of the pathophysiology of Diabetes Mellitus Type 2 is characterized by increased glucose production through gluconeogenesis in liver as a consequence of excessive supply of energy sources as described above. [79]

An imbalanced intra-mitochondrial acetyl-coA pool has also been connected with abnormalities of the energy metabolism of skeletal muscle in diabetic patients. Insulin seems to be unable to mediate the switch from lipid to glucose metabolism within the muscle leading to decreased glucose

utilization in the muscle cells. This can be explained by raised pyruvatedehydrogenase phosphorylation due to high acetyl-coA levels. [80] Also in terms of cardiomyopathy defect regulation of acetyl-coA pools has severe consequences. The uncoupling between glycolysis and gluconeogenesis, which is controlled by acetyl-coA, leads to accumulation of products like lactate and protons that contribute to decreased efficiency of actin and myosin in the ischemic heart. [80]

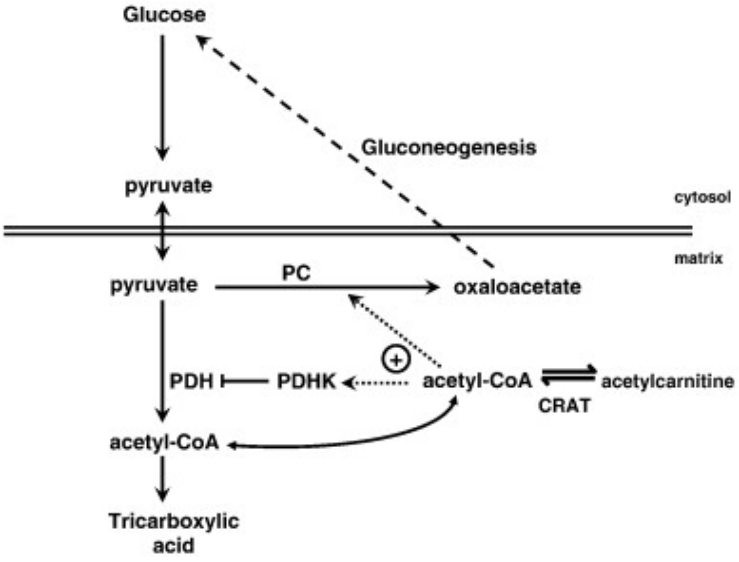


Figure 11: Central role of Acetyl-CoA in fatty acid and glucose metabolism in the liver (abbrevations: PC –pyruvate carboxylase, PDH pyruvate dehydrogenase, PDHK – pyruvate dehydrogenase kinase) from Zammit et al, 2009.

1.4 Medical and pharmacological aspects of L-Carnitine

1.4.1 Carnitine deficiencies

Defective fatty acid degradation has quite a big field of consequences. During fasting periods fatty acids are the main source of energy, but if this part of metabolism is defect, fatty acids accumulate in liver, skeletal and heart muscle. In liver inability of fatty acid catabolism leads to steatosis and decreased production of ketone bodies. During fasting brain tissue needs this ketone bodies as a consequence of glucose deficiency leading on the long run to loss of consciousness. Missing fatty acid degradation also leads do decreased acetyl-coA levels in liver, which normally induces the enzyme

pyruvate carboxylase as starting reaction of gluconeogenesis. If this branch of energy metabolism is also missing glucose, oxidation takes place without regeneration due to lack of gluconeogenesis leading to hypoglycaemia and finally to loss of consciousness. Accumulation of long-chain fatty acids and acylcarnitine-esters in heart muscle tissue leads to alteration of electric activity of the conduction system of the heart and further on to arrhythmia and cardiomyopathy. In muscle cells this accumulation of intermediates of the fatty acid metabolism leads to myopathy, hypotension and myoglobinuria.[81]

There are different deficiencies in the „carnitine-shuttle-system“, that can cause such symptoms.

1.4.1.1 Primary Carnitine Deficiency

Primary carnitine deficiency (OMIM 212140) is an autosomal recessive disorder caused by mutations in the *SLC22A5* gene coding for the cation/carnitine transporter OCTN2. This mutation leads to decreased ability of carnitine uptake into the cell across the plasma membrane. The following urinary loss of L-carnitine results in low plasma carnitine levels. Patients carrying such a mutation in this transporter present mainly metabolic and cardiac symptoms. [82]

The metabolic symptoms are almost observed in children under 2 years of age. These patients are lethargic and refuse eating. They show symptoms like hepatomegaly, hypoglycaemia and elevated CK (creatinin kinase). Treatment consists of intravenous administration of glucose. Without treatment primary carnitine deficiency would lead to coma and eventually death. [81]

Cardiomyopathy as a consequence of defect OCTN2 is mostly observed in older patients. Symptoms are associated with hypotension, an enlarged heart and decreased ventricular ejection fraction. Heterozygote carriers of mutated OCTN2 present cardiac hypertrophy. [83]

Diagnosis is confirmed via determination of plasma carnitine levels. Physiological carnitine levels in the plasma ranges between 25-50 µM,

OCTN2 deficient patients show free plasma carnitine levels below 5µM. To determine whether this low plasma carnitine level is caused by a defect OCTN2 transport protein activity is via a cell-based assay in fibroblasts. [84] Meanwhile analysis of OCTN2 is part of the newborn screening. 49 different mutations have been mapped within the SLC22A5 gene. Patients suffering from primary carnitine deficiency respond to dietary carnitine supplementation with high doses of 100-400 mg/kg/day. [81]

1.4.1.2 Secondary Carnitine Deficiency

Secondary deficiency of carnitine is characterized by increased excretion of carnitine in urine in form of acylcarnitine as a consequence of accumulating organic acids. [85] Secondary carnitine deficiency has many different causes: increased urinal loss of carnitine (Fanconi-Syndrom), pharmacological therapy with agents like pivampicillin or valproic acid [86], different inherited metabolic disorders (discussed below), haemodialysis, and carnitine poor diet. [87] L-carnitine supplementation in patients suffering from secondary carnitine deficiency leads to normalized plasma carnitine levels and improvements in several symptoms and complications like cardiac problems, muscle symptoms and hypotension. [88]

1.4.1.3 CPT-1 deficiency

CPT-1 deficiency (OMIM 255120) is an autosomal recessive metabolic disorder. Hypoketotic hypoglycemia elevated free fatty acids and liver enzymes characterize this disease. Plasma carnitine levels are increased. Onset of this disease is usually between birth and the age of 18 month. [81, 89] Children suffering from this disease show altered mental status and hepatomegaly. [81]

1.4.1.4 CACT deficiency

Deficiencies in the carnitine/acylcarnitine translocase (OMIM 212138) present metabolic misbalances like hypoketotic hypoglycemia especially after fasting periods, elevated creatinin kinases, hyperammonemia, very low free carnitine levels and elevated long-chain acylcarnitine

concentrations in the serum. Clinical symptoms comprise from seizures, irregular heartbeat and apnoea to skeletal muscle damage and cardiomyopathy. [81] A less severe type of CACT deficiency has been reported with delayed onset at the age of 15 month. [90] Since this disease is distinguishable from that caused by CPT2 deficiency only a fibroblast-based assay of CACT activity can confirm the diagnosis.[91] Therapy consists of frequent feeding with carbohydrates, low fat, of which most should be of medium-chain length to be independent from the carnitine shuttle. Additional L-carnitine supplementation is strongly recommended. [81, 92]

1.4.1.5 CPT-2 deficiency

CPT2 deficiency (OMIM 255110) is the most common inherited disorder caused by a defect in the mitochondrial long-chain fatty acid oxidation. Three different types of this disease have been characterized; the most common of those is the myopathic from, which mainly occurs in young adults and shows symptoms of rhabdomyolysis provoked by exercise, fasting or fever. [93] The two other types are the infantile (OMIM 600649) and the neonatal lethal forms (OMIM 608836). [94] The neonatal type presents shortly after birth with seizures, hepatomegaly, cardiomegaly, cardiac arrhythmia and neuronal migration defects. The infantile form usually has its onset between 6 and 24 months of age and shows similar symptoms to CACT deficient patients. [81] Final diagnosis can be confirmed by DNA mutation studies and fibroblast-based CPT2 activity assays.

Late-onset CPT2 deficiency is treatable with restriction of fat and long-chain fatty acids and increased dietary carbohydrate. Further on mild CPT2 deficiency is treatable with fibrates. [81, 95]

1.4.2 L-Carnitine and its analogues as pharmaceuticals in cardio-metabolic disorders

The above-described deficiencies of proteins involved in fatty-acid metabolism underline the importance of L-carnitine and the acyl-carnitine

shuttle system. One common symptom of most deficiencies concerning this system is connected to cardiomyopathy.

The first evidence for the therapeutic benefit of L-carnitine in the ischemic heart has been shown in pigs. [96] Especially propionyl carnitine leads to improved heart function after ischemia and heart failure. This can be explained by the fact that loss of L-carnitine takes place during ischemia. [97] Supplementation with L-carnitine in cases of ischemia and cardiac failure improves contractive function by modulating the acetyl-coA pool in the mitochondria by shifting the CRAT mediated reaction towards acetyl-L-carnitine. [17]

1.4.3 L-Carnitine and Diabetes Mellitus Type II

Preclinical experimental data also reported a possible therapeutic effect of L-carnitine on diabetic conditions. In a study with streptozotocin-treated diabetic rats the administration of L-carnitine (3g/kg) over 6 weeks leads to significant decrease of plasma glucose levels. Also the reversal of the diabetes associated symptoms of polydipsia and glocosuria was observable.[98]

A more recent study also showed the beneficial effect of L-carnitine on fructose induced diabetic metabolism in rats. The rat model was characterized by severe impairment of insulin sensitivity, increased hepatic gluconeogenesis, dyslipidemia and hyperglycemia. Administration of L-carnitine intraperitoneally leads to normalization of glucose and insulin levels. The plasma values of free fatty acids and triglyzerides also normalized after L-carnitine administration. The rats returned to a diabetic metabolic state after withdrawal of supplementary L-carnitine.[99]

1.5 PPAR-alpha and its regulative effect on fatty acid degradation

Peroxisome proliferator-activated receptor alpha is a member of a subfamily of nuclear receptors. Together with other members of this subfamily (PPARγ and PPARδ) PPARα modulates signalling pathways of

intermediary metabolism and inflammation outlining the clinical importance of these nuclear receptors. [100] PPARα gets activated by derivatives of fibric acid (like fenofibrate and bezafibrate) and lowers triglyzerides and raises HDL (High density lipoproteins). [101] The PPARs are a family of liganded-activated receptors, which form heterodimers with RXR (retinoic X receptor) that bind to specific response elements (PPREs) to alter transcription levels of target genes.

Polyunsaturated and saturated fatty acids are PPAR ligands. [102] Also a variety of other lipids have been identified as PPAR ligands like prostaglandins,[103] leukotrienes, acyl-CoA species. So far no endogenous PPAR ligand has been identified.

PPARα expression takes place at high levels in liver cells where it promotes fatty acid oxidation, lipid transport and gluconeogenesis. [104]

1.6 Goals of the project

The previous results lead to the question which factor mediates the "Carnitine-effect" on the CRAT gene, meaning the upregulation of this gene. For this aim we wanted to repeat the theoretical promoter analysis with TESS and also new program called Patch, in order to include new findings gathered in the last few years in the field of transcription factors. This led us to a novel-binding site, namely a HNF-3β (FoxA-2). Further literature research also led us to the transcription factors PPARα and LXRα/β. These factors had to be tested via supershift assays.

Another approach for identifying the transcription factor responsible for the L-carnitine response was found in southwestern-blotting. This technique allows estimating the size of a transcription factor binding to a specific site in a promoter. With further programs like *"molwsearch"* possible transcription factors can be determined.

Another experimental approach was the measurement of RNA-levels via RT-Real Time PCR, in order to quantify the mRNA levels in cells, which were cultured under different metabolic and pharmacological conditions.

Here we wanted to investigate the effect of L-carnitine on transcriptional activity of the CRAT gene in course of time and also we wanted to test the effect of different concentrations of L-carnitine on CRAT transcription in the murine liver cell line TIB73.

2 Methods

2.1 Cell culture

All experiments were performed with TIB73 murine liver cells.

2.1.1 Propagation of cells

The cells were grown in 100mm petri-dishes with 10ml of Dulbecco´s modified Eagles´s medium (DMEM) supplemented with 10% foetal calf serum and 1% Antibiotics (30mg/l penicillin, 50mg/l streptomycin sulphate). Cells were grown at 37°C with 100%humidity and 7.5% CO2 near to confluence and were split in order to allow continuous growth. Cell splitting was performed after removal of the medium and incubation of the cells with 6-7 drops of Trypsin/EDTA at 37° for some minutes. The de-attachment and mobilization of the cells was verified through observation under the microscope. The cells resuspended in 10ml DMEM+10%FCS and AB+G were counted in the CASEY-cell counter and seeded onto fresh 100mm petri dishes in desired density.

2.1.2 Freezing of the cells

Growing cells with a density of 2 million cells per petri dish were treated with Trypsin/EDTA to detach the cells from the petri dish. The cells were resuspended in 1.5ml DMEM+10%FCS+AB+G and transferred into a 1.8ml cryo-freezing tube. Afterwards 150µl of DMSO were added and the mixed with the cell suspension through inverting the tube. The cells were frozen at -80°C.

2.1.3 Thawing of cells

Frozen cells were slowly thawed on ice and finally transferred in a 100mm petri dish with 10ml of pre-warmed DMEM+10%FCS+AB+G and further on cultivated at 37°, 100%humidity and 7.5% CO2.

2.1.4 Metabolic conditions for cell cultivation

For the band shift assays, supershift assays, southwestern blot analysis, western blot analysis nuclear extracts were prepared from TIB73 mouse liver cells cultured under different metabolic conditions.

- **Normal (N):** TIB73 cells were grown in DMEM+10%FCS+Antibiotics +Glutamine until they reached confluence – meaning about 2-3 million cells on a 100mm petri dish.
- **Starvation (St):** TIB73 cells were grown in DMEM+0,5%FCS+Ab. Medium change had to be performed each day in order to offer essential nutrients.
- **Serum Induction (SI):** at first TIB73 cells were grown in Starvation Medium (DMEM+0,5%FCS+Ab) for 2 days. Afterwards the cells got serum induced, by adding DMEM+20%FCS+Ab for 24 hours.
- **0µM-L-carnitine (dialysed FCS):** TIB73 cells were grown in DMEM+10% FCS+AB+G for 24 hours. Afterwards these cells were cultivated in DMEM+ 10% dialyzed FCS+Ab+G
- **40µM-L-carnitine:** TIB73 cells grown on medium with dialysed FCS got induced with 40µM-L-carnitine for a desired amount of time.
- **80µM-L-carnitine:** TIB73 cells grown on medium with dialysed FCS got induced with 40µM-L-carnitine for a desired amount of time.
- **120µM-L-carnitine:** TIB73 cells grown on medium with dialysed FCS got induced with 40µM-L-carnitine for a desired amount of time.
- **5µM-dexamethasone:** TIB73 cells were grown till confluence reached 2/3 of the petri dish. These cells got supplemented with DMEM+10%FCS+Ab+G +5µM dexamethasone
- **10µM-dexamethasone:** TIB73 cells were grown till confluence reached 2/3 of the petri dish. These cells got supplemented with DMEM+10%FCS+Ab+G +10µM dexamethasone

2.1.5 Media and solutions for cell culture

DMEM (Dulbecco´s modified eagle medium) +10% FCS and antibiotics:
Powdered Dulbecco´s Modified Eagle Medium was dissolved in water

supplemented with 3,7g/l NaHCO3 and filtered using a sterile filter. DMEM (445ml) was supplemented with 5ml antibiotics (AB) and 50ml fetal calf serum (FCS, Gibco BRL).

Dialyzed FCS

Dialysis of FCS was performed against 1xPBS for 48 hours with 5 buffer changes.

10xPBS

80 g/l	NaCl
2 g/l	KCl
16.5 g/l	Na_2HPO_4
2 g	KH_2PO_4
adjust	to pH 7.4

2.2 Nuclear Extracts

The isolation of nuclear extracts was performed after the protocol described from Siu et al.[105]

1. Cells were cultivated on 100mm petri dishes till they reached confluence of 80-90%.
2. Cells were washed 3 times with 1xPBS.
3. Per dish 2ml of Cell Lysis Buffer were added.
4. Cells were dislodged from the petri dish using a rubber policeman and afterwards transferred into a 2ml eppendorf tube.
5. The cell suspension was centrifuged at 2,500g at 4°C for 5min.
6. The supernatant was removed without disturbing the pellet.
7. The pellet was resuspended in 500µl of Nuclear Extract Buffer and incubated at 4°C on a rocker platform (50 r.p.m)
8. The cell debris was removed by centrifugation at 12,000g for 10 min at 4°C.

9. The supernatant containing the nuclear extracts was transferred into a new Eppendorf tube and quick frozen in liquid nitrogen and afterwards stored at -80°C.
10. Protein concentration was measured right before use.

2.2.1 Bradford Assay

In order to measure protein concentration Bradford concentrate got diluted 1:5 with ddH_2O. 2µl of the nuclear extract solution were added to 1ml Bradford diluent and incubated at room temperature for 5 minutes and absorbance was measured at 595nm. BSA protein standards (1µg-10µg) were used for quantification.

2.2.2 Buffers and Solutions

Cell Lysis Buffer:

20 mM	HEPES pH 7.6
20 %	Glycerol
10 mM	NaCl
1.5 mM	$MgCl_2$
0.02 mM	EDTA
0.1%	Triton X
1 mM	DTT

Protease Inhibitors (Roche) and PMSF were added right before use.

Nuclear Extract Buffer:

20 mM	HEPES pH 7.6
20 %	Glycerol
500 mM	NaCl
1.5 mM	$MgCl_2$
0.02 mM	EDTA
0.1%	Triton X
1 mM	DTT

Protease Inhibitors (Roche) and PMSF were added right before use.

2.3 Band Shift Assays

For Band Shift analyses different ODN´s were designed:

- **SP1_2:**
  sense: 5' GCC ACC AAA GCT CCG CCC CGT GAG TC 3'
antisense: 5'GAC TCA CGG GGC GGA GCT TTG GTG GC 3'

- **RXR/RAR:**
  sense: 5' CCT ACC GTT GTG ACC CCG TGA CGG G 3'
antisense: 5' CCC GTC ACG GGG TCA CAA CGG TAG G 3'

- **HNFβ:**
  sense: 5' GTT TGT TTG TTT GTT TTG GTG 3'
antisense: 5' CAC CAA AAC AAA CAA ACA AAC 3'

- **PPARα_1:**
  sense: 5' GGC ACC TTG AAC CTA GAA CCG 3'
antisense: 5' CGG TTC TAG GTT CAA GGT GCC 3'

- **RXR_1:**
  sense: 5' AGC GCC TAC CGT TGT GAC CCC G 3'
antisense: 5' CGG GGT CAC AAC GGT AGG CGC T 3'

- **Creb:**
  sense: 5' CGT GAC GGG AGC CCA GCG CCG G 3'
antisense: 5' CCG GCG CTG GGC TCC CGT CAC G 3'

- **USF/CREB:**
  sense: 5' GAA CGC ACG TGA CCG CCT CG 3'
antisense: 5' CGA GGC GGT CAC GTG CGT TC3'

For supershift analysis following antibodies were used:

SP1: sc-420

PPARα: sc-9000 X

LXRα/β:sc-1201 X

Nurr77: sc-7013 X

Trap220: sc-74475

PPARγ: sc-7273

CREB: sc-186 X

2.3.1 Labelling of oligonucleotide probe – Spun column method

Oligonucleotides were marked radioactively with γ-^{32} phosphate by T-4 polynucleotide kinase reaction (PNK reaction)

Reaction mix:

1 µl	Oligo desoxynucleotide (100ng/µl)
5 µl	10x T4- polynucleotidkinase buffer
4 µl	γ-^{32} ATP
1.5 µl	T4-PNK
38.5 µl	ddH_2O
Ad 50 µl	Total volume

Reaction mix was incubated for 30 minutes at 37°C and stopped by addition of 50 µl of 1xTEN buffer. The whole reaction mix was transferred on spun column and centrifuged at 2.500rpm for 5 minutes.

Before and after centrifugation 1µl of the radioactive reaction mix was mixed with 99 µl of dH_2O and used for Cherenkov activity measurement to control the incorporation of γ-^{32} ATP.

Afterwards 2-3 µl of asODN was added and heated to 90°C. For proper annealing the reaction mix was kept at room temperature for 30 minutes before storage at 4°C.

2.3.2 Reaction mix for band shift and supershift assay

10 µl	2x Reaction Buffer
1 µl	Sonicated salmon sperm (5µg/ml)
1 µl	ssODN (γ-^{32} ATP)
x µl	10µg nuclear extract
y µl	ddH_2O
0.5-1-5 µl	antibody solution
Ad 20µl	Total volume

Reaction mix was incubated on ice in the dark for 30 minutes before loading the reaction mix onto an analytical non-denaturating polyacrylamide gel. Gel electrophoresis was performed at 100-120 V at 4°C in 1xTBE for 7-8 hours.

After electrophoresis the gel was transferred on Whatman paper, covered with PE foil and dried under vacuum for 2-4 hours and exposed to x-ray films for 24 hours up to 1 week.

2.3.3 Band shift gel

6.36 ml	30% Acrylamide-Bisacrylamide
36.84 ml	dH_2O
2.88 ml	10x TBE
240 µl	APS (10%)
48 µl	TEMED

2.3.4 Buffers and solutions

10x TBE (1000 ml)

108 g	TRIS
55 g	Boric acid
40 ml	EDTA (pH 8.0)

10x TEN

0.1 M	TRIS
0.01 M	EDTA (pH 8.0)
1 M	NaCl

5x Binding Buffer

20%	glycerol
100 mM	TRIS-HCl pH 8.0
300 mM	KCl
25 mM	$MgCl_2$
500 µg/ml	bovine serum albumine

30% Acrylamide-Bisacrylamide (100ml)

38 g	acryl amide
2 g	bisacrylamide
1 spoon	Dowex
Ad 100 ml	ddH_2O

2.4 Southwestern Blotting

This technique allows the determination of the molecular weight of factors binding to a specific oligonucleotide sequence. For this aim again ODN´s were labelled with radioactive γ-^{32}ATP. The protocol for this experimental approach was obtained from Siu et al. [105]

For southwestern analysis nuclear extracts were prepared as discussed above. First a denaturing SDS-page (10% SDS acrylamide gel) was performed to separate the proteins per size. Afterwards the proteins were transferred on to a nitrocellulose membrane by electro-blotting (wet blotting technique at 100 V at 4°C for 1 hour). For active binding of proteins to oligonucleotides proteins had to be renaturated. For this aim the nitrocellulose membrane was incubated with TNED buffer with 5% skim milk for 24 hours. The renatured membrane bound proteins were incubated

with the radioactive γ-^{32}labelled oligonucleotides of a specific DNA sequence in TNED buffer with 2.5% skim milk for 24 hours. After 3 washing steps with TNED buffer the blot was dried for some minutes at room temperature. The interaction is finally visualized via autoradiography.
The comparison of the resulting signals with the molecular weight marker (PageRuler ^{TM} – Prestained Protein Ladder) on the original nitrocellulose membrane allows the determination of the weight of the protein marked by the γ-^{32}labelled ODN. Subsequent use of the online program “molwsearch” from the website www.gene-regulation.com [106] allows the listing of possible transcription factors of a defined protein size.

2.4.1 Buffers and Solutions

10% SDS Separating Gel

dH_2O	2,03 ml
30% acrylamide	2,5 ml
1 M Tris, pH 8.8	2,81 ml
10% SDS	75 µl
10% APS	75 µl
Temed	7,5 µl

Stacking Gel

dH2O	2,55 ml
30% acrylamide	0,625 ml
1 M Tris, pH 8.8	0,475 ml
10% SDS	37,5 µl
10% APS	37,5 µl
Temed	2,5 µl

1x TNED Buffer

10 mM	TRIS HCl pH 7.5
50 mM	NaCl
0.1 MM	EDTA pH 8.0
1mM	DTT

5x Western Blot Running Buffer

100 mM	TRIS
1 M	glycine
1.2%	SDS

1x Western Blotting Buffer

25 mM	TRIS
190 mM	glycine
20%	methanol

2.5 Western Blot

For protein analysis 20µg of nuclear proteins were mixed with sample buffer and incubated at 95°C for 5 minutes. Samples were loaded on a 10% SDS polyacrylamide gel (SDS-PAGE). For orientation and size determination a prestained molecular weight marker was loaded. Electrophoresis was performed at 120V. Afterwards proteins were transferred onto a nitrocellulose membrane with wet blot technique. (250 mA for 2.5 hours).
Membranes were blocked after transfer with 1xPBS-T containing 5% skim milk at room temperature for at least one hour. Depending on manufacturers protocols primary antibody solution was prepared (1:100 up to 1:1000). Incubation of the blot with primary antibody was performed overnight at 4°C followed by 3 washing steps with 1xPBS-T. The blot was incubated with a 1:10.000 secondary antibody solution (HRP-conjugated secondary antibody) for at least one hour at RT and subsequently the blot was washed again 3 times with 1xPBS-T. For detection ECL solution was

prepared fresh as a 1:1 mixture of the two ECL solutions (Perkin Elmer Life Science). The blot was incubated with ECL solution for 2 minutes, followed by removal of the solution. The blot got exposed to radio films for various amounts of time (from 1 minute to 1 hour).

2.5.1 Buffers and solutions

Running buffer and transfer buffer were prepared as described for southwestern blotting.

Antibody:

For PPARα Western Blots sc-9000 anti-PPARα antibodies by Santa Cruz Biotechnology were used. Best results were obtained with a ratio of 1:500.

Sample buffer.

30 mM	TRIS HCl pH 6.8
30%	glycerol
10%	SDS
1mM	DTT
0.002%	bromophenol blue

Blocking Solution

1x	PBS
0.1%	Tween 20
5%	skim milk (powder)

2.6 RT-Real Time PCR

2.6.1 Cell Culture

2.6.1.1 Propagation of cells

The cells were grown in 100mm petri-dishes with 10ml of Dulbecco´s modified Eagles´s medium (DMEM) supplemented with 10% foetal calf serum and 1% Antibiotics (30mg/l penicillin, 50mg/l streptomycin sulphate).

Cells were grown at 37°C with 100%humidity and 7.5% CO2 near to confluence and were split in order to allow continuous growth. Cell splitting was performed after removal of the medium and incubation of the cells with 6-7 drops of Trypsin/EDTA at 37° for some minutes. The de-attachment and mobilization of the cells was verified through observation under the microscope. The cells were resuspended in 10ml DMEM+10%FCS + Ab+G, counted in the CASEY-cell counter and seeded onto fresh 100mm cell culture-dishes in desired density.

2.6.1.2 Metabolic conditions for cell cultivation

For the real-time analysis mRNA was isolated from TIB73 mouse liver cells cultured under different metabolic conditions.

- **Normal (N):** TIB73 cells were grown in DMEM+10%FCS+Antibiotics +Glutamine until they reached confluence – meaning about 2-3 million cells on a 100mm petri-dish.
- **Starvation (St):** TIB73 cells were grown in DMEM+0,5%FCS+Ab Medium change had to be performed on a daily basis in order to offer essential nutrients.
- **Serum Induction (SI):** at first TIB73 cells were grown in Starvation Medium (DMEM+0,5%FCS+Ab) for 2 days. Afterwards the cells got serum induced, by adding DMEM+20%FCS+Ab for 24 hours.
- **10µM fenofibrate:** normal grown cells (DMEM+10%FCS+Ab) were cultured till the requested density. Afterward these cells were supplemented with 10µM fenofibrate for 3 hours before harvesting.
- **20µM fenofibrate:** normal grown cells (DMEM+10%FCS+Ab) were cultured till the requested density. Afterward these cells were supplemented with 20µM fenofibrate for 3 hours before harvesting.
- **40µM fenofibrate:** normal grown cells (DMEM+10%FCS+Ab) were cultured till the requested density. Afterward these cells were supplemented with 40µM fenofibrate for 3 hours before harvesting.

- **0.5µM LXR agonist:** normal grown cells (DMEM+10%FCS+Ab) were cultured till the requested density. Afterward these cells were supplemented with 0.5µM GW3965 for 3 hours before harvesting.
- **1µM LXR agonist:** normal grown cells (DMEM+10%FCS+Ab) were cultured till the requested density. Afterward these cells were supplemented with 1µM GW3965 for 3 hours before harvesting.
- **1.5µM LXR agonist:** normal grown cells (DMEM+10%FCS+Ab) were cultured till the requested density. Afterward these cells were supplemented with 1.5µM GW3965 for 3 hours before harvesting.
- **5µM retinoic acid:** normal grown cells (DMEM+10%FCS+Ab) were cultured till the requested density. Afterward these cells were supplemented with 5µM retinoic acid for 3 hours before harvesting.
- **10µM retinoic acid:** normal grown cells (DMEM+10%FCS+Ab) were cultured till the requested density. Afterward these cells were supplemented with 10µM retinoic acid for 3 hours before harvesting.
- **15µM retinoic acid:** normal grown cells (DMEM+10%FCS+Ab) were cultured till the requested density. Afterward these cells were supplemented with 15µM retinoic acid for 3 hours before harvesting.

2.6.2 RNA-Isolation

RNA isolation was performed with the GeneJet RNA Purification Kit by Fermentas according the manufacturers protocols with small modifications:

- Growth medium was removed
- Cells were washed with 1xPBS followed by aspiration of PBS
- The cells were rubbed off with a rubber policemen in an adequate amount of PBS and transferred into a Eppendorf tube
- Cells were centrifuged at 1300 rpm for 5min
- Supernatant got discarded
- Pellet was resuspended in 600µl Lysis Buffer
- Cell suspension was vortexed for 10sec until it is well homogenized
- 360µl of 96% EtOH were added and mix sample by pipetting

- Up to 700µl of lysate were transferred to the Purification Column inserted in a collection tube
- This step was repeated until all of your lysate had been transferred into the column
- Flow-through was discarded and the purification column was placed the on a new collection tube (2ml)
- 700µl of Wash Buffer 1 were added
- Centrifugation with 10.000 rpm for 1'
- Flow-through was discarded
- 600µl of Wash Buffer 2 were added
- Centrifugation with 10.000 rpm for 1'
- 200µl of Wash Buffer 2 were added
- Centrifugation with 10.000 rpm for 2'
- Purification column got transferred to microcentrifuge tube
- 50µl of nuclease free water were added
- Centrifugation at 10.000 rpm for 1'
- RNA concentration was measured with NanoDrop photometer and stored at –80°

2.6.3 cDNA Synthesis

cDNA synthesis was performed after manufacturers protocols with the Revert Aid M-MuLV Reverse Transcriptase Kit:
3µg of RNA and 1µl of oligo dT-primers were added to DEPC treated water to a volume of 11µl. Subsequently the samples were heated to 70°C for 5 minutes followed by incubation on ice for another 5 minutes. In the next step 4µl of 5x reaction buffer by Fermentas, 2µl of dNTP mix (10mM stock), 1µl of RNase Inhibitors (Fermentas) and 1µl of DEPC treated water were added and incubated at 37°C for 5 minutes. Afterwards 1µl of Reverse Transcriptase (by Fermentas) was added and incubated at 42°C for 1 hour. The enzyme got deactivated afterwards by heating the samples to 70°C for 1 minute followed by an additional incubation on ice for 4 minutes. Samples were stored until use at -80°C.

2.6.4 Primers

Primers for PCR were designed by use of the web tool “Primer3”.

muβ-Actin:
sense 5'- GCGTGACATCAAAGAGAAG -3'
antisense 5'- AGGAGCCAGAGCAGTAATC -3'
T_a: Block-PCR 52°C, RT-PCR 55°C.

muCRAT:
sense 5'- GCTCAGCCTCCATAGACTCG -3'
antisense 5'- AGCAATGGCGTAAGAGGTGT -3'
T_a: Block-PCR 52°C, RT-PCR 55°C.

muCPT1a:
sense 5'- CCAGGCTACAGTGGGACATT -3'
antisense 5'- GAACTTGCCCATGTCCTTGT -3'
T_a: Block-PCR 50°C, RT-PCR 53°C.

muCACT:
sense 5'- TGGACACTGTTGCTGAGAGG -3'
antisense 5'- TTGGCCAAAGGTATCGAGTC -3'
T_a: Block-PCR 52°C, RT-PCR 55°C.

muOCTN2:
sense 5’- ACAGTATCCCGTTCGACACG -3’
antisense 5’- ACACCAGGTCCCAGTCTGTC -3’
T_a: Block-PCR 52°C, RT-PCR 55°C.

muCPT2
sense 5’- TCCTCGATCAAGATGGGAAC -3’
antisense 5’- GATCCTTCATCGGGAATCA -3’
T_a: Block-PCR 52°C, RT-PCR 55°C.

2.6.5 Block PCR and gel extraction

Classic block PCRs on gradient cyclers were performed with each designed primer-pairs in order to check their specifity and reactivity. After gel electrophoresis the PCR products were cut out of the gel, extracted after the manufacturer's protocol with the "Concert Gel Extraction System" by Gibco BRL and DNA concentration was measured. From this "DNA-stocks" defined DNA concentrations were diluted from 1µg/µl down to 1ag/µl as standards for the following real-time PCR.

2.6.6 Real-Time PCR

Real-Time PCR was performed based on LightCycler Fast Start DNA Master SYBR Green I.

2.6.6.1 Pipetting scheme for RT-PCR and protocol for Roche Capillary LightCycler

The following scheme depicts the volumes for one LightCycler reaction. LightCycler capillaries were precooled at 4°C in centrifuge adapters.

- The following components were prepared. Depending on number of reactions the amounts were multiplied and prepared as a mastermix in a 1.5ml eppendorf tube.

10.2µl	ddH_2O
1,3µl	$MgCl_2$
0.5µl	primer sense
0.5µl	primer antisense
1.5µl	SYBR Green Master Mix

- Components were gently mixed
- 14 µl of the mastermix were pipetted into the precooled capillaries
- 1µl of the sample cDNA was added
- The capillaries got sealed with a stopper

- Together with the precooled adapter the capillaries got placed in a microcentrifuge and spun down
- The capillaries were placed in the rotor of the LightCycler and the run was started

For each tested gene the annealing temperature was first optimised on a gradient cycler. For each gene a standard curve was obtained using the LightCycler. cDNA samples were diluted 1:100 right before use. All samples were measured at least in triplicates. The mean of these measurements was normalized to the housekeeping gene β-actin, which itself was measured three times. The RT-PCR results are based on relative quantification. [107]

2.6.7 Statistical Analysis

Continuous data was presented as mean and standard deviation. Differences between groups were assessed by means of impaired Students T-Test. Statistical analysis was performed with SPSS Statistics 17.0 (IBM, Armonk, USA).

3 Results

3.1 Results of the theoretical promoter analysis

```
        HNF-beta (Foxa2)
-435 TTTGTTTGTT TGTTTTGGTG AGGGGTGATG GAGGGAATAG GTATGGCAGC AGTATTCTTG CGGAGCAAGG
                        GR                                                    GR
-365 CCACCTGTCA TCTTCTTTGT GCATGCCTCC ACCCAAGCAA TCTGAGACAG TCCCCAGTCA ACAGTTGTGT
                           TREB                              USF   (CREB TGACCG)
-295 TCTCCTGCCA TTCCTAGAGT GACGTTCAAG GACACCAGTC AGACTTGAAC GCACGTGACC GCCTCGACAG
                                    HES-1              RXR-alpha/RAR-beta    CREB
-225 CTAAGGTGGC TTTCGGAGGC GGAGAGCACG AGCGGGACAG CGCCTACCGT TGTGACCCCG TGACGGGAGC
                                                        SP1
-155 CCAGCGCCGG GCTGCTAACA GCTGAGCTAG AGGTCCGTCT CCGCAAGCCC CGCCCTCTGC GGCCACTCCC
                                                                  SP1
 -85 CGGCTTGGGC CCACCCCGAG ATCGGGGCGA GCGGCCGGGG ACTGCGAGCC ACCAAAGCTC CGCCCCGTGA

 -15 GTCCTGCCCC ACCGGCAGCC CGCCGGGCCC GGTGGCCGCG GGGCCGCCTC TACGGCTAGC GGCAGTGACT
               PPARα
  +1 CGGCGACCTT GAACC
```

Figure 12: Result of the theoretical promoter analysis performed with Transcription Element Search System (TESS), a bioinformatical tool to search for transcription factor binding sites within a determined region. Using another tool named PATCH a second bioinformatical promoter analysis had been performed. (Figure adapted from Aniko Ginta Pordes[74])

As mentioned above a first specific bioinformatical analysis had already been performed some years ago.[74] In order to consider new research results this type of theoretical analysis had to be repeated and in fact several new possible binding sites were proposed by TESS. For a better understanding the following schematic figure of the CRAT promoter shows the different proposed transcription factor binding sites and their relation to two genes, which enclose this bidirectional promotor (*Crat* and *Ptpa*).

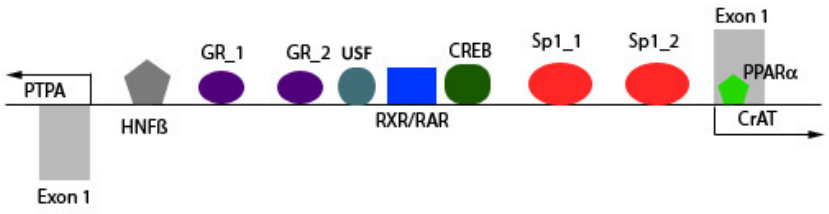


Figure 13: Schematic figure of proposed transcription factor binding sites after theoretical promoter analysis.

This re-evaluation of the CRAT promotor led to further analysis of the following putative binding sites, which had not been considered before:

- USF_CREB: TESS proposed binding sites for these two different transcription factors in a region from 234nt to 243nt upstream the promoter start. According to the data generated by TESS the two binding sites are overlapping. Since this region is located within the reporter-gene assay construct mCRAT-2 apart for the last nucleotide (nt243) and because CREB is a multifunctional transcription factor this site was included for further analysis.
- HNF3β: The binding site for the Hepatic Nuclear Factor 3 beta or also called FoxA2, is localized far out the mCRAT-2 construct, but based on the facts told by the literature that this factor is especially active in liver cells and it is of influence in cases of MODY (maturity onset diabetes of the young)[108] we decided to subject HNF3β further analysis.
- PPARα: The influence of PPARα on genes of the acylcarnitine transferase system has been reported repeatedly.[56],[48] PATCH search identified a PPARα binding site already within the first exon. This was reason enough for further investigation of this site.
- Although the hitherto analysis of the other proposed transcription factor binding sites (SP1, RXR/RAR, GR) did not give any hints about the regulative mechanism of Crat transcription under L-carnitine influence the re-analysis of these sites also had to be part of the thesis.

3.2 Analysis of the proposed SP1_2 binding site

SP1 an ubiquitously expressed transcription factor, can often be found in TATA less promoters mediating the basic transcription especially of housekeeping genes. Through its C(2)H(2) type zink-finger domain SP1 is able to activate or repress transcription of many genes. Recent evidence indicates that the whole SP-family has besides its “housekeeping” activity

also key functions as mediators of gene expression induced by insulin and other hormones.[109] SP1 is able to recruit the important factors TBP/TFIID, which allows SP1 to promote transcriptional initiation, but not elongation. This ability to recruit TFIID effectly explains the ability of SP1 to induce transcription in TATA-less promoters.[110]

TESS search proposed two distinct binding sites for SP1 within the CRAT promoter. In previous band shift experiments, published by Aniko Ginta Pordes in her diploma thesis [74], especially the SP1 site which is closer to the promoter start - further on named SP1_2 - showed stronger indications for being involved in the regulation of CRAT transcription. Nuclear extracts bound the SP1_2 site with a higher efficacy than the SP1_1 site resulting in a stronger band shift signal.

3.2.1 Southwestern blot

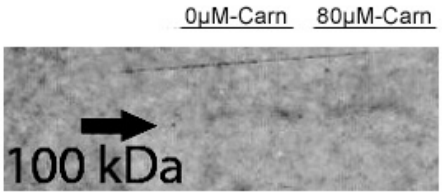


Figure 14: SP1 southwestern blot with signals proximate to the 100kDA marker.

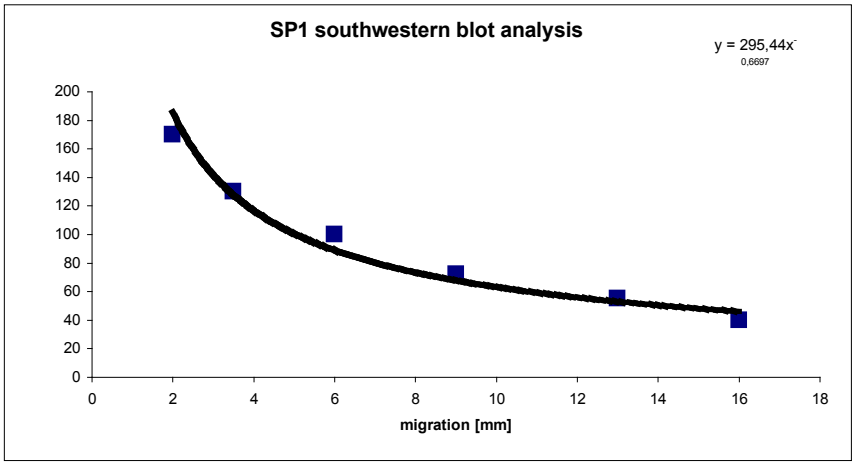


Figure 15: Size determination of the nuclear factor binding the ^{32}p labelled SP1_2 ODN through determination of migration length. Signal was detected 6mm downstream the migration start resulting in a molecular weight of the probed protein of 105kDa.

The southwestern analysis showed that the binding of the γ^{32}-ATP labeled ODN gave rise to a signal close to the 100kDa marker line. The subsequent "molwSearch" in the "Transfac database" tightened the suspicion that the ^{32}P-labeled oligonucleotide bound to SP1, which itself has a molecular weight of 105kDa.

3.2.2 Band Shift Assays

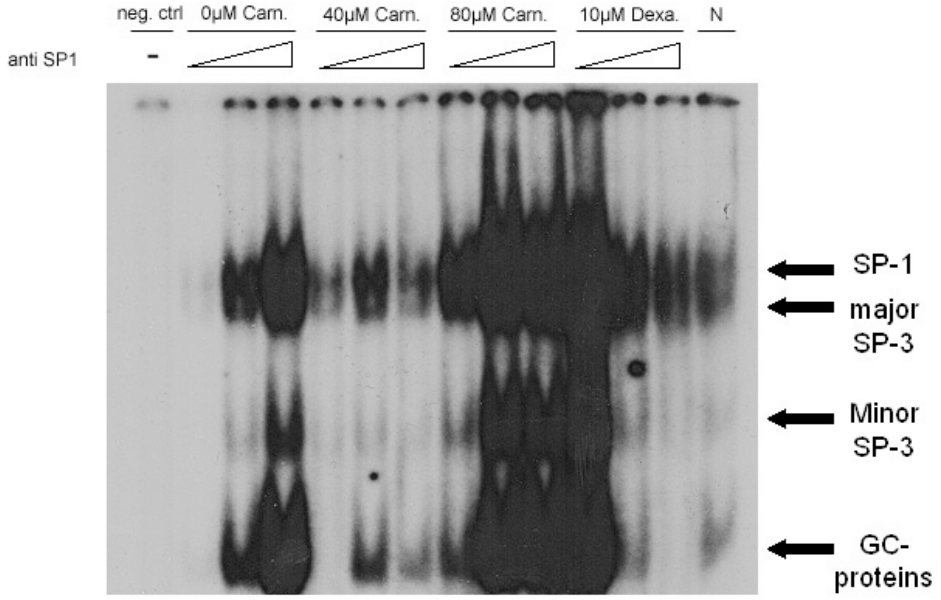


Figure 16: SP1 EMSA with rising L-carnitine and antibody concentrations

The outcome of this mobility shift experiment approved the fact that the analyzed sequence is a SP1 binding site. The typical band shift pattern of SP1 sites can be seen above. Not only SP1 itself is binding to this element, also major and minor SP3 and the GC-box proteins are able to bind this specific element in the CRAT promoter in vitro. [111] We did not observe an influence of L-carnitine on the binding affinity of the nuclear abstracts to the ^{32}P labelled DNA probe. This supports the thesis that SP1 very likely is involved in the basic transcription of the CRAT gene, but is not sensitive to L-carnitine supplementation.

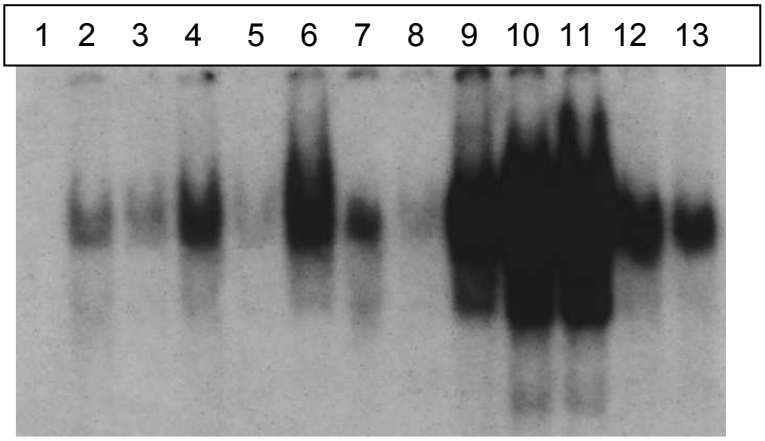


Figure 17: SP1 EMSA with supershift signal with extracts from dexamethasone-induced cells.

1	**negative control**	**8**	**serum induction**
2	**normal**	**9**	**dexamethasone + 1µl SP1 antibody**
3	**0-carnitine**	**10**	**starvation+ 1µl SP1 antibody**
4	**40-carnitine**	**11**	**Serum induction + 1µl SP1 antibody**
5	**80-carnitine**	**12**	**40-carnitine + 1µl SP1 antibody**
6	**10 dexamethasone**	**13**	**80-carnitine + 1µl SP1 antibody**
7	**starvation**		

This combined band shift and supershift experiment using the SP1-oligo with nuclear extracts from cells of different cultivation types showed a supershift in the lane 9. In this lane nuclear extracts from dexamethasone treated cells were loaded.

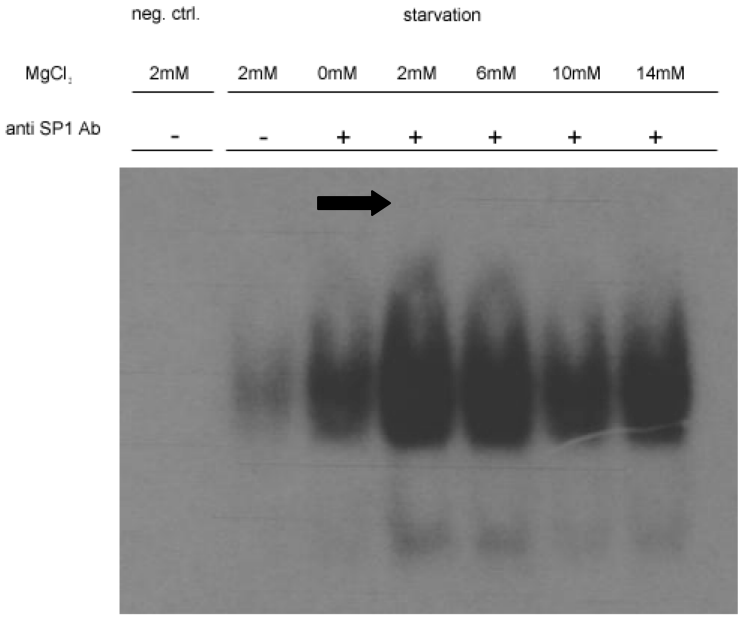


Figure 18: EMSA in order to determine optimal $MgCl_2$ concentration in the reaction mix with distinct supershift (marked by the arrow).

In the optimization experiments, where the ideal amount of $MgCl_2$ should be determined, also supershifts were observable. The optimal $MgCl_2$ concentration for supershifts in this in vitro system turned out to be 2mM per approach. Concluding from these two band shift experiments one can conclude that under starving and dexamethasone supplemented conditions more SP1 is available or in the proper modification to bind the radiolabeled SP1 promotor sequence element.

3.3 Analysis of the putative PPARα binding site

Since it is widely accepted that PPARα is involved in the regulation of many genes of the fatty acid metabolism and also of the carnitine-acyltransferase system, special attention was paid to identify a possible PPARα binding element within the promoter or within the first exons and introns. Finally Patch search predicted such a putative site in the first exon just nine nucleotides downstream the promoter start.

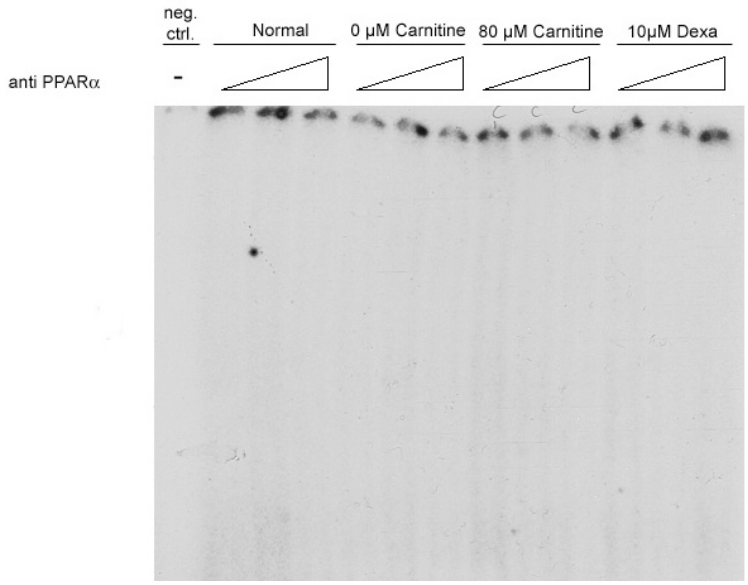


Figure 19: EMSA with putative PPARα binding site (located in the first exon of *Crat*)

The outcome of this band shift experiment was quite obvious. No murine nuclear factor was able to bind this DNA probe representing a theoretical PPARα binding site under the defined growth and incubation conditions.

3.4 Analysis of the HNF-3β site

Hepatocyte nuclear factor 3β is a very active transcription factor in liver cells and was proposed by TESS with the highest likelihood-ratio of all nuclear factors. Although the discussed binding site of this factor is localized far out of the 342nt region, which, according to previous data [74], it should be mainly responsible for carrying out the L-carnitine effect in CRAT gene expression. Therefore further analysis of this region was mandatory. HNF-3β was described to be a transcription factor, which promotes the transcription of genes that are involved in glucose homeostasis.[112] Since CRAT can be seen as a modulator between beta-oxidation and glucose utilization, due to its impact on the pyruvatedehydrogenase complex, a possible regulative influence of HNF-3β on CRAT transcription seemed quite comprehensible.

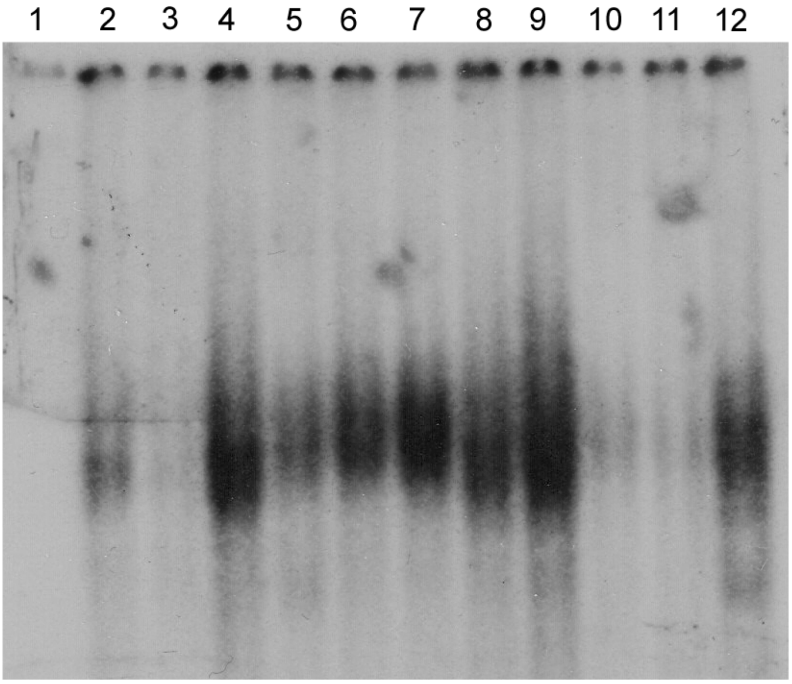


Figure 20: HNF-3β mobility shift assay

1	**Negative control**	**7**	**DMEM + dialysed 10%FCS+ 40µM L-Carn. – 4h**
2	**DMEM+10%FCS (N)**	**8**	**DMEM + dialysed 10%FCS+ 80µM L-Carn. – 4h**
3	**Starvation**	**9**	**DMEM + dialysed 10%FCS+ 120µM L-Carn. - 4h**
4	**Serum Induced**	**10**	**DMEM + dialysed 10%FCS + 80µM L-Carn. 24 h**
5	**N+10µM Dexamethasone**	**11**	**N+10µM Dexamethasone 24 hours**
6	**DMEM + dia. 10%FCS(0-C)**	**12**	**N+5µM Dexamethasone**

Apparently this distinct HNF-3β site is a target for nuclear factors. Mobility shift experiments with this sequence box resulted in a double-shift pattern, displaying that two different factors with different molecular weight are binding this site. Under starvation the band shift signal turned out to be weaker indicating decreased binding of nuclear proteins to the oligonucleotide. After treatment of the cells with dialyzed fetal calf serum, the binding of nuclear factors to this ODN was increased in comparisons to the “normal-status” (nuclear extracts from cells cultivated in DMEM+10%FCS+Ab). An influence of L-carnitine could not be observed as indicated in lanes 7-10.

Considering these results and the fact that the HNF-3β binding site is located far out of the 342nt-carnitine sensitive region we can exclude this regulative element from being included in the L-carnitine induced upregulation of the CRAT gene expression.
About possible regulative effects of HNF-3β on the gene regulation of PTPA further experiments would be interesting, but from the point of knowledge we have now, no serious prediction can be made whether this factor is a regulative element of the *Crat* or the *Ptpa* gene.

3.5 Analysis of the GR site

The glucocorticoid receptor (GR) is a member of the nuclear receptor superfamily, composing subfamily 3C.[113] The unbound receptor is located in the cytoplasm and moves after binding its substrate into the nucleus where it recognizes its consensus sequences. From this point on, the glucocorticoid receptor closely linked to its activating steroid has two major tasks. On the one hand it promotes the expression of anti-inflammatory genes and on the other hand it works as a repressor for pro-inflammatory genes.[114]
In this case again the connection of the GR elements to the energy metabolism – glucocorticoids elevate the blood sugar – was reason enough for a repetition of the analysis of this specific promoter element. A connection between L-carnitine and glucocorticoid receptors had already been reported. In vitro administration of pharmacological dosage of L-carnitine can activate GRα and subsequently modulate the transcription of glucocorticoid-responsive genes. This provides a way to explain the immunosuppressive properties of L-carnitine by mimicking glucocorticoid actions.[115]

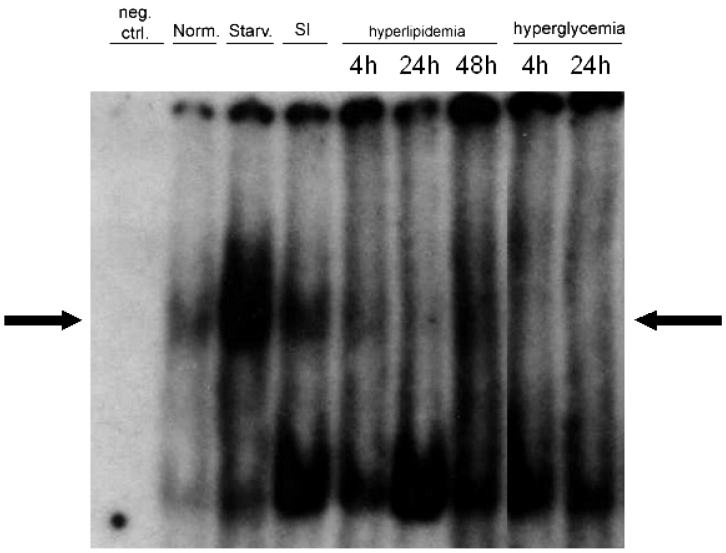


Figure 21: EMSA with the GR element from the *Crat* promoter

This mobility shift assay revealed an interesting phenomenon and also posed further questions. First of all one can see that under physiological conditions (DMEM+10%FCS+Ab), starvation and serum induction obviously 2 different factors or factor complexes can bind the GR-element representing oligonucleotide. But in those experimental approaches where the pathophysiological status of hyperlipidemia and hyperglycemia were simulated the upper band shift signal diminished. The homeostatic changes supported the disaggregation of the DNA-protein complex.

A GR mobility shift assay resulting in a double band shift, like the one illustrated above, seems to be a common pattern for glucocorticoid responsive elements. Gao et al. performed also EMSAs with a GR element, which produced a double band pattern. [116]

3.6 Analysis of the USF_CREB Site

TESS search also proposed a special site within the *CRAT* promoter where according to the program two overlapping binding sites for different factors are located. On the one side TESS propounded a USF-1 element. Upstream stimulating factor 1 is also an ubiquitously expressed

transcription factor, which has been linked with the etiology of familial combined hyperlipidemia and metabolic syndrome.[117]

On the other side also a possible binding site for the well-known transcription factor CREB has also been located within this specific area.

CREB is the final target of many signaling pathways and its effects are manifold. It plays an important role in neuronal plasticity and memory, neuronal development and is also involved in addictional behavior.[118]

Above that it is involved in many metabolic processes like fat metabolism and hepatic gluconeogenesis. Its regulative role in the response to nutrient and hormonal changes has also been described. [119],[120] In addition muscle cell energy homeostasis is regulated by CREB.

Via the AMP-activated protein kinase (AMPK) CREB gets phosphorylated and controls together with coactivators like PGC-1α the capacity of fatty acid oxidation.[121]

This pathway including CREB and PGC-1α is also involved in CPT1a transcriptional regulation, especially after starvation CPT1a expression is upregulated in response to glucagon release from the pancreas, a process that is mediated by cAMP.[31]

Analysis of the proposed DNA region revealed that the putative USF-1 binding site is located at the very beginning of the reportergene assay construct mCRAT-2 except for the nucleotide 243 upstream the promotor start which is just outside this construct. This sequence has been split during the generation of the constructs for the reportergene analysis. An important fact that has to be considered is that one has to be able to analyze the results of these experiments correctly.

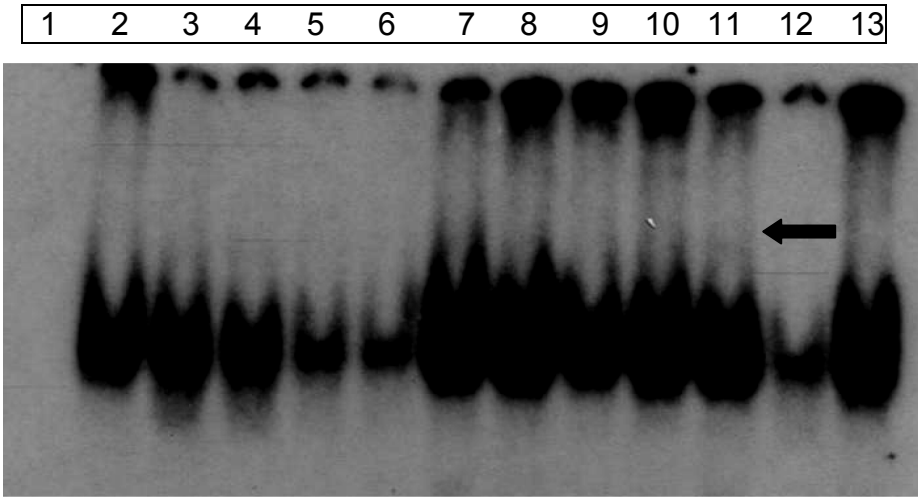


Figure 22: Mobility shift assay with the USF1-Creb ^{32}P-labelled ODN

1	**negative control**	**8**	**serum induction**
2	**normal**	**9**	**5 dexamethasone**
3	**0-carnitine**	**10**	**10 dexamethasone**
4	**40-carnitine**	**11**	**starvation + 1µl Creb antibody**
5	**80-carnitine**	**12**	**80-carnitine + 1µl Creb antibody**
6	**120-carnitine**	**13**	**10-dexamethasone + 1µl Creb antibody**
7	**starvation**		

The outcome of this experiment is quite meaningful. First of all it generates a typical single band shift signal that only one factor or factor complex is able to bind this specific DNA probe. A general look at the fluorographic radiofilm reveals that starving conditions led to strong binding of nuclear factors to the ODN probe. It is also evident that rising L-carnitine levels led to weakening of the signal. Another interesting result was that a weak supershift signal is visible in lane 11, where the nuclear extract of starved cells in combination with a CREB antibody was analyzed. So very likely this sequence element of the *Crat* promoter harbors a CREB binding site to which especially under starvation nuclear factors showed high affinity.

3.7 Analysis of the RXRα/RARβ binding site

From the very beginning of the investigation of the *Crat* promoter this element had been the most interesting and most promising one. Already my colleague *Ginta Pordes* published that this element showed the strongest band shift signals after L-carnitine induction[74]. But so far no supershifts or other more defining results could be achieved. Nevertheless this element stayed in the center of research for several reasons:

- This RXRα element is highly conserved and also present in the human *CRAT* promoter.[74]
- The retinoic x receptor α is able to form heterodimers with many other nuclear receptors which are involved in fatty acid metabolism like PPARα and LXRα.[122]
- Its role in insulin resistance has been described. This aspect appeared to be interesting since the accumulation of metabolites of a defective fatty acid degradation was also linked to insulin resistance.[123]

3.7.1 Band Shift and Supershift Assays

In the first experimental approaches with the RXRα/RARβ probe the focus had been set on possible factors interacting with this special region based on the results of the TESS search. From this starting point supershift assays with possible interacting factors of RXRα, which were defined as such by *Pubmed*, were performed.

3.7.1.1 RORα Supershift Assay

The first candidate in this screening was the RAR-related orphan receptor alpha (RORα), a member of the steroid hormone receptor superfamily. Studies with ROR deficient mice showed the importance of this receptor for several physiological processes. For instance its central role in

development of the cerebellum was described. It was also implicated that ROR is a key regulator in the maintenance of bone tissue and RORα deficient mice were more susceptible to arteriosclerosis.[124] Above that cholesterol and 7-dehydrocholesterol were identified as RORα ligands, which led to the awareness that this nuclear receptor is interfering with the regulation of lipid homeostasis.[125] These reasons made this factor also interesting for closer investigation in correlation with the *Crat* gene.

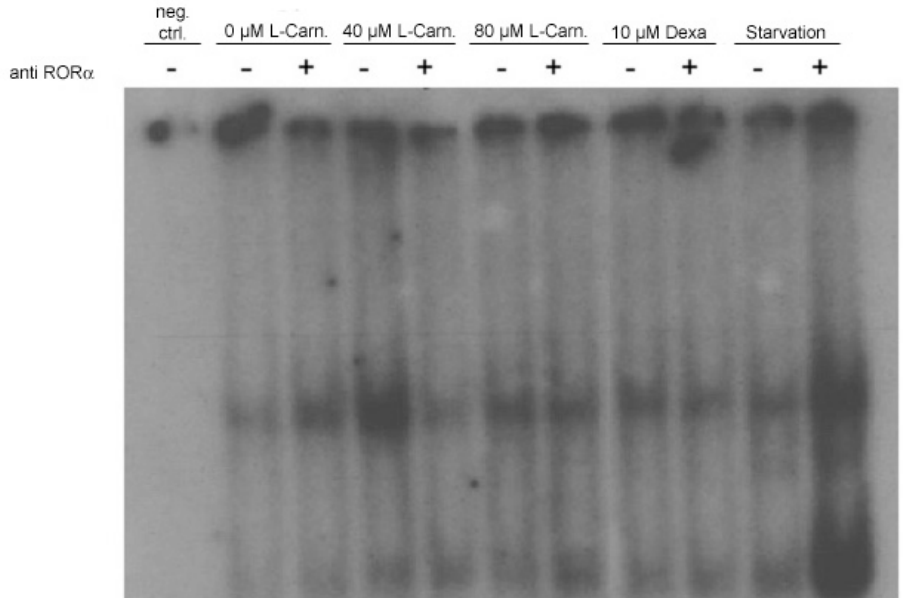


Figure 23: RXRα/RARβ EMSA with anti-RORα antibodies for supershift analysis

This experiment with the RXRα/RARβ ODN probe resulted in two distinct band shifts at different migration distances. A supershift as described for the SP1 and the CREB site could not be observed. The outcome from this approach is that this nuclear factor RORα can be excluded from binding to this promoter element.

3.7.1.2 PPARγ Supershift Assay

Another heterodimerization partner of RXR is the Peroxisome proliferator activated receptor gamma (PPARγ). This heterodimer binds a specific DNA sequence called PPAR Responsive Element (PPRE). [126]

After docking to this PPRE the binding of endogenous and exogenous factors like fatty acids and eicosonoids takes place leading to cofactor recruitment and subsequent transcriptional regulation.[127]

The PPARγ receptor plays a very important role in fat cell differentiation by inducing fatty-acid metabolism specific genes and promoting the formation of lipid-rich adipocytes.[128] Experiments with PPARγ deficient mice revealed the importance of this factor for the development of white and also brown adipocytes.[129]

This critical involvement suggested a possible regulative role in the transcriptional control mechanisms of *Crat* expression.

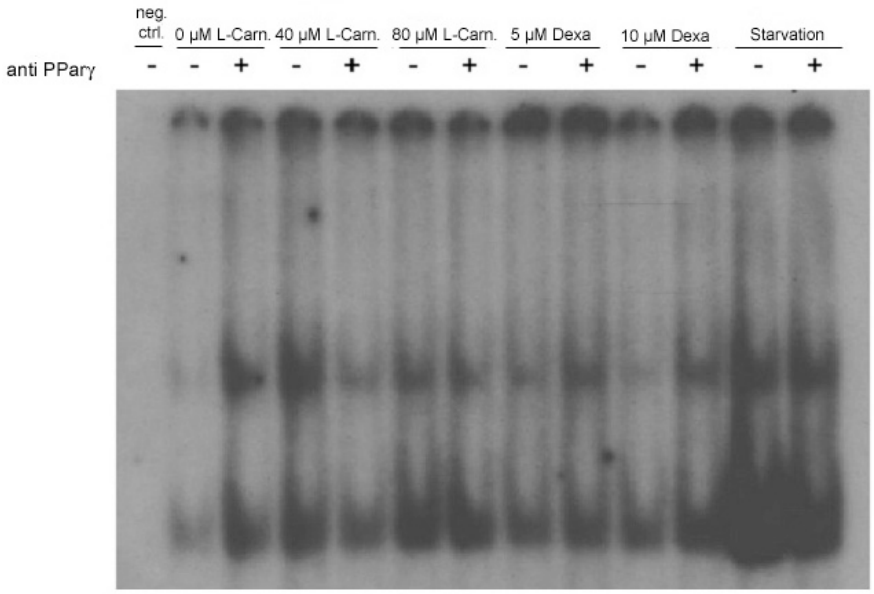


Figure 24: RXRα/RARβ EMSA with anti-PPARγ antibodies for supershift analysis

The outcome of this experiment is quite similar to the one of the RORα supershift assay. The EMSA resulted in a double-banded shift but no supershift signal indicating that PPARγ is not binding to this specific RXRα/RARβ probe from the *Crat* promoter.

3.7.1.3 PPARbP(TRAP220) Supershift Assay

The next factor, which had been tested in these first screening experiments, was the PPAR binding protein (Pbp), or also called TRAP220 or MED1. It is a multiprotein coactivator that is needed by DNA-binding transcription factors in order to activate the RNA-Polymerase II.[130]

Pbp is also able to interact with PPARγ, PPARα, RARα, RXRα, and TR-β in vitro. All mentioned factors are involved in the fatty acid metabolism. It has been proposed that Pbp is an important coactivator for the physiological functions of PPARs in energy homeostasis.[131]

Another important certainty, which made PPARbp very interesting in correlation with the *Crat* promoter, was the fact that the PPARbp gene itself gets upregulated by L-carnitine after artificial L-carnitine deficiency. *Elke Hofer-Litzlbauer* showed in her doctoral thesis with RNA-microarrays and reverse transcriptase RT-PCR that the mRNA levels of cells induced with 80 µM L-carnitine exhibited 12-fold higher levels in comparison to L-carnitine lacking cells.[132]

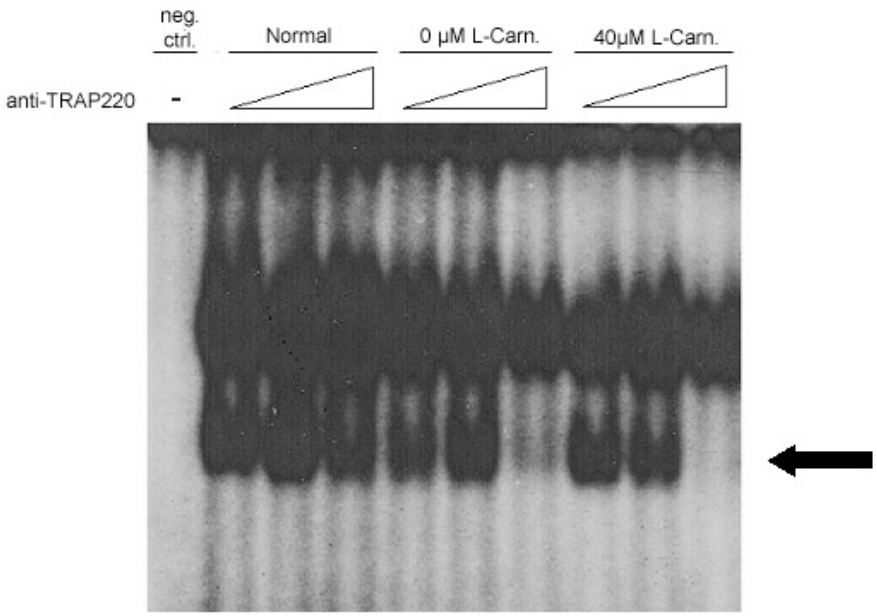


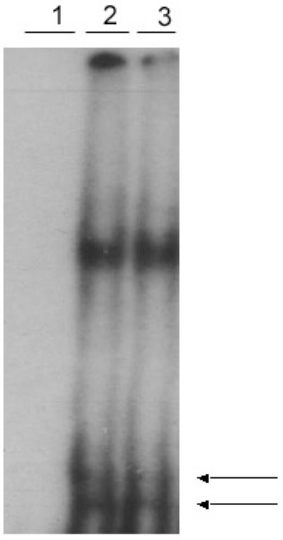
Figure 25: RXRα/RARβ EMSA with anti-TRAP220 antibodies for supershift analysis

The anti-TRAP220 antibody had quite an impact on the shifting features of the nuclear extracts in combination with the γ^{32}-phosphate labeled oligonucleotide. Rising antibody concentrations (from 0-4ng of a 200µg/ml

concentrated antibody stock solution) provoked a weakening of the lower band shift signal at 0µM L-carnitine (cells cultivated with dialyzed FCS) and nearly a disappearance of the lower signal at 40µM L-carnitine (cells cultivated with dialyzed FCS and supplemented with 40 µM L-carnitine for four hours).

3.7.1.4 Extended RXRα/RARβ Band Shift Assay

For better understanding of the band-shift patterns previous EMSAs presented, further experiments had to be performed with the aim to generate better resolution on the X-ray films. Because of this reason the gel electrophoretic conditions had to be changed and running time was extended for two hours.



1	neg. control
2	dialyzed FCS
3	dialyzed FCS + 40µM L-carnitine

Figure 26: EMSA with RXRα/RARβ dsODN probe after elongated gel electrophoresis

As one can observe on the X-ray film depicted above, mobility shift assays with the RXRα/RARβ probe resulted in shifts with three distinct bands. Elongated gel electrophoresis showed that the lower shifting signal, which diminished in the TRAP220 supershift assay, was in reality the joint product of two distinct band shifts. Obviously the difference in molecular weight of these two factors causing this double band is quite small.

3.8 Evaluation and conclusion of the first screening experiments

Since these experiments resulted in band shifts with three distinct bands, but no supershift could be observed, the used oligonucleotide representing the promoter-site of interest had to be re-evaluated and examined with another bioinformatical tool. The analysis with the search tool Patch[106] revealed that a hitherto not known transcription-factor binding site was included in this oligonucleotide, namely a c-AMP responsive element (CREB).

CCTACCGT TG**TGACCC**CG *TGACGG*G

Figure 27: The sequence marked in red represents our "old" RXRα/RARβ element. The green one marks the sequence that Patch identified as a CREB binding site.

Another result of the Patch search was that it proposed further interaction-partners for the RXRα/RARβ locus.

Factor	Sequence	direction
PPAR-alpha RXR-alpha	TGAACC	Sense
VDR	TGACC	Sense
RAR-alpha1 RAR-beta2 RAR-gamma1	GTTCA	antisense
RAR-beta RXR-alpha RXR-beta	GGTTCA	antisense
RXR-alpha VDR	GGTTCA	antisense

Table 1:Putative interacting transcription factors for the RXRα/RARβ locus.

Due to these results the "old" RXRα/RARβ ODN probe had to be split and the following oligonucleotides were synthesized in order to analyze the two different binding sites separately.

5’ AGCGCCTACCGTTGTGACCCCG 3’ RXR_2 Sense
5’ CGTGACGGGAGCCCAGCGCCGG 3’ CREB_1 sense

Figure 28: Oligonucleotides RXR_2s and CREB_1s

3.8.1 Southwestern Blot

The main “suspect” remained the RXR site since this factor is able to correspond with many different putative interaction partners. A special experimental approach, the so called ”Southwestern Blot” analysis, allowed to determine the molecular weight of the factors binding this specific oligonucleotide, which again had been labeled with radioactive ^{32}P-ATP.

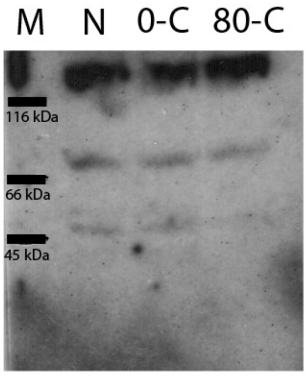


Figure 29: Southwestern Blot shows the binding of the radioactive-labelled RXR_2 probe to specific proteins

For southwestern analysis of the RXRα element nuclear extracts from cells of 3 different cultivation conditions (normal, dialyzed FCS, dialyzed FCS+80µM L-carnitine) were analyzed. The radiographic analysis of the blot revealed three distinct bands in each lane.

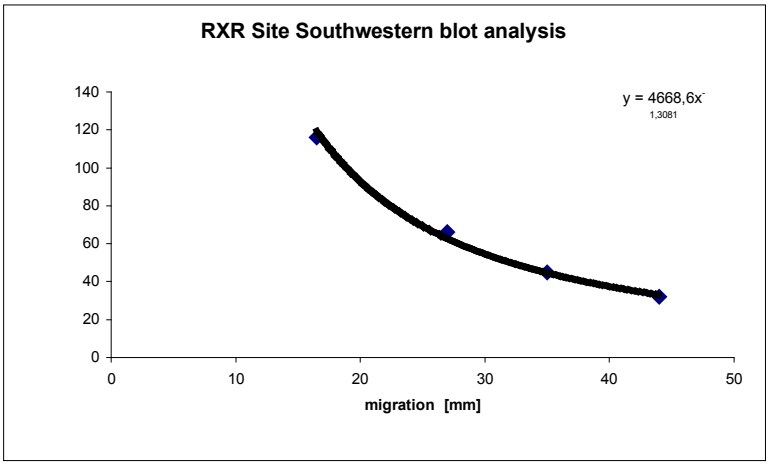


Figure 30: Size determination of the proteins on behalf of migration length of the proteins after denaturing SDS-Page

To determine the molecular weight of these three proteins a standard curve was calculated by using the migration length of the molecular weight markers. (Fermentas PageRuler Unstained Protein ladder). After determination of the migration distance of the unknown proteins their size could be determined:

Band 1	145kDa
Band 2	70 kDa
Band 3	51 kDa

Table 2: Molecular weight of the RXR_2 probe binding proteins

The subsequent "*molwSearch"* using the "*Transfac Database"* from the website www.gene-regulation.com presented LXRα and PPARα as candidate factors within a size range from 51-53kDa for the lowest protein signal, c-MYB and c-MYC as putative factors for the ~70kDa band and EVI-1 for the ~145kDa band signal.

These proposed factors and their possible metabolic functions had been further on examined by using the *OMIM* database and *Pubmed*:

- **Peroxisome proliferator-activated receptor α (PPARα) and liver x receptor α and β (LXRα/β)** are reciprocal regulators of the fatty acid metabolism.[133] More importantly their central role in the

regulation of other genes of the carnitine acyltransferase system made those two factors highly interesting for also being involved in the carnitine induced gene expression of *Crat* and the rest of the connected “carnitine-shuttle” system (*Cpt1a, Cact, Cpt2*).[22],[56], [48]

- Activation of the proto-oncogene **EVI-1** (Ecotropic viral integration site 1) gene expression has been described for being related to murine myeloid leukaemia.[134] This factor is also able to induce a myelodysplatic syndrome in mice. [135]
- **c-Myc** is a transcription factor which has been identified as a proto-oncogene, which can activate and repress transcription in combination with c-Max as a heterodimer. Due to its central role in cell functions during mitosis and cell growth deregulation of c-Myc can lead to genetic alterations, whereas constitutive activity of c-Myc is involved in the genesis of a manifold variety of cancers.[136]
  Especially the Burkitt’s lymphoma, a Non-Hodgkin lymphoma, had been connected with hyperactivity of c-Myc. [137]
  The influence of c-Myc on the liver metabolism was described in so far, that hyperactive c-Myc down-regulates PPARα controlled genes belonging to the fatty acid catabolism. On the other side it raises the transcriptional rate of genes controlled by SREBP-1 that promote the anabolism of fatty acids and cholesterol. [138, 139]

3.9 Analyses of the oligonucletides RXR_2 and CREB_1

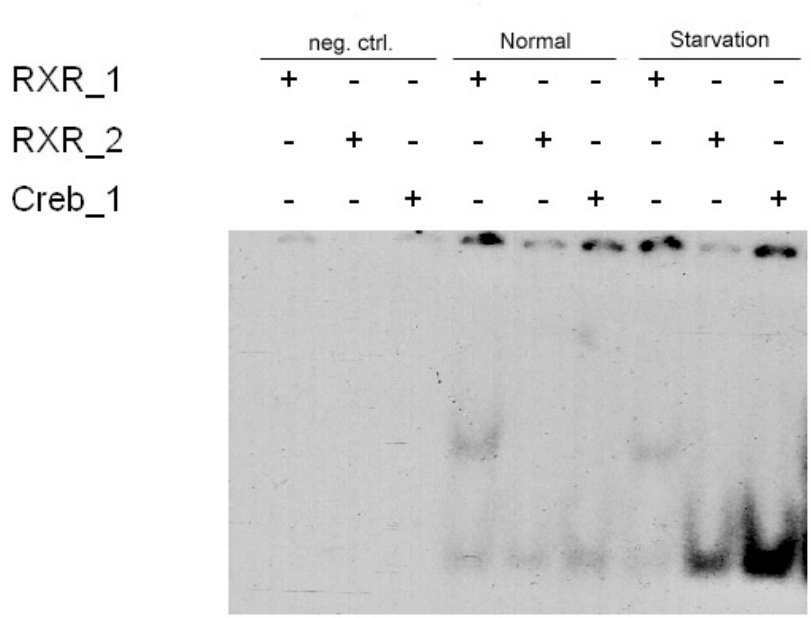


Figure 31: comparative EMSA of the three dsODN probes RXR_1, RXR_2 and CREB_1

This experiment confirmed the thesis that the RXR_1 probe (the "old" RXRα/RARβ element) really contained binding sites for two different transcription factors or factor complexes.

As the figure illustrates, the nuclear extracts with the probes RXR_2 and CREB_1 showed band shift signals at the same height, whereas the shift experiments with the RXR_1(RXRα/RARβ) oligonucleotide probe resulted in two distinct signals. The upper band shift represents some kind of a "supershift" as a result of the binding of two different factors to the RXR_1 oligonucleotide, each binding to its specific DNA motif leading to more bound protein and further on to shorter migration during gel-electrophoresis.

3.9.1.1 PPARα supershift assay with the RXR_2 ODN

The first supershift assay with the newly synthesized RXR_2 element was performed with extracts of normal treated, dialyzed and L-carnitine supplemented cells and anti-PPARα antibodies.

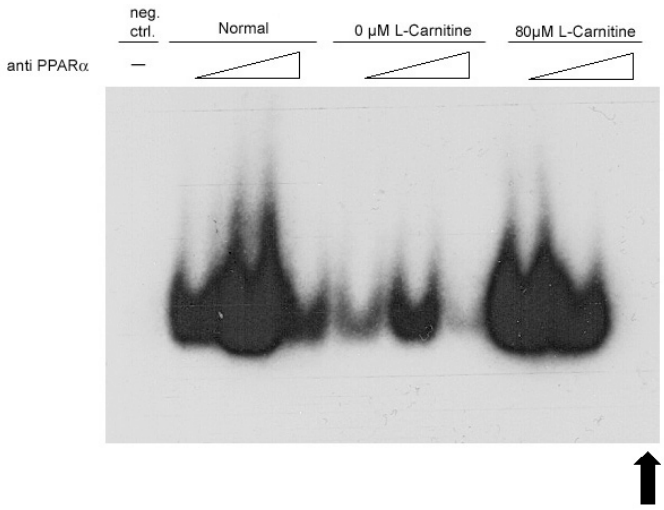


Figure 32: RXR_2 EMSA and anti-PPARα antibodies for supershift analysis

Increasing antibody concentration led to a weakening of the band shift signal under carnitine deficient conditions and especially after supplementation with 80 µM L-carnitine. The antibody reduced or hampered the formation of the protein-DNA complex indicating that PPARα is part of this complex. A classical supershift band was not visible, but according to the literature, reduction of the signal after application of the antibody is equivalent to a supershift signal. Obviously the antibody blocked the DNA binding domains of the PPARα protein. [140]

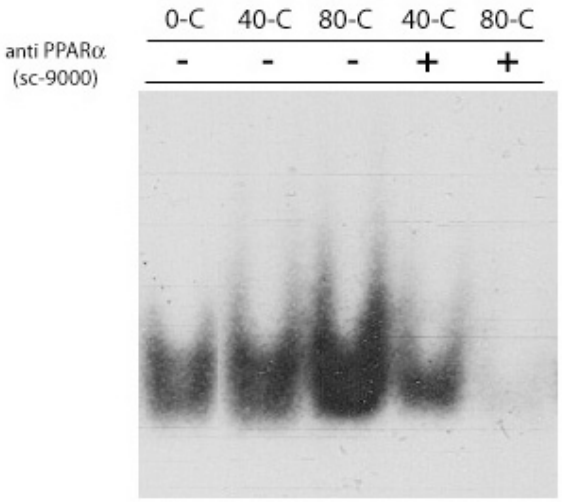


Figure 33: PPARα supershift analysis with RXR_2 probe resulting in a reduced formation of the DNA-protein complex

A very good insight into the binding features of nuclear extracts to the RXR_2 ODN gave the supershift approach depicted above. Obviously, as expected from previously generated data by our lab, increasing L-carnitine concentrations in the growth-media during cultivation of the TIB73 cells led to stronger affinity of the proteins to the examined promoter element. The double band shift pattern got stronger with increasing L-carnitine concentrations. Application of anti-PPARα antibody solution resulted in the characteristic reduction of the band shift signal. At 40 µM L-carnitine only the upper signal diminished but at highest L-carnitine and antibody concentrations the whole signal got nearly extinguished.

3.9.1.2 LXRα/β supershift assay

LXRα/β, antagonistic factors to PPARα in the regulation of energy metabolism genes, are also able to build heterodimers with RXRα. Thus the possible involvement in the regulation and mediation of the "L-carnitine" effect should also be explored with band and supershift assays.

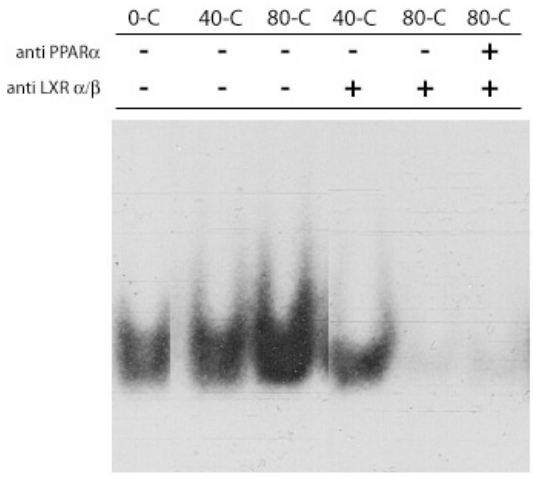


Figure 34: combined LXRα-PPARα supershift analysis with RXR_2 probe resulting in a reduced formation of the DNA-protein complex

The administration of the LXRα/β antibody seemed to have a similar effect on the binding affinity of the nuclear extracts to the ^{32}P labelled dsODN. With increasing L-carnitine concentration and constant anti-LXRα/β antibody the band shift signal got weaker again and almost disappeared.

First at 40 µM L-carnitine again the upper signal diminished first and secondly at 80µM L-carnitine also the lower band shift signal got weaker. In presence of both antibodies, namely anti-PPARα and LXRα/β, the effect remained the same and the shift signal disappeared almost completely.

3.10 PPARα Western Blot

In a Western Blot analysis the effect on PPARα expression itself under L-carnitine influence had to be examined. Again nuclear extracts from differently treated cells were used in order to see if time dependent administration of L-carnitine would lead to upregulation of PPARα protein in the nucleus.

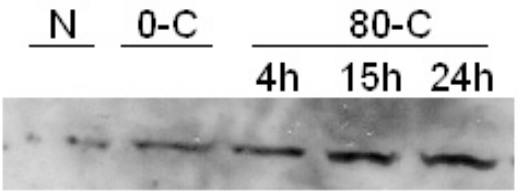


Figure 35: PPARα Western Blot illustrating the increase of PPARα with time after L-carnitine administration

This blot provided more indications that the PPARs, especially PPARα, and L-carnitine metabolism are directly connected and influence each other. Clearly visible is a time-dependent PPARα expression, after 24 hours after administration of 80 µM L-carnitine increased protein synthesis did take place that could explain the stronger blotting signals at the time points 15h and 24h.

3.11 Real-Time RT PCR

In previous projects the influence of L-carnitine on the transcription rate of genes involved in the L-carnitine metabolism had been shown in different human cell lines by RT-real time PCR[141]. *Aniko Ginta Pordes* showed in her diploma thesis by luciferase reporter gene assays the effect of L-carnitine on CRAT gene expression in the murine liver cell lines TIB73 and NIH3T3.[74] Therefore one task for this thesis was to support this "L-carnitine effect" in murine cells by real-time RT-PCR analysis. For this aim

Crat transcription levels had to be tested under the influence of L-carnitine. In order to compare these expression levels also with other genes of the carnitine acyltransferase family (*Cpt1a, Cpt2, Cact, Octn2*) had to be analysed.

Further on also the *Crat* gene expression under different metabolic conditions like as hyperlipidemia, hyperglycemia, and starvation were of interest. These aspects also supported the research goal to search for other inductors and inhibitors of *Crat* gene expression.

3.11.1 Real Time analysis of Carnitine Acetyltransferase (*Crat*) gene expression

3.11.1.1 Concentration dependent influence of L-Carnitine on *Crat* gene expression

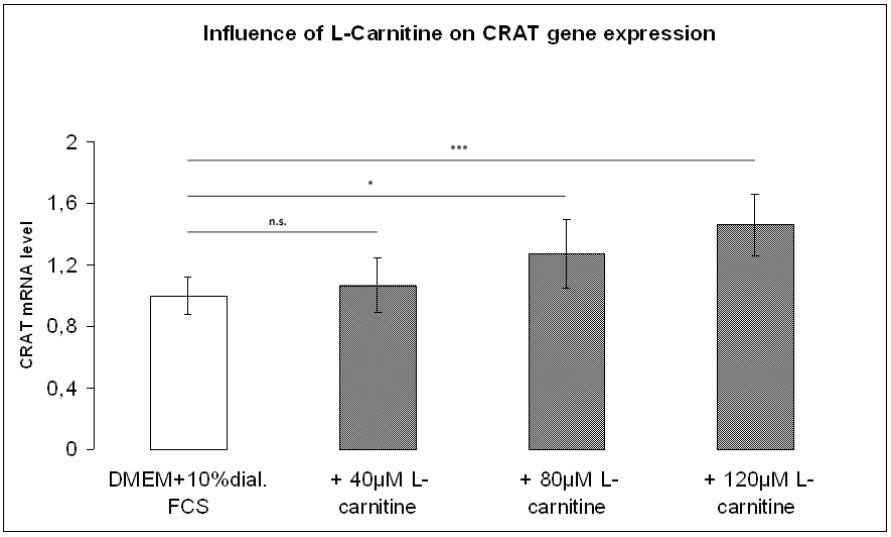


Figure 36: Real Time Analysis after administration of rising L-carnitine concentrations (0-120µM) for 4 hours (n=4)

As expected due to knowledge from previous experiments *Crat* mRNA levels increased with higher L-carnitine levels. Already at a supplemented concentration of 40 µM L-carnitine a tendency for induced *Crat* transcription is observable, although the value of 1.12 ($p>0.05$) did not reach statistical significance in a students-t test.

Cells induced with 80µM L-carnitine already showed significant higher *Crat* mRNA levels in comparison to those cells grown under L-carnitine deficiency. A 1.27-fold increase could be reached ($p<0.05$).
1.46-fold was the increase of *Crat* mRNA expression in TIB73 cells under supplementation with already unphysiologically high 120µM L-carnitine ($p<0.001$).

3.11.1.2 Time dependent CRAT gene expression after supplementation of 80 µM L-carnitine

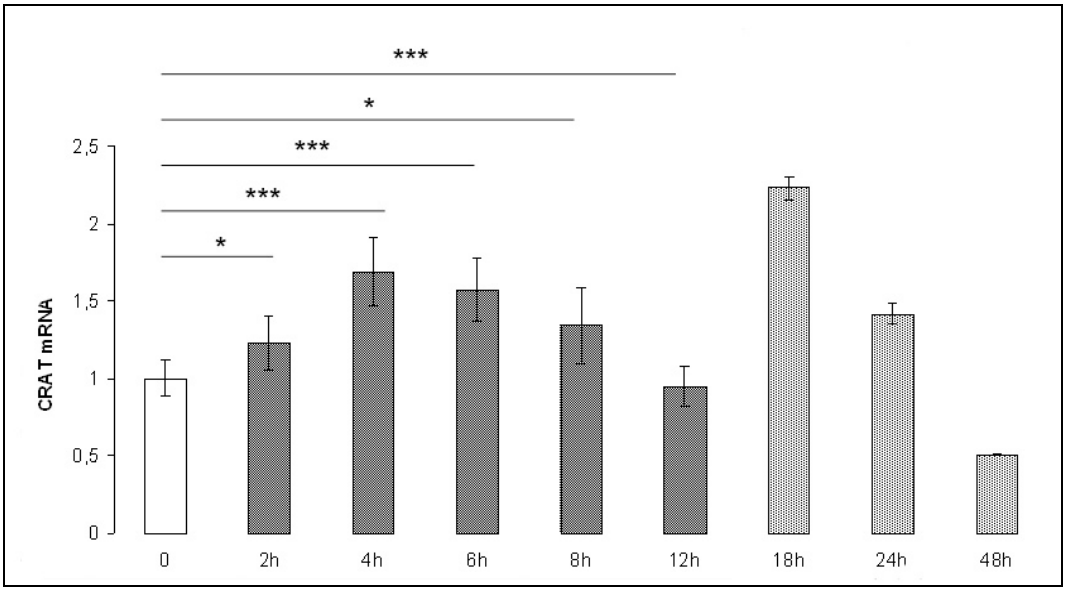


Figure 37: Time dependent transcript levels of the murine *Crat* gene after initial administration of 80µM L-carnitine (n=4)

The analysis of *Crat* transcription in dependence of time shows for the first twelve hours a typical Gauß-shaped curve of transcript induction. After 4 hours the highest mRNA levels of the *Crat* transcript were reached. Afterwards the Crat mRNA steady state level decreases, either due to the lack of transcription or constant degradation, reaching a minimum after 12 hours. After 15 hours of incubation re-administration of new 80 µM L-carnitine had been performed and a peak occurred again after 18 hours of initial carnitine supplementation.

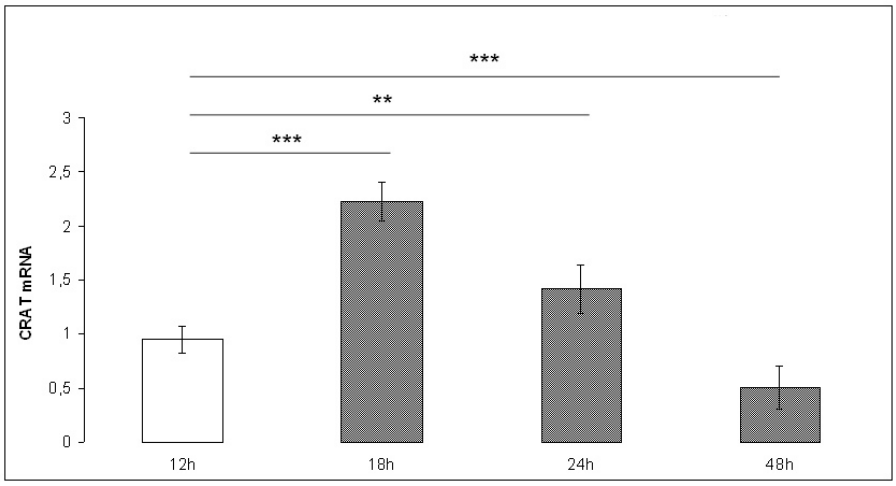


Figure 38: Results of Real Time PCR after second administration of 80 µM L-carnitine 15 hours after the initial one (n=4)

The re-administration of L-carnitine 15 hours after the initial dosis resulted in even higher mRNA levels (2.23 fold, $p<0.001$) after 3 hours of reinduction (or 18 hours of total induction). 6 hours later (24 hours after first L-carnitine administration and 8h after reinduction) still an increased *Crat* mRNA level of 1.41 ($p<0,001$) was measured.

3.11.2 Pharmacological aspects of CRAT gene expression

The regulative effect of PPARα on genes of the carnitine acyltransferase family was reported several times [142]. Since the data from the band shift assays described before also delivered strong indications that PPARα and LXRα are regulative factors for the transcription of *Crat*, agonists for the two transcription factors became part of our investigations. Fibrates like fenofibrate and clofibrate offered themselves as candidates for PPARα agonists.

3.11.2.1 Influence of fenofibrate on *Crat* mRNA levels

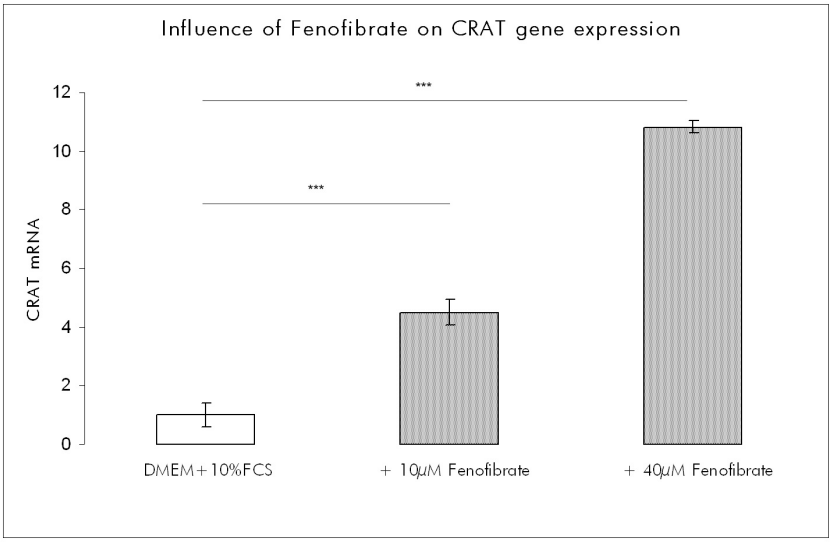


Figure 39: Impact of fenofibrate on Crat mRNA transcription levels (n=4)

Quite impressive was the induction of the *Crat* transcription after administration of the PPARα agonist fenofibrate. Cells treated with this hyperlipidemic drug showed already at 10µM a 5-fold increase in *Crat* mRNA after 3 hours of treatment in comparison to non-treated cells ($p<0.001$). The administration of 40µM fenofibrate even resulted in a 11-fold higher mRNA level ($p<0.001$).

3.11.2.2 Influence of Clofibrate on *Crat* mRNA levels

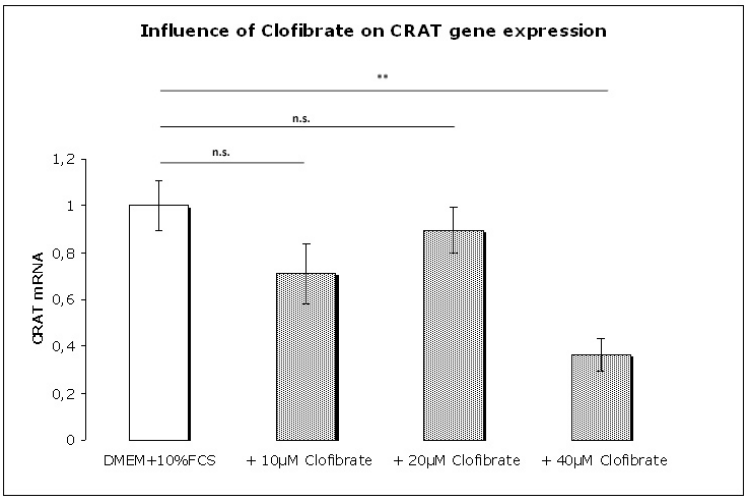


Figure 40: Effects of clofibrate on *Crat* gene expression (n=3)

The treatment of cells with clofibrate and the following analysis of the mRNA levels showed a completely different outcome than described above. Increasing clofibrate levels led to reduction of *Crat* transcription resulting in a decrease of the mRNA level 0.25-fold at a concentration of 40 µM clofibrate.

An important observation to mention in connection with these experimental data is the induction of apoptotic processes in cell culture cells checked under the microscope during the treatment. TIB73 showed also kind of fat mobilization and vacuolization during clofibrate treatment. Shortly after administration of this hyperlipidemic drug drops of lipid aggregates or grease were observable in the media.

3.11.2.3 Influence of the LXR-agonist GW3965 on *Crat* mRNA levels

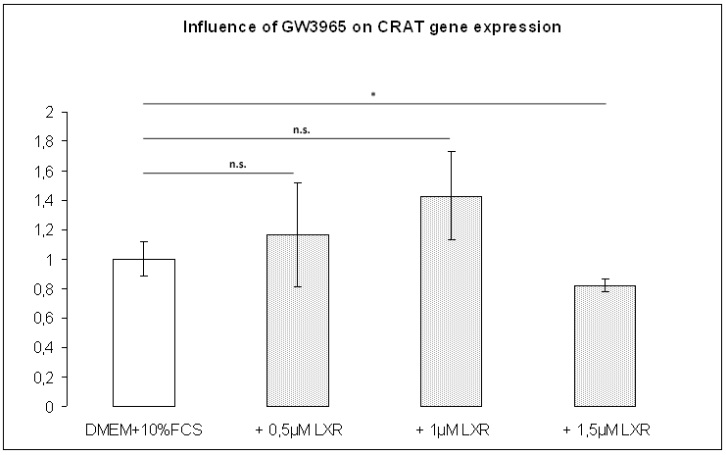


Figure 41: Impact of the LXRα agonist GW3965 on *Crat* transcription levels (n=3)

Cells treated with GW3965, a LXRα agonist, showed at lowest levels (0.5µM and 1µM) non-significant indications of increased *Crat* mRNA levels ($p>0.05$), but at the highest GW3965 level used (1,5 µM) an inhibitory effect (decrease to 0.8 in comparison with untreated cells) was observable ($p<0.05$).

3.11.2.4 Influence of Retinoic Acid on *Crat* mRNA levels

Retinoic acid is a substrate for the RAR nuclear receptor. Since theoretical promoter analysis proposed at first a RXRα/RARβ element, a possible influence of this compound on *Crat* expression had to be examined.

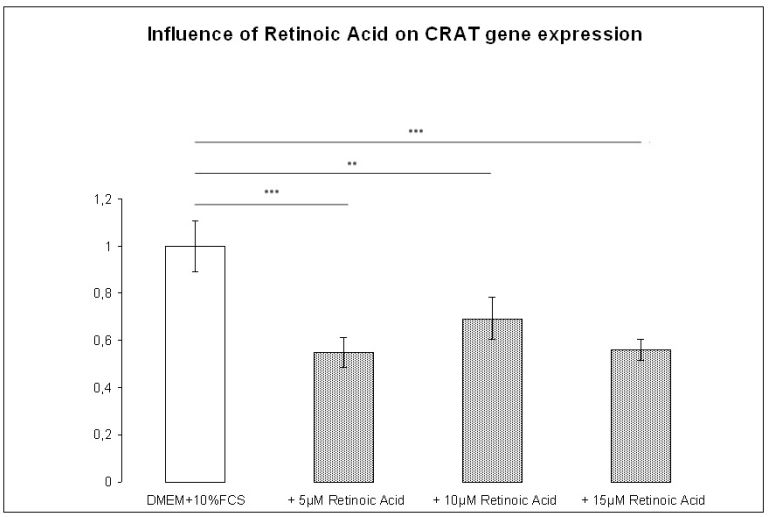


Figure 42: Retinoic acid and its influence on *Crat* mRNA levels

Already the administration of small amounts of retinoic acid, in this case 5µM, caused a significant decrease of *Crat* mRNA levels and reached only half the amount than the control did (0.53, $p<0.001$). This level kept quite constant with increasing concentrations from 10 µM (0.69, $p<0.01$) to 15 µM retinoic acid (0,56, $p<0.001$).

3.11.2.5 Metabolic influences on *Crat* gene expression

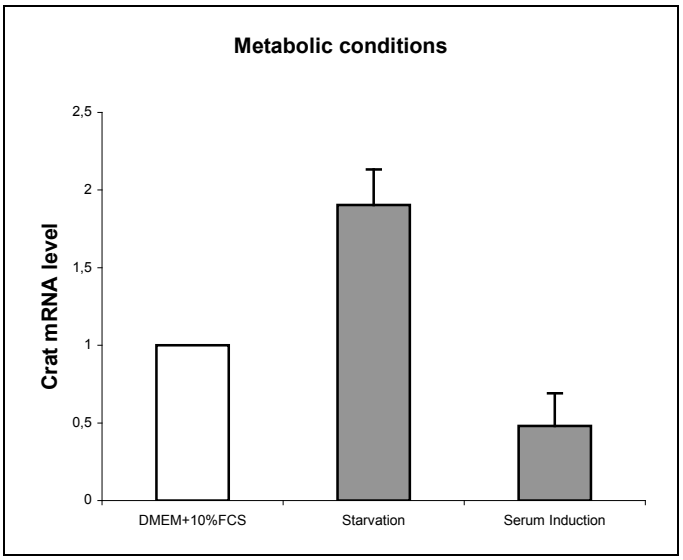


Figure 43: Crat mRNA levels under different metabolic influences

Analysis of the mRNA levels of the simulated metabolic conditions "starvation" and "serum induction" revealed increased transcription levels of *Crat* under starvation conditions (up to 2-fold induction). In serum induced murine liver cells *Crat* transcription got downregulated to a level of 0.48 fold in comparison to normally grown cells (DMEM+10%FCS).

3.11.3 Comparison of transcription levels between the different carnitine acyltransferase genes during L-carnitine deficiency and supplementation

The transcript levels of human carnitine acyltransferases are L-carnitine dependent. Under L-carnitine deficiency the mRNA levels of the three transferases *CRAT*, *CPT1a* and *CPT2* were 2-3 fold down regulated in human cell systems in vitro.

L-carnitine supplementation can restore original transcript levels or even raise them.[141] These findings had to be tested and confirmed in the murine liver cell line TIB73 as well.

A time dependent analysis of *mCrat* levels is shown above. In order to get an understanding whether other members of the carnitine-shuttle system react similarly, transcription levels of the murine homologues to the genes described above had to be determined. In addition to the carnitine acyltransferases we included the transport proteins *Cact* and *Octn2* in this analysis.

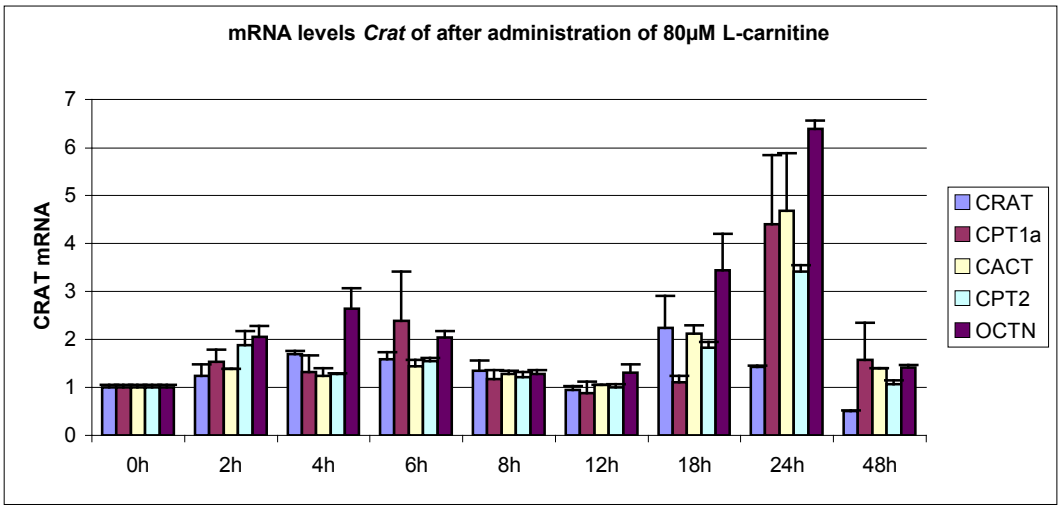


Figure 44: time course of mRNA levels of genes of the "carnitine-shuttle-system" after initial administration of 80µM L-carnitine and re-induction after 15h. (n=5)

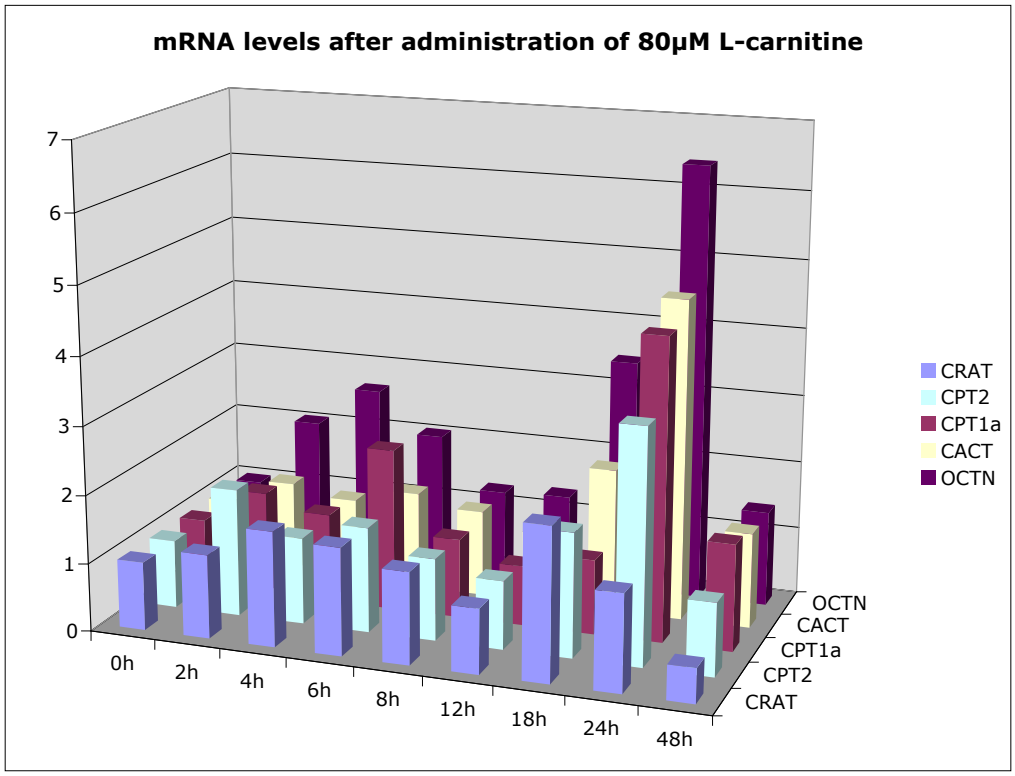


Figure 45: 3-D figure of mRNA levels of "carnitine-shuttle" genes after 80µM L-carnitine supplementation (n=5)

The mRNA levels of the 5 compared genes respond quite similar to L-carnitine administration with only slight differences. After one initial application of 80µM L-carnitine *Octn2* and *Crat* reached their highest levels of mRNA expression after 4 hours, whereas *Cpt2* reacted faster and had its highest transcript level already after 2 hours of L-carnitine supplementation. *Cpt1a* and *Cact* mRNA induction reached their highest levels after 6 hours. Afterwards a constant decrease of the mRNA steady state levels reached its initial amount after 12 hours.

Octn2 showed the strongest response to L-carnitine by raising its transcript level 2.6 fold ($p<0.001$) after 4 hours. Also the induction of the other four genes was statistically significant (*Crat* as described above; *Cact*: 1.4 fold after 6 hours, $p<0.001$; *Cpt1a*: 2.38 fold increase after 6h, $p<0.001$; *Cpt2*: 1.8 fold increase after 2 hours, $p<0.001$).

Very impressive was the transcriptional behavior of these genes after a second administration of 80µM L-carnitine 15 hours after the first one. Again *Octn2* responded the strongest with a 6.3 fold higher mRNA level in comparison with the untreated cells ($p<0.001$). The transcript level increased not that fast as they already did after the first administration of L-carnitine. 9 hours after the second L-carnitine “shot” and 24 hours after the first one, *Octn2* mRNA level reached its absolute highest peak mRNA level at all.

Also the transcripts of *Cpt1a*, *Cpt2* and *Cact* reach their highest levels 9 hours after re-induction. (*Cpt1a*: 4.4 fold increase, $p<0.001$; *Cpt2*: 3.4 fold increase, $p<0.001$, *Cact*: 4.6 fold increase $p<0.001$).

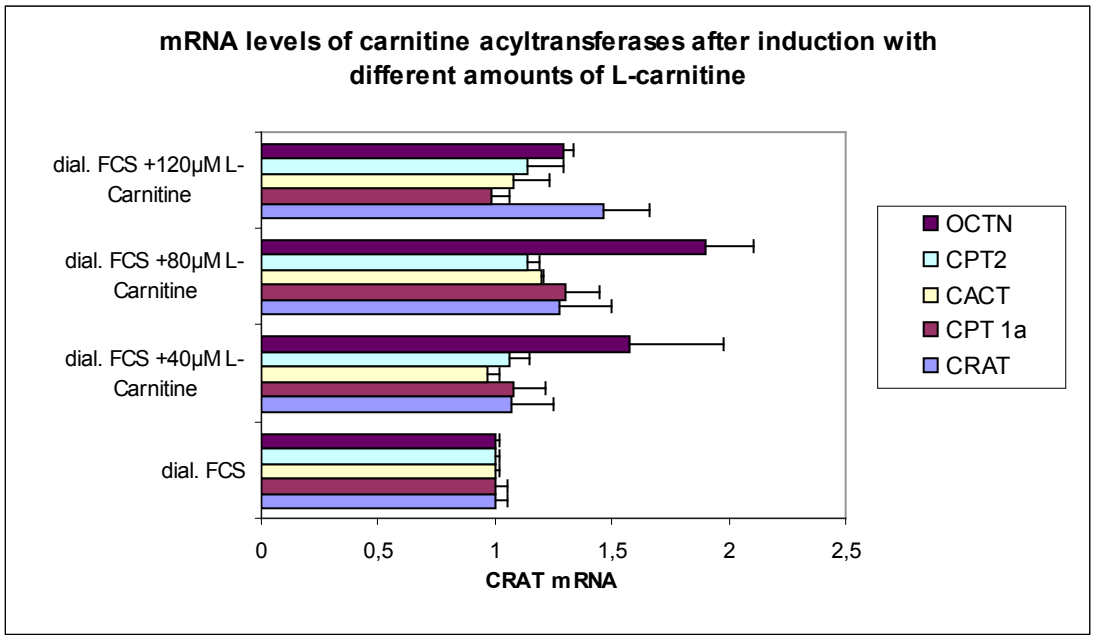


Figure 46: comparative expression levels of *Crat* mRNA after administration of different concentrations of L-carnitine for 4 hours.

As described above *Crat* expression reached its highest level with supplementation of 120µM L-carnitine. As Figure 46 clearly illustrates that the genes of the four other members of the carnitine-shuttle system showed their biggest response after the administration of 80µM L-carnitine. Higher levels of L-carnitine in the media seem to have already inhibitory effects on transcription in this case.

3.12 Pharmacological effects of fenofibrate, GW3965 and retinoic acid on transcription levels of Cpt1a, Cact, Octn2 and Cpt2

3.12.1 Influence of fenofibrate on transcription-levels of "carnitine shuttle" genes

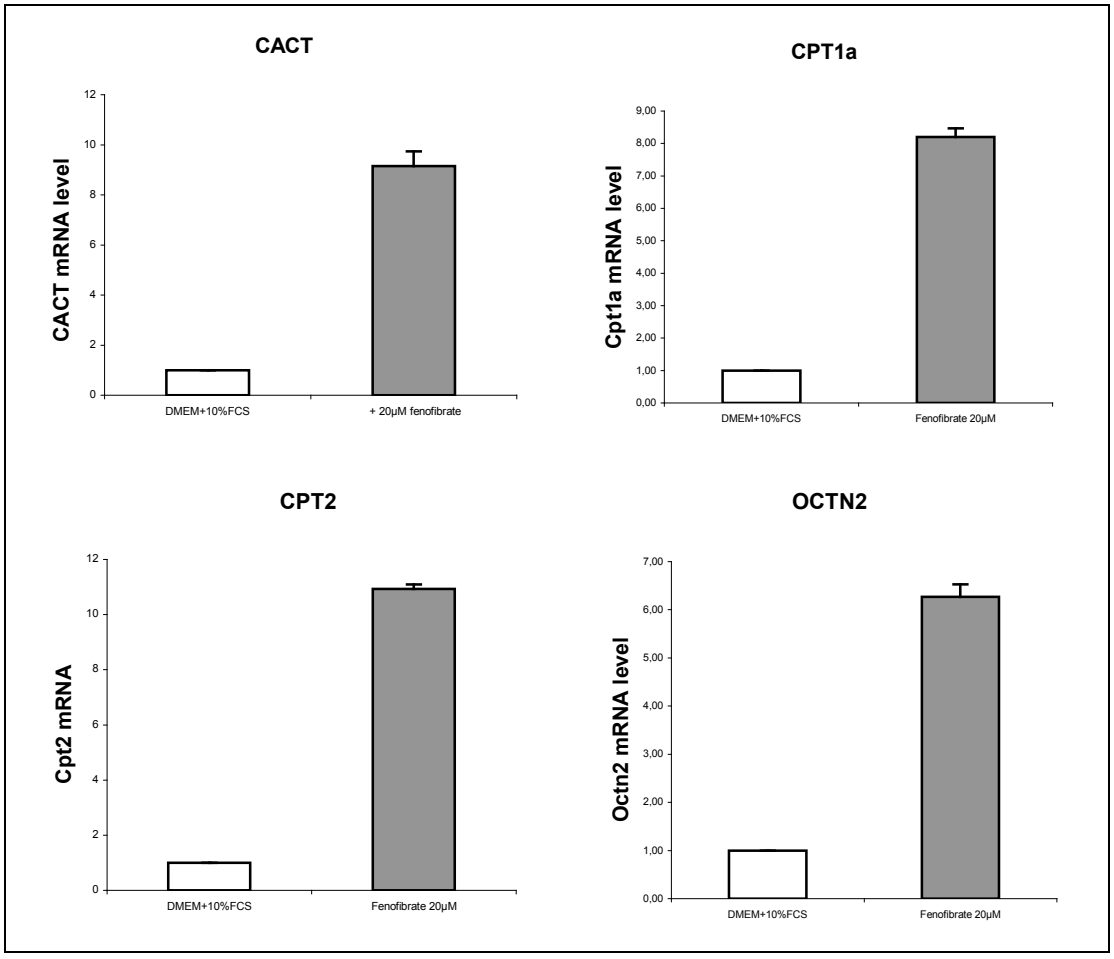


Figure 47: mRNA levels of "carnitine-shuttle genes" after administration of the PPARα-agonist fenofibrate

As the knowledge from the literature predicted, all members of the murine carnitine-shuttle system are inducible by PPARα agonists, in this case by the anti-hyperlipidemic drug fenofibrate (20µM). For all the members depicted above the PPARα sensitive PPRE have been defined and mapped, except for CRAT.

3.12.2 Influence of GW-3965 on transcription-levels of "carnitine shuttle" genes

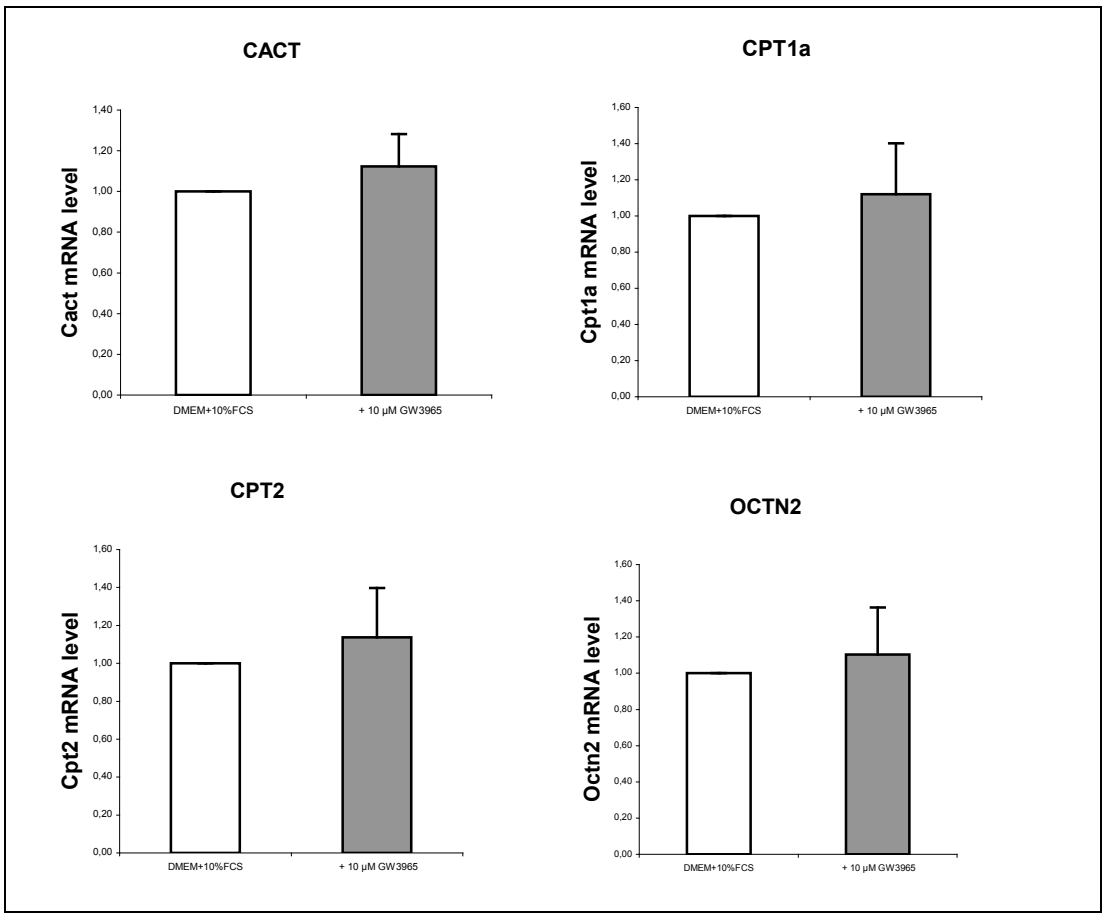


Figure 48: mRNA levels of "carnitine-shuttle genes" after administration of the LXR-agonist GW3965

Liver X Receptors α and β have been described as reciprocal factors to PPARα. For this reason the impact of the LXR agonist GW3965 on the murine carnitine-shuttle system was tested by RT-PCR. As the figure above indicates, GW3965 has no significant impact on the transcription levels of the discussed genes.

3.12.3 Influence of the retinoic acid on transcription-levels of "carnitine shuttle" genes

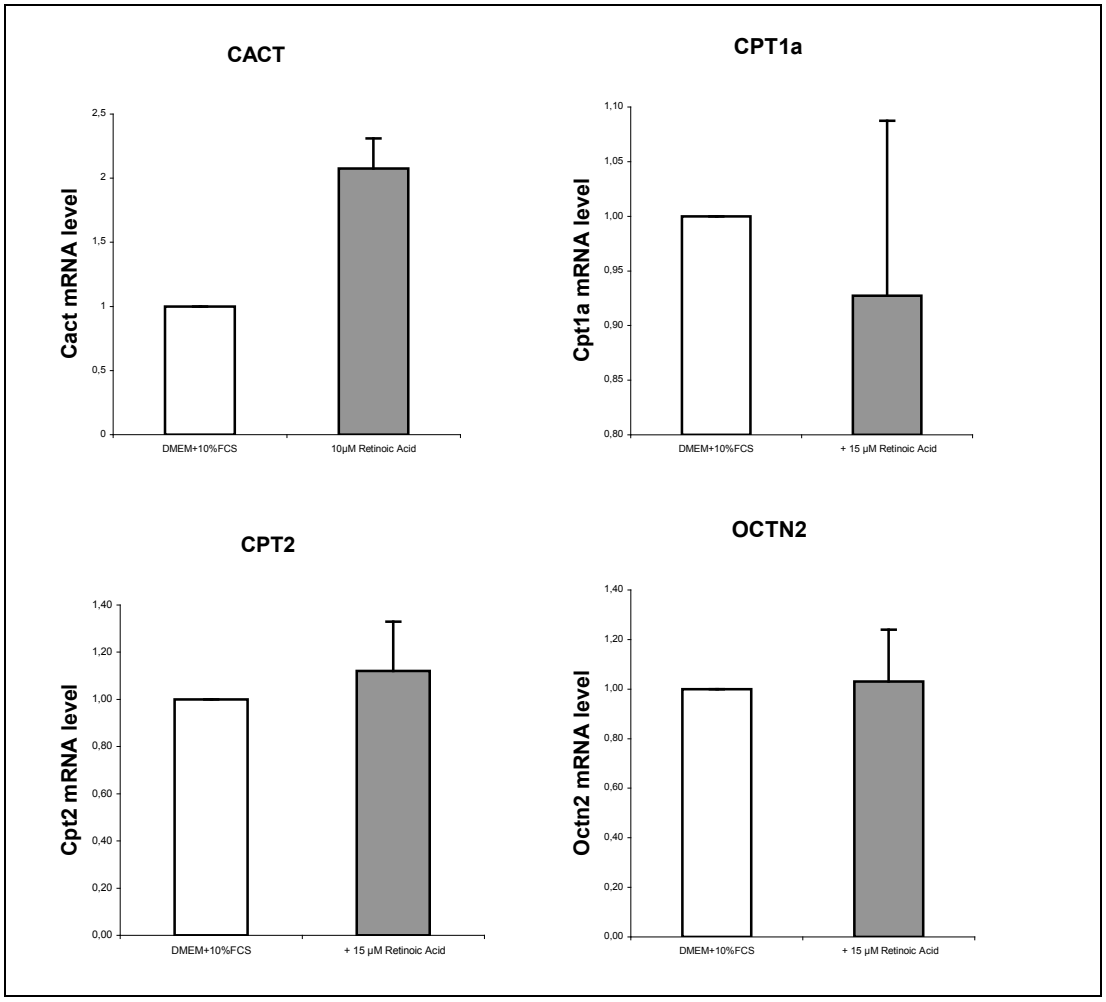


Figure 49: mRNA levels of "carnitine-shuttle genes" after retinoic-acid supplementation

As already mentioned in the introduction it was shown that 9-cis retinoic acid is able to induce transcription of CACT [53]. Since PPARα forms heterodimers with the Retinoid X Receptorα (RXRα) possible influences of retinoic acid on transcriptional regulation of the genes of the carnitine shuttle system. Whereas retinoic acid has an inhibitory effect on murine *Crat,* it enhances *Cact* transcription (2-fold induction). The other members of the carnitine-shuttle system *Cpt2, Octn2* and *Cpt1a* were not significantly influenced by retinoic acid.

4 Discussion

One major goal of this project was the characterization of the *Crat* promoter. As it could be shown in this thesis mobility shift assays are powerful tools to examine the binding of nuclear factors to specific promoter elements. Especially in combination with southwestern blotting, possible candidate factors can be curtailed more precisely due to the fact that the molecular weight can be defined.

With this combined tools it was possible to show that the *Crat* promoter harbours a functional Sp1 binding site. As the analysis of this specific promoter element with supershift assay showed, it is very likely that this site enables basic transcription of *Crat.* Especially under starvation conditions and after dexamethasone induction this Sp1 site seemed to be very active.

Another element, namely a CREB binding site, which is located about 210nt upstream this Sp1 site showed also response to starvation conditions. Supershift signals indicated the binding of CREB to this sequence after 24 hours of starvation. The results of the RT-PCR analysis that showed a 1.9 fold increase of *Crat* expression under starving conditions support the above-described findings.

The main aim of this thesis was the identification of the factor/s mediating the previously described “carnitine effect”.[74, 132, 141] Many experiments, all conducted in our laboratory, showed the increase of transcription levels of many different genes after administration of L-carnitine after artificially induced carnitine deficiency. With this work we could provide evidence that the nuclear receptors PPARα, LXRα/β and PPARbP are involved in the upregulation of *Crat* gene expression after carnitine administration. The analysis of a specific sequence in the Crat promoter, which had been defined by bioinformatical analysis as a RXR/RAR binding site, led to these findings. Southwestern blot analysis revealed the size of protein factors binding to this promotor element and herewith named possible candidates. These candidates were closer examined via supershift assays. These band shift experiments resulted in an interesting and unexpected weakening of

band shift signals with rising carnitine concentration in presence of antibodies against PPARα, LXRα/β and PPARbP. Supershift assays with other candidate factors like RORα, Nurr77 or RARγ did not cause any significant change of the band shift pattern.

Cross-talk between these factors had been described several times.[133, 143, 144] Especially LXRα and PPARα form an important signalling axis controlling metabolic and energy homeostatic pathways.[145]

Both factors, PPARα and LXRα form heterodimers with RXR. PPARα/RXR heterodimers bind to PPREs and enhance the expression of genes responsible for fatty acid oxidation and transport. [146] LXRα/RXR heterodimers bind to LXREs. LXRα and LXRβ are both dominant activators of SREBP-1c, which regulates lipid homeostasis.[147] LXRs are important for expression of fatty acid synthesis genes.[148]

PPARα and LXRα/SREBP-1c are reciprocal regulators of fatty acid metabolism. PPARα inhibits the formation of LXRα/RXR heterodimers and vice versa. Also the direct interaction between LXRα and PPARα was shown via two-hybrid system. [133] So if metabolism requires fatty acid oxidation PPARα enhanced oxidation of fatty acids and simultaneously inhibits LXR signalling and subsequently fatty acid synthesis and storage. If the opposite metabolic pathway is needed LXRα becomes the dominant factor and inhibits PPARα signalling leading to fatty acids synthesis and storage. [143] A theoretical mechanism and interaction of L-carnitine is depicted below in Figure 50.

Our band shift experiments implicate the interference of L-carnitine with this RXR/PPARα/LXRα signalling axis.

The importance of PPARα in *Crat* gene expression was supported by RT-PCR data. Administration of fenofibrate, a PPARα agonist led to 11-fold upregulation of Crat transcription (Figure 39). But no influence of LXR on *Crat* transcription was observable after the administration of GW3965, a LXRα agonist (Figure 41).

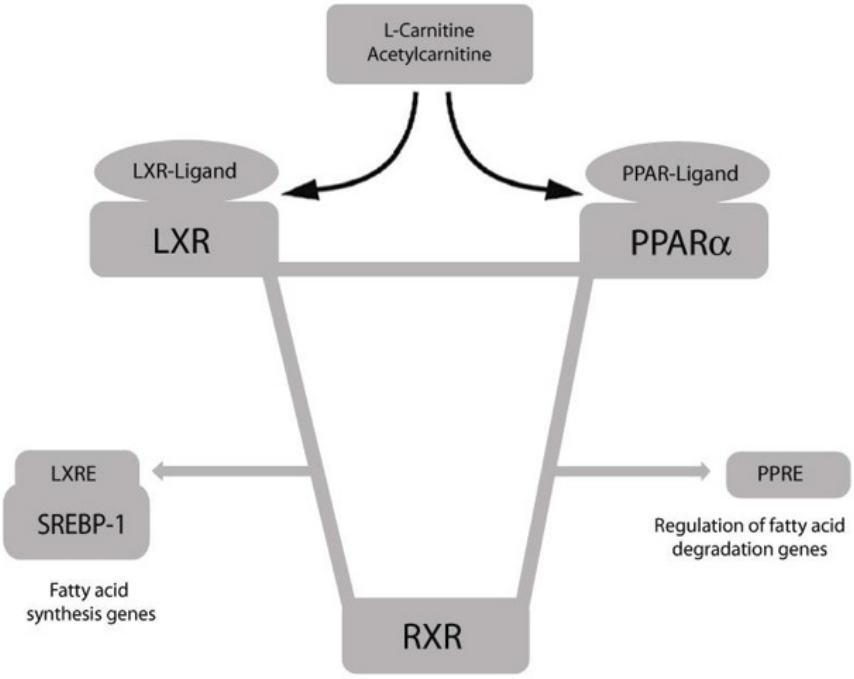


Figure 50: Model of the PPAR/LXR signalling axis and the possible influence of L-carnitine (adapted from Ide et al, 2004)

The direct interaction of PPARα with the discussed promoter sequence had been indicated through the PPARα supershift assay as depicted in Figure 33. Although there is no distinct supershift signal visible, this kind of "supershift" namely the weakening of the band shift signal was described and interpreted as a proof for interaction of certain proteins with the labelled ODN of interest. [140]

The examined sequence can be seen as an imperfect PPRE (PPAR responsive element). The accepted PPRE consensus sequence was shown to be a direct repeat of TGACCG separated by 1 nucleotide (DR1) flanked by an A/T rich sequence. [149] Based on the results we propose an imperfect PPRE with one mismatch in each consensus sequence and two nucleotides separating these repeats: TGACCCCGTGACGG. Binding of PPARα/RXRα heterodimers to such imperfect PPREs and functionality was shown before for instance in the *CPT2* and *OCTN2* promoters. [56, 63]

The third factor, which showed indications of being of influence to Crat expression after L-carnitine supplementation had been PPARbP or Trap220

(Figure 25). Interestingly again a weakening of the band shift signal could be observed with rising L-carnitine concentration in the cultivation medium and antibody concentration in the band shift approach. This outcome very nicely fits to the results from a microarray analysis a different approach, where PPARbP (PPAR binding protein) itself got upregulated by L-carnitine. The administration of 80µM L-carnitine after artificially induced carnitine deficiency led to 12.04-fold increase of PPARbP mRNA levels.

PPARbP was needed as a cofactor by PPARα for transcriptional regulation of liver specific genes. [150]

These results described above indicate that PPARα and L-carnitine form a tight entangled reciprocal signalling system. This theory is also supported by the fact, that PPARα itself gets upregulated by L-carnitine as the western blot depicted in Figure 35. Further it was reported that PPARα plays a key role in the regulation of carnitine homeostasis. It regulates genes, which are involved in carnitine synthesis and uptake. [151]

This close relation between these two compounds leads to the question, whether there is direct interaction. It will be the aim of future investigations if L-carnitine is able to bind PPARα directly or if other factors are needed for interaction. There is evidence that L-carnitine is able to bind directly to nuclear receptors like the glucocorticoid-receptor. [115]

Possible future investigations may also lead into the field of gender medicine. As it has been reported, PPARα regulated genes are less expressed in females. [152] Since PPARα and L-carnitine showed this tight connection also L-carnitine might exert different effects in males and females.

L-carnitine and its possible positive effects in the treatment of Diabetes Mellitus Type 2 have been discussed throughout the last decades. As mentioned in the introduction (see chapter 1.3) the ratio between free acetyl-coA + free L-carnitine and acetylcarnitine + free coA is important for regulation of balance of glycolysis and gluconeogenesis. A dysbalance of this system leads to increased hepatic gluconeogenesis, one pathologic

aspect of Diabetes Mellitus Type II. So shifting this ratio toward acetylcarnitine might lead to improved glucose metabolism.

The importance of *CRAT* was already shown in human myocytes. Overexpression of this enzyme in human skeletal muscle cells increased glucose uptake and attenuated lipid-induced suppression of glucose oxidation. This implicated that carnitine insufficiency and reduced CRAT activity might be reversible components of the metabolic syndrome. [153]

In the context of L-carnitine and Diabetes Mellitus Type II it was also reported that L-carnitine and acetyl-L-carnitine are effective compounds in improving insulin-mediated glucose disposal. Glucose uptake and oxidation was improved. [154] The authors of this study provided two possible mechanisms for this effect: on the one side the regulation of the cellular trafficking of acetylcarnitines and acylcarnitines could play a role and on the other side the synthesis of glycolytic and gluconeogenetic key enzymes were influenced by L-carnitine and acetyl-L-carnitine.

Here we provide another aspect of the beneficial actions of L-carnitine in context of DM Type II and the metabolic syndrome. In previous research projects was shown that L-carnitine has a direct influence on the transcription rates of many genes throughout the whole genome. Among these we find the genes of the acylcarnitine transferase system. Also human *CRAT* and its murine homologue *Crat* are inducible by L-carnitine.[132, 141] So administration of L-carnitine leads to increased levels of CRAT and provides free L-carnitine. These increased levels of CRAT and L-carnitine provide the basis that more acetyl-groups can be stored in the acetylcarnitine-pools simultaneously reducing acetyl-coA levels. Further more acetyl-coA groups are used in the Krebs cycle and gluconeogenesis is reduced.

This hypothesis would also support the possible influence of fibrates on glucose metabolism. Since we provide evidence, that *Crat* is upregulated by fenofibrate. This would implicate that fibrates do not only improve fatty acid and lipoprotein metabolism but also may be beneficial in treatment of DM Type II and the metabolic syndrome.

5 References

1. Vaz, F.M. and R.J.A. Wanders, *Carnitine biosynthesis in mammals.* Biochem. J., 2002. **361**: p. 417-429.
2. Strijbis, K., F.d.r.M. Vaz, and B. Distel, *Enzymology of the carnitine biosynthesis pathway.* IUBMB Life, 2010. **62**(5): p. 357-362.
3. Tanphaichitr, V. and H.P. Broquist, *Role of Lysine and N-Trimethyllysine in Carnitine Biosynthesis.* Journal of Biological Chemistry, 1973. **248**(6): p. 2176-2181.
4. Rebouche, C.J. and A.G. Engel, *Tissue distribution of carnitine biosynthetic enzymes in man.* Biochimica et Biophysica Acta (BBA) - General Subjects, 1980. **630**(1): p. 22-29.
5. Rebouche, C., *Carnitine function and requirements during the life cycle.* The FASEB Journal, 1992. **6**(15): p. 3379-3386.
6. Rebouche, C.J., *Kinetics, Pharmacokinetics, and Regulation of l-Carnitine and Acetyl-l-carnitine Metabolism.* Annals of the New York Academy of Sciences, 2004. **1033**(1): p. 30-41.
7. Rebouche, C.J. and C.A. Chenard, *Metabolic Fate of Dietary Carnitine in Human Adults: Identification and Quantification of Urinary and Fecal Metabolites.* The Journal of Nutrition, 1991. **121**(4): p. 539-546.
8. Tamai, I., et al., *Molecular and Functional Identification of Sodium Ion-dependent, High Affinity Human Carnitine Transporter OCTN2.* Journal of Biological Chemistry, 1998. **273**(32): p. 20378-20382.
9. Koepsell, H., B.M. Schmitt, and V. Gorboulev, *Organic cation transporters*, in *Reviews of Physiology, Biochemistry and Pharmacology*. 2004, Springer Berlin Heidelberg. p. 36-90.
10. Koepsell, H. and H. Endou, *The SLC22 drug transporter family.* Pflügers Archiv European Journal of Physiology, 2004. **447**(5): p. 666-676.
11. McArthur, M.J., et al., *Cellular uptake and intracellular trafficking of long chain fatty acids.* Journal of Lipid Research, 1999. **40**(8): p. 1371-1383.
12. Paul A., W., *Fatty acid activation.* Progress in Lipid Research, 1997. **36**(1): p. 55-83.
13. Kerner, J. and C. Hoppel, *Fatty acid import into mitochondria.* Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 2000. **1486**(1): p. 1-17.
14. Soupene, E. and F.A. Kuypers, *Mammalian Long-Chain Acyl-CoA Synthetases.* Experimental Biology and Medicine, 2008. **233**(5): p. 507-521.
15. Murthy, M.S. and S.V. Pande, *Malonyl-CoA binding site and the overt carnitine palmitoyltransferase activity reside on the opposite sides of the outer mitochondrial membrane.* Proceedings of the National Academy of Sciences, 1987. **84**(2): p. 378-382.
16. Zammit, V.A., *Carnitine acyltransferases: functional significance of subcellular distribution and membrane topology.* Progress in Lipid Research, 1999. **38**(3): p. 199-224.
17. Zammit, V.A., et al., *Carnitine, mitochondrial function and therapy.* Advanced Drug Delivery Reviews, 2009. **61**(14): p. 1353-1362.

18. Turkaly, P., J. Kerner, and C. Hoppel, *A 22 kDa polyanion inhibits carnitine-dependent fatty acid oxidation in rat liver mitochondria.* FEBS Letters, 1999. **460**(2): p. 241-245.
19. Faye, A., et al., *Rat Liver Carnitine Palmitoyltransferase 1 Forms an Oligomeric Complex within the Outer Mitochondrial Membrane.* Journal of Biological Chemistry, 2007. **282**(37): p. 26908-26916.
20. Indiveri, C., A. Tonazzi, and F. Palmieri, *Identification and purification of the carnitine carrier from rat liver mitochondria.* Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1990. **1020**(1): p. 81-86.
21. Brunner, S., et al., *Cloning and characterization of murine carnitine acetyltransferase: evidence for a requrement during cell cycle progression.* Biochem. J., 1997. **322**: p. 403-410.
22. van der Leij, F.R., et al., *Genomics of the Human Carnitine Acyltransferase Genes.* Molecular Genetics and Metabolism, 2000. **71**(1-2): p. 139-153.
23. Ramsay, R.R., R.D. Gandour, and F.R. van der Leij, *Molecular enzymology of carnitine transfer and transport.* Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 2001. **1546**(1): p. 21-43.
24. McGarry, J.D. and N.F. Brown, *The Mitochondrial Carnitine Palmitoyltransferase System - From Concept to Molecular Analysis.* European Journal of Biochemistry, 1997. **244**(1): p. 1-14.
25. Price, N.T., et al., *A Novel Brain-Expressed Protein Related to Carnitine Palmitoyltransferase I.* Genomics, 2002. **80**(4): p. 433-442.
26. Sierra, A.Y., et al., *CPT1c Is Localized in Endoplasmic Reticulum of Neurons and Has Carnitine Palmitoyltransferase Activity.* Journal of Biological Chemistry, 2008. **283**(11): p. 6878-6885.
27. Gao, S., et al., *Important roles of brain-specific carnitine palmitoyltransferase and ceramide metabolism in leptin hypothalamic control of feeding.* Proceedings of the National Academy of Sciences, 2011. **108**(23): p. 9691-9696.
28. Gooding, J.M., M. Shayeghi, and E.D. Saggerson, *Membrane transport of fatty acylcarnitine and free l-carnitine by rat liver microsomes.* European Journal of Biochemistry, 2004. **271**(5): p. 954-961.
29. Stephens, T., G. Cook, and R. Harris, *Effect of ph on malonyl-coA inhibition of carnitine palmitoyltransferase I.* Biochem J, 1983. **212**(2): p. 521-24.
30. Cook, G.A., et al., *Differential Regulation of Carnitine Palmitoyltransferase-I Gene Isoforms (CPT-I alpha and CPT-Ibeta) in the Rat Heart.* Journal of Molecular and Cellular Cardiology, 2001. **33**(2): p. 317-329.
31. Louet, J.-F.o., et al., *The Coactivator PGC-1 Is Involved in the Regulation of the Liver Carnitine Palmitoyltransferase I Gene Expression by cAMP in Combination with HNF4beta and cAMP-response Element-binding Protein (CREB).* Journal of Biological Chemistry, 2002. **277**(41): p. 37991-38000.
32. Mangelsdorf, D.J. and R.M. Evans, *The RXR heterodimers and orphan receptors.* Cell, 1995. **83**(6): p. 841-850.
33. Puigserver, P., et al., *A Cold-Inducible Coactivator of Nuclear Receptors Linked to Adaptive Thermogenesis.* Cell, 1998. **92**(6): p. 829-839.

34. Puigserver, P., et al., *Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1[alpha] interaction.* 2003. **423**(6939): p. 550-555.
35. Rosenfeld, M.G. and C.K. Glass, *Coregulator Codes of Transcriptional Regulation by Nuclear Receptors.* Journal of Biological Chemistry, 2001. **276**(40): p. 36865-36868.
36. Zhang, Y., et al., *Peroxisomal Proliferator-activated Receptor-gamma Coactivator-1alpha (PGC-1alpha) Enhances the Thyroid Hormone Induction of Carnitine Palmitoyltransferase I (CPT-Ialpha).* Journal of Biological Chemistry, 2004. **279**(52): p. 53963-53971.
37. Sadana, P., et al., *Regulation of carnitine palmitoyltransferase I (CPT-Ialpha) gene expression by the peroxisome proliferator activated receptor gamma coactivator (PGC-1) isoforms.* Molecular and Cellular Endocrinology, 2007. **267**(1-2): p. 6-16.
38. Napal, L., P.F. Marrero, and D. Haro, *An Intronic Peroxisome Proliferator-activated Receptor-binding sequence Mediates Fatty Acid Induction of the Human Carnitine Palmitoyltransferase 1A.* Journal of Molecular Biology, 2005. **354**(4): p. 751-759.
39. Park, E.A. and G.A. Cook, *Differential regulation in the heart of mitochondrial carnitine palmitoyltransferase-I muscle and liver isoforms.* Molecular and Cellular Biochemistry, 1998. **180**(1): p. 27-32.
40. Cook, G. and E. Park, *Expression and regulation of carnitine palmitoyltransferase-Ialpha and -Ibeta genes.* Am J Med Sci., 1999. **318**(1): p. 43-48.
41. Song, S., et al., *Peroxisome proliferator activated receptor alpha (PPARalpha) and PPAR gamma coactivator (PGC-alpha) induce carnitine palmitoyltransferase IA (CPT-1A) via independent gene elements.* Molecular and Cellular Endocrinology, 2010. **325**(1-2): p. 54-63.
42. Le May, C.d., et al., *Fatty Acids Induce L-CPT I Gene Expression through a PPARalpha-Independent Mechanism in Rat Hepatoma Cells.* The Journal of Nutrition, 2005. **135**(10): p. 2313-2319.
43. van der Leij, F.R., et al., *Structural and Functional Genomics of the CPT1B Gene for Muscle-type Carnitine Palmitoyltransferase I in Mammals.* Journal of Biological Chemistry, 2002. **277**(30): p. 26994-27005.
44. Wang, G.-L., M. Moor, and M. Jeanie, *A region in the first exon/intron on rat carnitine palmitoytransferase 1b is involved in encancement of basal transcription.* Biochem J, 2002. **362**: p. 609-618.
45. Yu, G.-S., Y.-C. Lu, and T. Gulick, *Co-regulation of Tissue-specific Alternative Human Carnitine Palmitoyltransferase Ib Gene Promoters by Fatty Acid Enzyme Substrate.* Journal of Biological Chemistry, 1998. **273**(49): p. 32901-32909.
46. Djouadi, F., C. Weinheimer, and D. Kelly, *The role of PPAR alpha as a "lipostat" transcription factor.* Adv Exp Med Biol., 1999. **466**: p. 211-220.
47. Moore, M.L., et al., *GATA-4 and Serum Response Factor Regulate Transcription of the Muscle-specific Carnitine Palmitoyltransferase I beta in Rat Heart.* Journal of Biological Chemistry, 2001. **276**(2): p. 1026-1033.
48. Gutgesell, A., et al., *Mouse carnitine-acylcarnitine translocase (CACT) is transcriptionally regulated by PPAR[alpha] and PPAR[delta] in liver cells.*

Biochimica et Biophysica Acta (BBA) - General Subjects, 2009. **1790**(10): p. 1206-1216.
49. Baillet, L., et al., *Elucidation of the Mechanism by Which (+)-Acylcarnitines Inhibit Mitochondrial Fatty Acid Transport.* Journal of Biological Chemistry, 2000. **275**(47): p. 36766-36768.
50. Burke, T.W. and J.T. Kadonaga, *Drosophila TFIID binds to a conserved downstream basal promoter element that is present in many TATA-box-deficient promoters.* Genes & Development, 1996. **10**(6): p. 711-724.
51. Rakhshandehroo, M., et al., *Comprehensive Analysis of PPARalpha-Dependent Regulation of Hepatic Lipid Metabolism by Expression Profiling.* PPAR Resarch, 2007. **2007**(26839).
52. Tachibana, K., et al., *Gene expression profiling of potential peroxisome proliferator-activated receptor (PPAR) target genes in human hepatoblastoma cell lines inducibly expressing different PPAR isoforms.* Nuclear Receptor, 2005. **3**(1): p. 3.
53. Iacobazzi, V., et al., *Statins, fibrates and retinoic acid upregulate mitochondrial acylcarnitine carrier gene expression.* Biochemical and Biophysical Research Communications, 2009. **388**(4): p. 643-647.
54. Convertini, P., et al., *Role of FOXA and Sp1 in mitochondrial acylcarnitine carrier gene expression in different cell lines.* Biochemical and Biophysical Research Communications, 2011. **404**(1): p. 376-381.
55. Montermini, L., et al., *Identification of 5' regulatory regions of the human carnitine palmitoyltransferase II gene.* Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression, 1994. **1219**(1): p. 237-240.
56. Barrero, M.J., et al., *Control of human carnitine palmitoyltransferase II gene transcription by peroxisome proliferator-activated receptor through a partially conserved peroxisome proliferator-responsive element.* Biochem. J., 2003. **369**(3): p. 721-729.
57. Bruce D., G., *Genomic structure of and a cardiac promoter for the mousecarnitine palmitoyltransferase II gene.* Genomics, 1993. **18**(3): p. 651-655.
58. Hashimoto, T., et al., *Defect in Peroxisome Proliferator-activated Receptor alpha-inducible Fatty Acid Oxidation Determines the Severity of Hepatic Steatosis in Response to Fasting.* Journal of Biological Chemistry, 2000. **275**(37): p. 28918-28928.
59. Bremer, J., *Carnitine--metabolism and functions.* Physiological Reviews, 1983. **63**(4): p. 1420-1480.
60. Reuter, S.E., et al., *Determination of the reference range of endogenous plasma carnitines in healthy adults.* Annals of Clinical Biochemistry, 2008. **45**(6): p. 585-592.
61. van Vlies, N., et al., *PPARalpha-activation results in enhanced carnitine biosynthesis and OCTN2-mediated hepatic carnitine accumulation.* Biochimica et Biophysica Acta (BBA) - Bioenergetics, 2007. **1767**(9): p. 1134-1142.
62. Luci, S., et al., *PPARalpha agonists up-regulate organic cation transporters in rat liver cells.* Biochemical and Biophysical Research Communications, 2006. **350**(3): p. 704-708.

63. Maeda, T., et al., *Regulation of Octn2 Transporter (SLC22A5) by Peroxisome Proliferator Activated Receptor Alpha.* Biological & Pharmaceutical Bulletin, 2008. **31**(6): p. 1230-1236.
64. Wen, G., R. Ringseis, and K. Eder, *Mouse OCTN2 is directly regulated by peroxisome proliferator-activated receptor alpha (PPARalpha) via a PPRE located in the first intron.* Biochemical Pharmacology, 2010. **79**(5): p. 768-776.
65. D'Argenio, G., et al., *Colon OCTN2 Gene Expression Is Up-regulated by Peroxisome Proliferator-activated Receptor gamma in Humans and Mice and Contributes to Local and Systemic Carnitine Homeostasis.* Journal of Biological Chemistry, 2010. **285**(35): p. 27078-27087.
66. Lancaster, C.S., et al., *Cisplatin-Induced Downregulation of OCTN2 Affects Carnitine Wasting.* Clinical Cancer Research, 2010. **16**(19): p. 4789-4799.
67. Markwell, M.A.K., et al., *The Subcellular Distribution of Carnitine Acyltransferases in Mammalian Liver and Kidney.* Journal of Biological Chemistry, 1973. **248**(10): p. 3426-3432.
68. Colucci, W.J. and R.D. Gandour, *Carnitine acetyltransferase: A review of its biology, enzymology, and bioorganic chemistry.* Bioorganic Chemistry, 1988. **16**(3): p. 307-334.
69. Cordente, A.G., et al., *Redesign of Carnitine Acetyltransferase Specificity by Protein Engineering.* Journal of Biological Chemistry, 2004. **279**(32): p. 33899-33908.
70. Kalaria, R. and S. Harik, *Carnitine acetyltransferase activity in the human brain and its microvessels is decreased in Alzheimer's disease.* Ann. Neurol., 1992. **32**(4): p. 583-586.
71. Maurer, I., S. Zierz, and H. Möller, *Carnitine acyltransferases are not changed in Alzheimer disease.* Alzheimer Dis Assoc. Disord., 1998. **12**(2): p. 71-76.
72. Melegh, B., et al., *Muscle carnitine acetyltransferase and carnitine deficiency in a case of mitochondrial encephalomyopathy.* Journal of Inherited Metabolic Disease, 1999. **22**(7): p. 827-838.
73. Brevetti, G., et al., *Muscle carnitine deficiency in patients with severe peripheral vascular disease.* Circulation, 1991. **84**(4): p. 1490-1495.
74. Pordes, A.G., *The CRAT (Carnitine Acetyltransferase) - Promoter under conditions of carnitine deficiency and supplementation*, in *Department for medical Biochemistry*. 2005, Medical University Vienna: Vienna.
75. Utter, M., D. Keech, and S. MC, *A possible role for acetyl CoA in the control of gluconeogenesis.* Adv Enzyme Requ., 1964. **2**: p. 49-68.
76. Jitrapakdee, S., et al., *Structure, mechanism and regulation of pyruvate carboxylase.* Biochem J, 2008. **413**(3): p. 369-87.
77. Randle, P.J., *Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years.* Diabetes/Metabolism Reviews, 1998. **14**(4): p. 263-283.
78. Roche, T. and Y. Hiromasa, *Pyruvate dehydrogenase kinase regulatory mechanisms and inhibition in treating diabetes, heart ischemia, and cancer.* Cellular and Molecular Life Sciences, 2007. **64**(7): p. 830-849.
79. DeFronzo, R.A., *Pharmacologic Therapy for Type 2 Diabetes Mellitus.* Annals of Internal Medicine, 1999. **131**(4): p. 281-303.

80. Wu, P., et al., *Mechanism responsible for inactivation of skeletal muscle pyruvate dehydrogenase complex in starvation and diabetes.* Diabetes, 1999. **48**(8): p. 1593-1599.
81. Longo, N., C. Amat di San Filippo, and M. Pasquali, *Disorders of carnitine transport and the carnitine cycle.* American Journal of Medical Genetics Part C: Seminars in Medical Genetics, 2006. **142C**(2): p. 77-85.
82. Angelini, C., L. Vergani, and A. Martinuzzi, *Clinical and Biochemical Aspects of Carnitine Deficiency and Insufficiency: Transport Defects and Inborn Errors of beta-Oxidation.* Critical Reviews in Clinical Laboratory Sciences, 1992. **29**(3-4): p. 217-242.
83. Koizumi, A., et al., *Genetic Epidemiology of the Carnitine Transporter OCTN2 Gene in a Japanese Population and Phenotypic Characterization in Japanese Pedigrees with Primary Systemic Carnitine Deficiency.* Human Molecular Genetics, 1999. **8**(12): p. 2247-2254.
84. Scaglia, F., et al., *Defective urinary carnitine transport in heterozygotes for primary carnitine deficiency.* Genet Med., 1998. **1**(1): p. 34-39.
85. Rinaldo, P., D. Matern, and M.J. Bennett, *Fatty Acid Oxidation Disorders.* Annual Review of Physiology, 2002. **64**(1): p. 477-502.
86. Famularo, G., et al., *Carnitine deficiency: Primary and secondary syndromes.*, in *Carnitine Today*, D.S. CFG, Editor. 1997: Austin, TX.
87. Flanagan, J., et al., *Role of carnitine in disease.* Nutrition & Metabolism, 2010. **7**(1): p. 30.
88. Matera, M., G. Bellinghieri, and V. Savica, *History of L-carnitine: implications for renal disease.* J Ren Nutr., 2003. **13**(1): p. 2-14.
89. Bougneres, P., J. Saudubray, and J. Girard, *Fasting hypoglycemia resulting from hepatic carnitine palmitoyl transferase deficiency.* J Pediatr., 1981. **98**(5): p. 742-746.
90. Rubio-Gozalbo, M., et al., *Carnitine-acylcarnitine translocase deficiency: case report and review of the literature.* Acta Paediatrica, 2003. **92**(4): p. 501-504.
91. Rubio-Gozalbo, M.E., et al., *Carnitine acylcarnitine translocase deficiency, clinical, biochemical and genetic aspects.* Molecular Aspects of Medicine. **25**(5-6): p. 521-532.
92. Iacobazzi, V., et al., *Response to therapy in carnitine/acylcarnitine translocase (CACT) deficiency due to a novel missense mutation.* American Journal of Medical Genetics Part A, 2004. **126A**(2): p. 150-155.
93. Thuillier, L., et al., *Correlation between genotype, metabolic data, and clinical presentation in carnitine palmitoyltransferase 2 (CPT2) deficiency.* Human Mutation, 2003. **21**(5): p. 493-501.
94. Isackson, P.J., et al., *CPT2 gene mutations resulting in lethal neonatal or severe infantile carnitine palmitoyltransferase II deficiency.* Molecular Genetics and Metabolism, 2008. **94**(4): p. 422-427.
95. Bonnefont, J.-P., et al., *Bezafibrate for an Inborn Mitochondrial Beta-Oxidation Defect.* New England Journal of Medicine, 2009. **360**(8): p. 838-840.
96. Liedtke, A.J., S.H. Nellis, and G. Copenhaver, *Effects of Carnitine in Ischemic and Fatty Acid Supplemented Swine Hearts.* The Journal of Clinical Investigation, 1979. **64**(2): p. 440-447.

97. Broderick, T.L., H.A. Quinney, and G.D. Lopaschuk, *L-carnitine increases glucose metabolism and mechanical function following ischaemia in diabetic rat heart.* Cardiovascular Research, 1995. **29**(3): p. 373-378.
98. Rodrigues, B., H. Xiang, and J. McNeill, *Effect of L-carnitine treatment on lipid metabolism and cardiac performance in chronically diabetic rats.* Diabetes, 1988. **37**(10): p. 1358-1364.
99. Rajasekar, P. and C.V. Anuradha, *Fructose-induced hepatic gluconeogenesis: Effect of L-carnitine.* Life Sciences, 2007. **80**(13): p. 1176-1183.
100. Bensinger, S.J. and P. Tontonoz, *Integration of metabolism and inflammation by lipid-activated nuclear receptors.* 2008. **454**(7203): p. 470-477.
101. Barter, P.J. and K.-A. Rye, *Is There a Role for Fibrates in the Management of Dyslipidemia in the Metabolic Syndrome?* Arteriosclerosis, Thrombosis, and Vascular Biology, 2008. **28**(1): p. 39-46.
102. Forman, B.M., J. Chen, and R.M. Evans, *Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and gamma.* Proceedings of the National Academy of Sciences, 1997. **94**(9): p. 4312-4317.
103. Kliewer, S.A., et al., *Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma.* Proceedings of the National Academy of Sciences, 1997. **94**(9): p. 4318-4323.
104. Bernal-Mizrachi, C., et al., *Dexamethasone induction of hypertension and diabetes is PPAR-[alpha] dependent in LDL receptor-null mice.* Nat Med, 2003. **9**(8): p. 1069-1075.
105. Siu, F.K.Y., L.T.O. Lee, and B.K.C. Chow, *Southwestern blotting in investigating transcriptional regulation.* Nature Protocols, 2008. **3**(1): p. 51-58.
106. http://www.gene-regulation.com/cgi-bin/pub/programs/molwsearch/molwsearch.cgi.
107. Frank, S. and P. Bernard, *Profiling Breast Cancer Using Realt-Time Quantitative PCR*, in *Rapid cycle real-time PCR:Methods and Application: quantification*, C. Wittwer, M. Hahn, and K. Kaul, Editors. 2004, Springer Verlag.
108. Zhu, Q., et al., *Identification of missense mutations in the hepatocyte nuclear factor-3beta gene in Japanese subjects with late-onset Type II diabetes mellitus.* Diabetologia, 2000. **43**(9): p. 1197-200.
109. Solomon, S.S., et al., *A critical role of Sp1 transcription factor in regulating gene expression in response to insulin and other hormones.* Life Sciences, 2008. **83**(9-10): p. 305-312.
110. Inken, W., *Sp1: Emerging roles - Beyond constitutive activation of TATA-less housekeeping genes.* Biochemical and Biophysical Research Communications, 2008. **372**(1): p. 1-13.
111. Schwarzmayr, L., et al., *Regulation of the E2F-associated phosphoprotein promoter by GC-box binding proteins.* The International Journal of Biochemistry & Cell Biology, 2008. **40**(12): p. 2845-2853.

112. Wang, H., et al., *Foxa2 (HNF3ß) Controls Multiple Genes Implicated in Metabolism-Secretion Coupling of Glucose-induced Insulin Release.* Journal of Biological Chemistry, 2002. **277**(20): p. 17564-17570.
113. Lu, N.Z., et al., *International Union of Pharmacology. LXV. The Pharmacology and Classification of the Nuclear Receptor Superfamily: Glucocorticoid, Mineralocorticoid, Progesterone, and Androgen Receptors.* Pharmacological Reviews, 2006. **58**(4): p. 782-797.
114. Rhen, T. and J.A. Cidlowski, *Antiinflammatory Action of Glucocorticoids - New Mechanisms for Old Drugs.* New England Journal of Medicine, 2005. **353**(16): p. 1711-1723.
115. Alesci, S., et al., *L-Carnitine: a nutritional modulator of glucocorticoid receptor functions.* The FASEB Journal, 2003. **17**(11): p. 1553-1555.
116. Gao, R.-l., et al., *Effects of notoginosides on proliferation and upregulation of GR nuclear transcription factor in hematopoietic cells.* 2007. **28**(5): p. 703-711.
117. Shoulders, C.C. and R.P. Naoumova, *USF1 implicated in the aetiology of familial combined hyperlipidaemia and the metabolic syndrome.* Trends in Molecular Medicine, 2004. **10**(8): p. 362-365.
118. Barco, A. and H. Marie, *Genetic Approaches to Investigate the Role of CREB in Neuronal Plasticity and Memory.* Molecular Neurobiology, 2011: p. 1-20.
119. Psarra, A.-M.G. and C.E. Sekeris, *Nuclear receptors and other nuclear transcription factors in mitochondria: Regulatory molecules in a new environment.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2008. **1783**(1): p. 1-11.
120. Sarawut, J., *Transcription factors and coactivators controlling nutrient and hormonal regulation of hepatic gluconeogenesis.* The International Journal of Biochemistry & Cell Biology, 2011.
121. Thomson, D.M. and W.W. Winder, *AMP-activated protein kinase control of fat metabolism in skeletal muscle.* Acta Physiologica, 2009. **196**(1): p. 147-154.
122. Lefebvre, P., Y. Benomar, and B. Staels, *Retinoid X receptors: common heterodimerization partners with distinct functions.* Trends in Endocrinology & Metabolism, 2010. **21**(11): p. 676-683.
123. Sudgen MC, H.M., *Role of nuclear receptors in the modulation of insulin secretion in lipid-induced insulin resistance.* Biochem Soc Trans., 2008. **36(Pt 5)**: p. 891-900.
124. Jetten, A.M., *Recent Advances in the Mechanisms of Action and Physiological Functions of the Retinoid-Related Orphan Receptors (RORs).* Current Drug Targets - Inflammation & Allergy, 2004. **3**(4): p. 395-412.
125. Boukhtouche, F., J. Mariani, and A. Tedgui, *The "CholesteROR" Protective Pathway in the Vascular System.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2004. **24**(4): p. 637-643.
126. Gurnell, M., *PPARgamma and metabolism: insights from the study of human genetic variants.* Clinical Endocrinology, 2003. **59**(3): p. 267-277.
127. Forman, B.M., et al., *15-Deoxy-delta12,14-Prostaglandin J2 is a ligand for the adipocyte determination factor PPARgamma.* Cell, 1995. **83**(5): p. 803-812.

128. Tontonoz, P., E. Hu, and B.M. Spiegelman, *Stimulation of adipogenesis in fibroblasts by PPARgamma, a lipid-activated transcription factor.* Cell, 1994. **79**(7): p. 1147-1156.
129. Barak, Y., et al., *PPARgamma Is Required for Placental, Cardiac, and Adipose Tissue Development.* Molecular Cell, 1999. **4**(4): p. 585-595.
130. Sato, S., et al., *A Set of Consensus Mammalian Mediator Subunits Identified by Multidimensional Protein Identification Technology.* Molecular Cell, 2004. **14**(5): p. 685-691.
131. Zhu, Y., et al., *Isolation and Characterization of PBP, a Protein That Interacts with Peroxisome Proliferator-activated Receptor.* Journal of Biological Chemistry, 1997. **272**(41): p. 25500-25506.
132. Hofer-Litzlbauer, E., *Biochemical and genetical consequences of carnitine deficiency caused by downregulation of the carnitine acyltransferase genes.*, in *Department of Medical Biochemistry, Division of Molecular Biology*. 2005, Medical University of Vienna: Vienna. p. 140.
133. Ide, T., et al., *Cross-Talk between Peroxisome Proliferator-Activated Receptor (PPAR) alpha and Liver X Receptor (LXR) in Nutritional Regulation of Fatty Acid Metabolism. II. LXRs Suppress Lipid Degradation Gene Promoters through Inhibition of PPAR Signaling.* Molecular Endocrinology, 2003. **17**(7): p. 1255-1267.
134. Morishita, K., et al., *Activation of EVI1 gene expression in human acute myelogenous leukemias by translocations spanning 300-400 kilobases on chromosome band 3q26.* Proceedings of the National Academy of Sciences, 1992. **89**(9): p. 3937-3941.
135. Buonamici, S., et al., *EVI1 induces myelodysplastic syndrome in mice.* The Journal of Clinical Investigation, 2004. **114**(5): p. 713-719.
136. Lebofsky, R. and J.C. Walter, *New Myc-anisms for DNA Replication and Tumorigenesis?* Cancer Cell, 2007. **12**(2): p. 102-103.
137. Taub, R., et al., *Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells.* Proceedings of the National Academy of Sciences, 1982. **79**(24): p. 7837-7841.
138. Riu, E., *Overexpression of c-myc in diabetic mice restores altered expression of the transcription factor genes that regulate liver metabolism.* Biochem J, 2002. **368**(3): p. 931-37.
139. Riu, E., et al., *Overexpression of c-myc in the liver prevents obesity and insulin resistance.* The FASEB Journal, 2003.
140. Dayoub, R., et al., *Foxa2 (HNF-3[beta]) regulates expression of hepatotrophic factor ALR in liver cells.* Biochemical and Biophysical Research Communications, 2010. **395**(4): p. 465-470.
141. Godarova, A., et al., *L-Carnitine regulates mRNA Expression Levels of the Carnitine Acyltransferases - CPT1A, CPT2 and CRAT.* Chemical Monthly, 2005. **136**: p. 1349-63.
142. Gerondaes, P., A.K.G. M.M, and A. Loranne, *Fatty acid metabolism in hepatocytes cultured with hypolipidaemic drugs.* Biochem.J., 1988. **253**: p. 161-167.
143. Yoshikawa, T., et al., *Cross-Talk between Peroxisome Proliferator-Activated Receptor (PPAR) alpha and Liver X Receptor (LXR) in*

Nutritional Regulation of Fatty Acid Metabolism. I. PPARs Suppress Sterol Regulatory Element Binding Protein-1c Promoter through Inhibition of LXR Signaling. Molecular Endocrinology, 2003. **17**(7): p. 1240-1254.
144. Thomas, J., et al., *A chemical switch regulates fibrate specificity for peroxisome proliferator-activated receptor alpha (PPARalpha) versus liver X receptor.* J Biol Chem, 2003. **278**(4): p. 2403-10.
145. Caton, P.W., et al., *PPARalpha-LXRalpha as a novel metabolostatic signalling axis in skeletal muscle that acts to optimize substrate selection in response to nutrient status.* Biochem J, 2011. **437**(3): p. 521-30.
146. Leone, T.C., C.J. Weinheimer, and D.P. Kelly, *A critical role for the peroxisome proliferator-activated receptor alpha (PPARalpha) in the cellular fasting response: the PPARalpha-null mouse as a model of fatty acid oxidation disorders.* Proc Natl Acad Sci U S A, 1999. **96**(13): p. 7473-8.
147. Repa, J.J., et al., *Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta.* Genes Dev, 2000. **14**(22): p. 2819-30.
148. Shimomura, I., et al., *Nuclear sterol regulatory element-binding proteins activate genes responsible for the entire program of unsaturated fatty acid biosynthesis in transgenic mouse liver.* J Biol Chem, 1998. **273**(52): p. 35299-306.
149. IJpenberg, A., et al., *Polarity and Specific Sequence Requirements of Peroxisome Proliferator-activated Receptor (PPAR)/Retinoid X Receptor Heterodimer Binding to DNA.* Journal of Biological Chemistry, 1997. **272**(32): p. 20108-20117.
150. Jia, Y., et al., *Transcription Coactivator PBP, the Peroxisome Proliferator-activated Receptor (PPAR)-binding Protein, Is Required for PPARÎ±-regulated Gene Expression in Liver.* Journal of Biological Chemistry, 2004. **279**(23): p. 24427-24434.
151. Wen, G., et al., *Mouse gamma-butyrobetaine dioxygenase is regulated by peroxisome proliferator-activated receptor alpha through a PPRE located in the proximal promoter.* Biochem Pharmacol, 2011. **82**(2): p. 175-83.
152. Ciana, P., et al., *A Novel Peroxisome Proliferator-Activated Receptor Responsive Element-Luciferase Reporter Mouse Reveals Gender Specificity of Peroxisome Proliferator-Activated Receptor Activity in Liver.* Molecular Endocrinology, 2007. **21**(2): p. 388-400.
153. Noland, R.C., et al., *Carnitine insufficiency caused by aging and overnutrition compromises mitochondrial performance and metabolic control.* J Biol Chem, 2009. **284**(34): p. 22840-52.
154. Mingrone, G., *Carnitine in type 2 diabetes.* Ann N Y Acad Sci, 2004. **1033**: p. 99-107.

Appendix

Abbreviations

Ab	antibiotics
APS	ammoniumpersulfate
ATP	adenosine triphosphate
BSA	bovine serum albumine
CACT	carnitine/ acylcarnitine transferase
coA	coenzyme A
CPT1	carnitine palmytoyltransferase 1
CPT2	carnitine palmytoyltransferase 2
CRAT	carnitine acetyltransferase
CROT	carnitine octanoyltransferase
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxy nucleotide triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetracetic acid
FCS	fetal calf serum
h	hours
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LXR	liver X receptor
mA	milliampere
mg	milligram
$MgCl_2$	magnesiumchloride
ml	milliliter
mol	
nt	nucleotides
OCTN2	organic cation/carnitine transporter 2
PATCH	attern search program using TRANSFAC 6.0
PBS	phosphate buffered saline

PMSF	phenylmethanesulfonylfluoride
PNK	polynucleotide kinase
PPAR	peroxisome proliferator-activated receptor
RXR	retinoic X receptor
SDS	sodium dodecyl sulfate
TBE	tris-borate-EDTA buffer
TEMED	tetramethylethylenediamine
TESS	transcription element search system
TE	Tris/EDTA
TRIS	tris(hydroxymethyl)aminomethane
V	volt
µl	microliters
µM	micromolare
PCR	polymerase chain reaction
RT-PCR	real-time polymerase chain reaction

Abstract

L-carnitine, an amino acid-like compound synthesized from lysine and methionine, is essential for energy metabolism. It mediates the transport of long-chain fatty acids prone for ß-oxidation across the inner mitochondrial membrane into the matrix. Chip-screen analyses performed in our lab revealed that L-carnitine has a distinct regulative effect on the transcriptional activity on a significant part of the whole genome (Litzlbauer et. al, 2006). Especially the mRNA expression levels of human carnitine acyltransferase genes CRAT, CPT1A, CPT2 are regulated by L-carnitine (Godárová et al, 2005). We could provide evidence that the murine homologues CRAT, CPT1a, CPT2 and OCTN2 are similarly regulated on transcriptional level by L-carnitine. qPCR analysis of the murine carnitine acetyltransferase (CRAT) gene under supplementation of 80 µM L-carnitine for 4 hours resulted in a 2-fold increase on mRNA level. Reporter gene-assays with constructs carrying different segments of the promoter of the murine CRAT gene localized the “carnitine-effect” regulating element within a 342bp region. Within this region several putative transcription factor bindings sites could be identified with bioinformatical tools. The most promising transcription factor binding sites are SP1, PPARα, RXRα and CREB. Analysis of the retinoic X receptor alpha (RXRα) element with band shift and supershift assays revealed the influence of the transcription factors PPARα, LXRα and PPARbP on CRAT gene regulation under increasing L-carnitine concentration. Additional Southwestern Blot analysis supported the association of these promoter elements to a hypothetical transcription factor complex of the RXR locus. qPCR analysis of CRAT mRNA expression under PPARα-agonist supplementation supports the findings that PPARα is involved in the L-carnitine mediated gene regulation of CRAT. This indicates that CRAT gene-expression is a direct pharmacological target of these hyperlipidemic drugs.

Zusammenfassung

L-Carnitine ist ein semi-essentieller Nährstoff welcher aus den Aminosäuren Methionin und Lysin synthetisiert und mit der Nahrung aufgenommen wird. Besonders im Energiestoffwechsel erfüllt L-Carnitine wichtige Aufgaben. Es ermöglicht den Transport von langkettigen Fettsäuren (C16-C18) aus dem Zytosol in die mitochondrielle Matrix, wo diese Fettsäuren im Rahmen der β-Oxidation zu Acetyl-CoA abgebaut und folglich dem Krebszyklus zugeführt werden.

L-Carnitine hat jedoch auch einen Einfluss auf die transkriptionelle Aktivität von verschiedensten Genen im gesamten Genom. (Litzlbauer et. al, 2006). Besonders die Menge an mRNA der humanen Acyltransferase-Gene CRAT, CPT1a und CPT2 werden nach Gabe von L-Carnitine hochreguliert. (Godárová et al, 2005). In dieser Diplomarbeit war es mir möglich diesen Effekt auch in der murinen Leberzelllinie TIB73 nachzuweisen. Durch quantitiative Real-Time PCR konnte ein bis zu 2-facher Anstieg der mRNA Menge des Gens der Carnitine-Acetyltransferase 4 Stunden nach Gabe von 80µM L-Carnitine nachgewiesen werden.

In Vorarbeiten von Aniko Ginta Pordes konnte mittels Reportergen-Assays diejenige Gensequenz eingegrenzt werden welche für diesen „Carnitine-Effekt“ verantwortlich ist. Diese ist innerhalb von 342nt proximal des ersten Exons lokalisiert. Innerhalb dieser Sequenz konnten mittels theoretischer Promoteranalyse zahlreiche Bindungsstellen möglicher Transkriptions-faktoren definiert werden. Darunter fanden sich Consensus-Sequenzen für nukleare Faktoren wie Sp1, RXR, PPAR und Creb.

Mittels MobilityShift Assays wurden diese Kandidaten überprüft. Die deutlichsten Effekte wurden bei der Analyse eines RXR Elements beobachtet, wobei in Supershift Experimenten besonders nach Gabe von PPARα und LXRα Antikörpern ein Abschwächungseffekt ausgemacht werden konnte. Der Einfluss von PPARα auf die Regulation der Expression des CRAT Gens konnte weiters mittels qPCR nachgewiesen werden, nachdem die Gabe von Fibraten zu eindeutigem Ansteigen der Expressionslevels von CRAT führte.

Curriculum vitae

Personal Information

Name:	Klemens Kienesberger
Address:	Nussdorferstrasse 36/5 1090 Vienna Austria E-Mail: a0400111@unet.univie.ac.at
Nationality:	Austrian

Education

10/2005 – 2/2012	**Studies of Molecular Biology** University of Vienna
10/2008 - present	**Studies of Human Medicine** Medical University of Vienna
1995-2003	BG/BRG Bad Ischl
1991-1995	Primary School, VS Pfandl, Bad Ischl

Laboratory Experience

07/2009-09/2009	Rotation Student at Institute of Medical Biochemistry, Division Molecular Biology, Group Hofbauer
11/2009-12/2009	Rotation Student KILM (Klinisches Institut Labormedizin), Group MedUni Wien Biobank
08/2010-09/2010	Rotation Student at Institute of Immunology, Group Ellmeier
11/2010-11/2011	Diploma Student at Institute of Medical Biochemistry, Division Molecular Biology, Group Hofbauer

Congresses and Conferences

09/2011	Posterpresentation at ÖGMBT Annual Meeting 2011, Puch/Urstein, Salzburg

Medical Internships

07/2010	Internship Department for Surgery, LKH Bad Ischl
07/2011	Internship Department for Internal Medicine, LKH Bad Aussee

Other Work Experience

09/2010 - present	Assistant to the course instructor; training course for medical assistants, Ärztekammer Wien
03/2005 – 10/2008	Seller and supplier at ice cream parlor “Giovanni”; Bad Ischl
05/2008 – 11/2008	Coordinator of museum educational service – “Oberösterreichische Landesausstellung” – Strobl/Wolfgangsee
11/2004 – 05/2005	Freelance collaborator, labour magazine “mein job”, Vienna
09/2003	Traineeship Museum of Contemporary History and Concentration Camp Memorial Ebensee
07/2002 and 07/2003	Summer internship Eurothermen Resort, Bad Ischl

Alternative civilian service

10/2003 – 09/2004	Alternative civilian service, Museum of Contemporary History and Concentration Camp Memorial Ebensee

Skills

Computer skills	Word, Excel, Powerpoint, SPSS, Adobe PhotoShop, Access, FlowJo,

Interests and Hobbies

Winter Sports, Basketball,
History, medical science
Gastroenterology and Endocrinology

Vienna, February 2012 Klemens Kienesberger