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Regulation of carnitine palmitoyltransferase Ia under inflammatory conditions in rat mesangial and primary liver cells

Dissertation

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I
Introduction

1.1 Metabolism of fatty acids

1.1.1 Role of fatty acids

Fatty acids (FAs) are a major source of energy for many tissues or organs in animals, especially for muscle, kidney and liver. Produced by lipolysis mostly from adipose tissue, FAs are transported bound to plasma albumin in the blood and are taken up by tissues via transport proteins present in the plasma membrane. In lipogenic tissues like the liver, white adipose tissue (WAT) and kidney, FAs can be synthesised *de novo* from glucose following glycolysis. These tissues are therefore major targets for the regulation of gene expression which is crucial for metabolism.
Inside cells, FAs have various targets depending on the tissue and its metabolic functions. For instance, they can be elongated, desaturated, oxidised for energy production, peroxidised, exchanged with phospholipids and are substrates for eicosanoid biosynthesis. Long-chain fatty acids (LCFAs) are critically important in cellular homeostasis as they are involved in a wide variety of processes including post-translational modifications of proteins, cell signalling, membrane permeability, and regulation of transcription.

1.1.2 Mitochondrial β-oxidation

The theory of ß-oxidation started in 1904, when Knoop could demonstrated that the oxidation of FAs begins at carbon atom 3, the β-carbon, causing it to yield FAs shortened by two carbon atoms (Vance and Vance, 2002).
The ß-oxidation of FAs is a complex pathway involving in the case of saturated LCFAs at least 16 proteins which are associated with the inner mitochondrial membrane and matrix. It is a central metabolic process supplying electrons to the respiratory chain and thus energy for the aerobic organisms, and it is of particular importance for cardiac and skeletal muscles. Moreover, a number of other tissues, primarily the liver but also the kidney, smal intestine and WAT, can utilise the

products of ß-oxidation for the formation of ketone bodies (acetoacetate and ß-hydoxybutyrate) which are important fuels for extrahepatic organs (e.g. brain).

1.1.3 The mitochondrial carnitine palmityltransferase system

After entry into the cell and before their catabolism, LCFAs are activated to acyl-CoA esters by acyl-CoA synthetases localised in the mitochondrial outer membrane. Acyl-CoA esters cannot directly cross the mitochondrial inner membrane but require carnitine and three proteins including carnitine palmitoyltransferase-I (CPT-I), the carnitine acylcarnitine carrier (CAC), and carnitine palmitoyltransferase-II (CPT-II) for this process (Fig. 1).

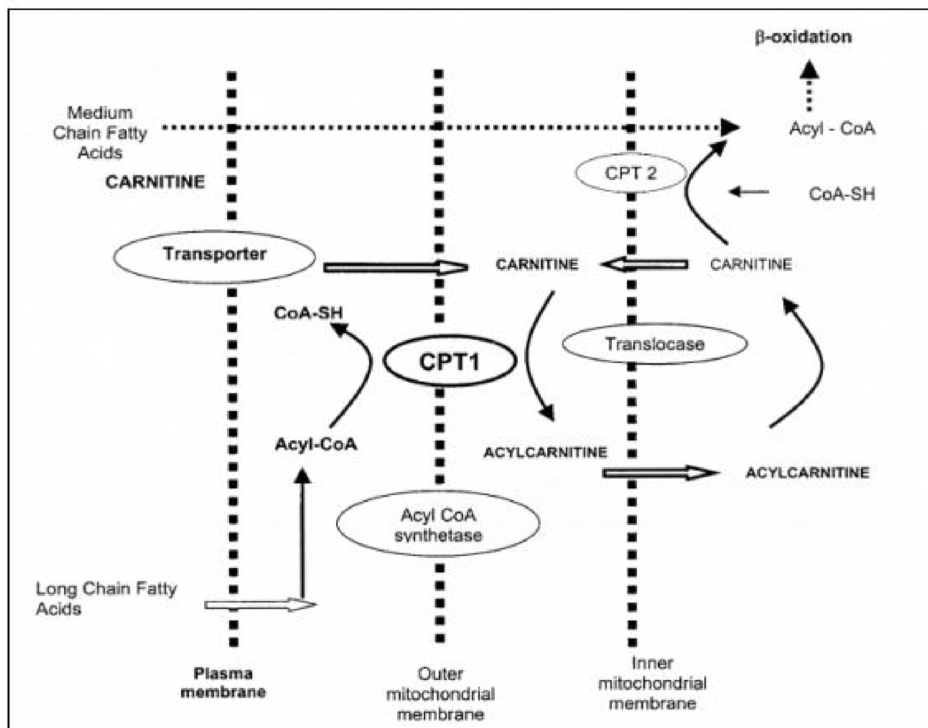


Figure 1.1 Mitochondrial carnitine palmitoyltransferase systhem . (Taken from Prasad *et al.,* 2001).

The first enzyme, CPT-I is localised in the outer-mitochondrial membrane and converts the fatty acyl-CoA ester to it's carnitine ester. The carnitine ester is subsequently transported across the mitochondrial inner membrane by CAC. Once inside the mitochondrial lumen, CPT-II, which is bound to the mitochondrial inner membrane facing the mitochondrial matrix, reconverts the carnitine ester to the CoA ester, which can then serve as a substrate for ß-oxidation.

1.1.4 Carnitine palmitoyltransferase-I

Carnitine palmitoyltransferase-I (CPT-I) is an enzyme that catalyses the regulatory step of LCFA translocation into the mitochondrial matrix. The other components of the fatty acid-translocating system are generally not considered to play a significant regulatory role in the transport of LCFAs into the mitochondrial matrix.

It is generally accepted that the oxidation of LCFA is regulated at the level of CPT-I through different mechanisms: (1) changes in CPT-I activity, (2) changes in the concentration of malonyl-CoA. Malonyl-CoA is the product of the reaction catalysed by key enzyme of FA synthesis, acetyl-CoA carboxylase (ACC) and is a physiological inhibitor of CPT-I, (3) changes in CPT-I sensitivity to malonyl-CoA inhibition (Girard *et al.*, 1992). The last two, so caled malonyl-CoA- dependent mechanisms of regulation are generally regarded as short-term controls of CPT-I activity and differ from long-term regulation in response to alterations in the nutritional and hormonal status of the animal. During the last years, however, a novel mechanism of short-term control of CPT-I has been proposed. This is the malony-CoA-independent stimulation of CPT-I, which involves modulation of interactions between CPT-I and cytoskeletal components. This modulation may rely on the Ca^{+2}/calmodulin-dependent protein kinase II (CM-PKII) cascade and 5'-AMP-activated protein kinase (AMPK) activation. Several observations suggested that the control of CPT-I is a concerted action of malonyl-CoA-dependent and -independent mechanisms (Valesco *et al.,* 1997; Valesco *et al.*, 1998).

The results of previous studies suggested that the regulation of CPT-I gene expression is dependent on hormonal (e.g. glucagon, insulin, thyroid hormones) and nutritional factors (e.g. FA, carnitine). It is known that rat liver CPT-I gene expression (and enzyme activity) increases dramaticaly after birth, and also during the translation from the fed to starved state in the adult animals (Girard *et al.,* 1992; Thumelin *et al.,* 1994). In both these situations there is an increase of plasma glucagon, cAMP, thyroid hormones and decrease of plasma insulin. Under these conditions, there is also a marked increases in CPT-I activation and CPT-I mRNA and protein level. Another potential regulator of CPT-I and FA oxidation may be hydrogen ion accumulation. It was found that small changes in pH-level from 7.0 to 6.8 inhibited CPT-I activity by 50% (Starritt *et al.,* 2000). Such changes in pH-level can be observed during intensive exercise and hypoxia (Jeukendrup, 2002).

1.1.5 Hepatic carnitine palmitoyltransferase I isoform

Two kineticaly different isoforms, namely hepatic CPT-Iα and muscle CPT-Iβ, have been described with distinct tissue distributions and are encoded by different genes localised on chromosome 11q13 and 22q13, respectively (Britton *at al.,* 1997).

The full length cDNA encoding rat and human liver CPT-Iα has been isolated and characterised (Esser *et al.,* 1993; Britton *et al.,* 1995). The rat cDNA contains an open reading frame of 2319 bases, predicting a 773-amino acid protein of 88 kDa. It was shown that CPT-Iα protein adopts a bitopic location within the mitochondrial outer membrane; it has two transmembrane domains, and both the N- and C-termini are exposed on the cytosolic side of the membrane, whereas the linker region between the transmembrane domains protrudes into the intermembrane space (Fraser *et al.,* 1997) (Fig. 1.2.).

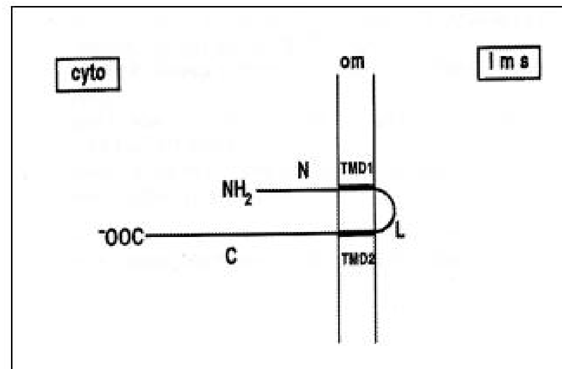


Figure 1. 2 Topology of CPT-Iα within the mitochondrial outer membrain.
TMD, transmembrane domains; Cyto, cytosolic side; Ims, intramembrane space; OM, mitochondrial outer membrane. (Taken from Fraser *et al.,* 1997).

CPT-Iα is found in most cells including heart, liver and renal mesangial cells, however, is not present in skeletal muscle cells and white adipocytes. CPT-Iß is expressed in skeletal muscle, brown and white adipocytes and heart (Cook *et al.,* 2001). The regulation of these isoforms is considerably different in terms of enzyme activity and gene expression. For instance, the sensitivity of CPT-Iα to malonyl-CoA inhibition is increased by insulin and decreased by thyroid hormone, while the sensitivity of CPT-Iß is not altered by these hormones. Interestingly, it was shown that development as well as hormonal and nutritional control of CPT-Iα have

different effects in liver and heart (Cook *et al.,* 2001). This result suggested that CPT-Iα is regulated also in a tissue- specific manner. However, little is known about differences in CPT-Iα regulation in liver and renal mesangial cells (or another renal cells), especially under inflammatory conditions. The present study was focused on only the characterisation of the CPT-Iα isoform in rat mesangial cells and primary hepatocytes.

1.1.6 Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHMG-CoA synthase)

HMG-CoA synthase catalyses the condensation of acetoacetyl-CoA and acetyl-CoA to form HMG-CoA plus free CoA. Two HMG-CoA synthase isoforms were localised in the cytosolic and mitochondrial compartments and characterised as products of two different genes (Ayte *et al.,*1990; Gil-Go`Imez *et al.,* 1993). The mitochondrial HMG-CoA synthase is an important control site of ketogenensis, a mitochondrial process by which acetyl-CoA, mostly derived from the ß-oxidation of fatty acids, is converted through four reactions into acetoacetate, ß-hydroxybutyrate and acetone, all of which are ketone bodies (Fig.1.3).

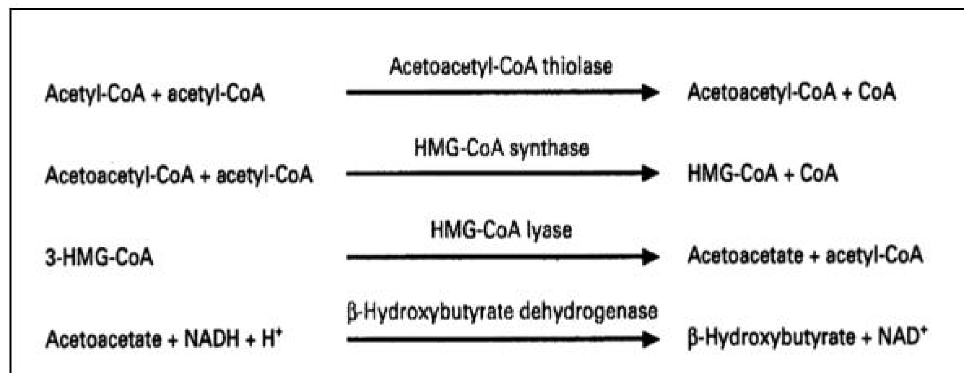


Figure 1. 3 Schematic representation of the HMG-CoA pathway. (Taken from Hegardt , *et al.,* 1999).

The utilisation of fatty acids for ketogenensis is also largely controlled by CPT-Iα in hepatocytes (McGarry and Brawn, 1997) and other tissues (Bla`zquez *et al.,* 1998) and the promoters of both enzymes contain similar DNA regulatory elements necessary to modulate the expression by cAMP, insulin and fatty acid (Hegardt, 1998; Hegardt, 1999; Chatelain *et al*., 1996).

1.2 Nitric oxide (NO)

Nitric oxide (NO) is a multifunctional mammalian effector that is involved in numerous biological processes, such as neurotransmission, vasodilation, immune modulation, and regulation of apoptosis (Krönke *et al.,* 1995; Huwiler and Pfeilschifter, 2003; Beck *et al.,* 1999).

It is synthesised from L-arginine by the enzyme nitric-oxide synthase (NOS), which requires the cofactor tetrahydrobiopterin (BH_4) for maximal activity. NOS catalyses the oxidation of the terminal guanidino group atom of L-arginine to form L-citrulline and NO. Details of this mechanism are controversial, but it is known that NOS enzymes combine oxygenase and reductase activities associated with distinct structural domains. The oxygenase domain contains heme, while the reductase domain binds calcium-calmodulin, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and NADPH. It is believed that the flavins accept electrons from NADPH and transfer them to the heme iron which binds O_2 and catalyses the stepwise oxidation of L-arginine to NO and citrulline.

Three isoforms of NOSs have been identified and categorised into constitutive and inducible isotypes. Two distinct constitutively expressed NOSs, endothelial NOS (eNOS) and neuronal (nNOS), produce nanomolar amounts of NO. A third isoform, inducible (iNOS), is expressed in response to inflammatory cytokines in many cell types including hepatocytes and mesangial cells, and produces micromolar concentrations of NO.

The result of many of the actions (e.g. vasodilation) of NO leads to the formation of cGMP (Lucas *et al.,* 2000). NO, formed by the cells, binds to a heme moiety on the soluble guanylyl cyclase and activates this enzyme to produce cGMP from GTP. The resulting increase of intracellular cGMP alters the activity of three main target proteins: (1) cGMP-regulated ion channels (2) cGMP-regulated phosphodiesterases, and (3) cGMP-dependent protein kinases (cGKs) (Schmidt *et al.,* 1993). Moreover, in smooth muscle cells, the activation of cGKs leads to the inhibition of calcium influx and subsequently to a decrease in smooth muscle tension, causing vasodilation.

Furthermore, NO affects gene expression by modulating transcription factors (e.g. NF-κB, AP-1), activating the MAPK cascade and in high concentrations directly causing damage of DNA (Beck KF *et al.*, 1999). NO and its reaction products can

also modify protein functions through S-nitrosylation of thiol groups or nitration of tyrosine residues of proteins (Stamler, 1994).

1.2.1 NO and inflammation

Many acute inflammatory diseases are associated with a massive production of NO. In particular, inflammatory kidney diseases and hepatic injuries are accompanied by changes in the expression patterns of cytokines, growth factors and many more inflammatory mediators (Huwiler and Pfeilschifter, 1999 [a; b]).
iNOS is rapidly upregulated in response to the endotoxin lipopolysaccharide (LPS), or proinflammatory cytokines (such as tumor necrosis factor-α (TNF-α), interleukin-1ß (IL-1ß) or interferon-γ or combinations of these) within hours in hepatocytes and mesangial cells. These stimuli often act synergistically to induce iNOS expression, however, IL-1ß alone is an effective stimulator of iNOS in rat mesangial cells and hepatocytes (Pfeilschifter and Schwarzenbach, 1990). In rat mesangial cells and hepatocytes, the cytokine- mediated upregulation of iNOS gene transcription requires the presence of transcriptional factor NF-κB. NF-κB can bind to κB-regulatory elements to enhance or to induce gene transcription and is a central regulator of a number of genes involved in cell adhesion, inflammatory responses, apoptosis, differentiation and proliferation (Eberhardt *et al.,* 1998; Diaz-Guerra *et al.,* 1996).

1.2.2 NO in the regulation of fatty acid metabolism

The regulation of CPT-I by NO was first described in 1996. After feeding of the rats with a specific inhibitor of NO synthase N^G-monomethyl-L-arginine L-NMMA, a significant decrease in ketogenesis was observed. Furthermore, in these rats, hyperlipidemia and hypercholesteremia was dramaticaly induced. These changes were explained by a decrease in CPT-I activity and subsequently, in a massive disorder of lipid metabolism (Khedara *et al.,* 1996). More recently, it was shown that the NO/cGMP signalling pathway is involved in the stimulation of CPT-Iα activity in rat liver cells. The NO donors and cGMP analogs inhibited ACC, and thus, decreased the level of malonyl-CoA. Because malonyl-CoA is a potent inhibitor of CPT-I, its depletion led to a significant activation of CPT-Iα and increase in the rate of FA oxidation (Garcia- Villafranca *et al.,* 2002).

1. 3 Hypoxia

Hypoxia is a condition by which tissues and cells are insufficiently supplied with oxygen. Thereby, acute responses often result in changes in the activity of preexisting proteins, while chronic responses cause changes in gene expression. Gene products that augment O_2 supply and enhance cell survival include erythropoietin which increases the proliferation of erythrocytes, the angiogenic factor VEGF which stimulates growth of new capillaries, and induction of glycolytic enzymes which are important for ATP production.

Hypoxia is the physiologic trigger that activates the hypoxia-inducible factor (HIF), a transcription factor which is also up-regulated by certain transition metals (Co^{2+}, Ni^{2+}, Mn^{2+}) and by iron chelation (desferrioxamine (DFO)) (Wang and Semenza, 1993; Goldberg e*t. al.,* 1988). It has been proposed that Co^{+2} is incorporated into newly synthesised heme, thereby locking the O_2 sensor into a deoxy configuration and therefore mimicking the effects of hypoxia.

Under hypoxic conditions, HIF-1 binds to hypoxia-responsive element (HRE) on the promoter or enhancer of various hypoxia-inducible genes (Rees *et al.,* 2001; Kakinuma *et al.,* 2001) including erythropoietin, vascular endothelial factor, glucose transport proteins, and glycolytic enzymes, as well as genes involved in iron metabolism.

HIF is a heterodimer that is composed of a HIF-Iα and HIF-Iß subunit. Hypoxia leads to the accumulation of HIF-Iα, dimerisation with HIF-Iß and to binding of this dimer to HIF-responsive elements (HRE) (Huang and Bunn, 2003). For some genes (*e.g.* glucose transporter-1), hypoxia-inducible expression is critically dependent on HIF-Iα, whereas for other genes (e.g. heme oxigenase-1, CPT-Iß) hypoxia-inducible changes in their expression appeared to be largely independent of HIF-Iα expression (Janice *et al.,* 2001).

In mesangial cells and hepatocytes, hypoxia can develop due to different physiological circumstances. For instance, during normal development, glomeruli might be relatively ischemic/ hypoxic because of incompletely developed capillaries (Yuan *et al.*, 2000). Chronic hypoxia is known to affect mesangial cell proliferation, matrix production and secretion of signaling molecules (Sahai *et al.*, 1997; Kim *et al.*, 1996). In liver, an oxygen gradient exists due to the unidirectional blood flow and to the oxygen-consuming metabolic processes of the cells. This gradient was

proposed to be an important regulator of the expression of genes encoding the key enzymes of the carbohydrate metabolism (Jungrmann and Kitetzmann, 1996).

1.3.1 Hypoxia and inflammation

Hypoxia plays an important role in many pathophysiological conditions including ischemia, cancer, stroke. For instance, it has been shown that HIF-Iα and VEGF are upregulated in the myocardium during heart failure or acute coronary occlusion (Kakinuma *et al.,* 2001; Huang and Bunn, 2003). In renal inflammatory diseases where the capillary is obliterated or distracted, glomeruli can be regarded as ischemic /hypoxic (Nangaku *et al.,* 1997). Just as HIF has a critical role to play in growth and progression, it is beneficial in vascular remodelling, where HIF-dependent genes are activated.

Recent studies have shown that HIF-Iα is not only accumulated by hypoxia, but is also activated in normoxic cells in response to a variety of mediators, including IL-1ß, growth factor (VEGF) and TNFα (Stiehl *et al.,* 2001). However, the induction of HIF-Iα by hypoxia is much more pronounced than by growth factors and cytokines (Huang and Bunn 2003). On the other hand, hypoxia activates HIF-I-dependent transcription factors to induce pro-inflammatory genes like interleukin-8 (IL-8), IL-6, cyclooxygenase-2 or secretoric phospholipases A_2 (Michiels *et al*., 2002).

1.3.2 Metabolic adaptation to hypoxia

Energy metabolic responses have evolved to permit preservation of cellular function in hypoxic environments. One critical adaptive response involves the suppression of cellular energy consumption and improved efficiency of oxygen utilization during ATP production. Under hypoxic conditions, decreased oxygen consumption is achieved in part by increasing cellular glycolytic capacity while down-regulating mitochondrial fatty acid ß-oxidation. Despite recent progress in characterising the regulation of cellular glucose utilization in response to hypoxia, little is known about the mechanisms involved in hypoxia-mediated suppression of mitochondrial flux. Although, cardiac myocytes have been the subject of many investigations concerning the regulation of CPT-I and fatty acid oxidation, there are many discrepancies and open questions. For instance, it has been shown that CPT-Iα activity is significantly stimulated in hypoxic myocytes and suggesting that FAs are the preferred substrate in hypoxic/ischemic heart (Wang *et al.,* 1998). However, a more recent study demonstrated that under hypoxia, LCFA-mediated transcriptional

activation of CPT-1ß is blocked by the PPAR/RXRα pathway and causes an accumulation of lipids in cardiac myocytes (Huss *et al.,* 2001). Even less clear is the regulation of CPT-Iα in liver and kidney under hypoxia, with no detailed studies available so far.

1.4 Phospholipases

Phospholipases A_2 (PLA_2) are widely distributed enzymes whose primary action is the hydrolysis of glycerophospholipids at the sn-2 position to yield free fatty acids, including arachidonic acid (AA), and lysophospholipids. These enzymes are classified in high-molecular-weight (85 kDa) cytosolic PLA_2s ($cPLA_2$s or group IV PLA_2s), low-molecular-weight (13-16 kDa) secretory PLA_2s ($sPLA_2$s), calcium-independent $iPLA_2$s (85 kDa) and PAF acetylhydrolases (PAF-AH) (Kudo and Murakami, 2002). Cytosolic PLA_2s show a marked preference for AA over other fatty acids and are activated by Ca^{2+} and by phosphorylation (with the exception of the calcium-independent isoform $cPLA_2$-γ). $cPLA_2$s are located in the cytosol but translocate to the perinuclar and Golgi membranes when the intracellular Ca^{2+} rises above about 0.5 µM. AA released from membrane phospholipids by the action of $cPLA_2$s is then used for the biosynthesis of eicosanoids as important lipid second messengers. Another intracellular form of PLA_2s is the family of Ca^{2+}-independent PLA_2s ($iPLA_2$s), which are responsible for removing highly saturated FAs from phospholipids in order to incorporate more unsaturated FAs. e.g. AA into cellular phospholipids (phospholipid acyl chain remodeling) (Winstead *et al.,* 2000). How $iPLA_2$s bind to membranes, and whether they cycle between the membrane and soluble fractions, remains to be determined. Another group of Ca^{2+}-independent PLA_2 enzymes of 30-40 kDa, care the platelet activating factor-acetylhydrolases (PAF-AHs). These enzymes have the capability to hydrolyse and inactivate the lipid mediator PAF, a phosphatidylcholine species that has a potent pro-inflammatory activity (Stafforini *et al.,* 1997). The PLA_2-family also includes a growing number of secreted enzymes, so-called secreted PLA_2s ($sPLA_2$). Several $sPLA_2$s have been characterised and classified into different groups (group IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XII) (Murakami and Kudo, 2004). Previous studies have shown that these enzymes possess distinct physiological functions and display distinct, but yet partially overlapping tissue distribution patterns. $sPLA_2$s are disulfide-rich proteins that display little FA chain specificity and hydrolyse the ester bond of

glycerophospholipids in the presence of mM concentrations of Ca^{2+} (Murakami and Kudo, 2004).

Both $cPLA_2$ and $sPLA_2$ have been implicated in various physiological and pathological functions including lipid digestion, release of proinflammatory mediators, cell proliferation, ischemic injury, inflammatory disease, cancer, and anti-bacterial defence.

1.4.1 Properties of group IIA PLA_2 ($sPLA_2$ IIA) and its expression in inflammation

$sPLA_2$-IIA is constitutively expressed in various tissues related to the immune response (e.g. spleen, thymus), as well as in the intestine and liver cells. Inflammatory effector cells such as neutrophils and mast cells store $sPLA_2$-IIA in their secretory granules and release it following cell activation. Renal mesangial and liver cells also secrete $sPLA_2$-IIA, however, only in response to pro-inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNFα), lipopolysaccharide (LPS) and cAMP-elevating or -mimicing agents. Moreover, an increase in $sPLA_2$ mRNA level in hypoxic endothelial cells was detected (Michels, *et al*, 2002). It has been clearly demonstrated that the secreted enzyme participates in the propagation of chronic inflammatory processes and allergic diseases such as rheumatoid arthritis (Seilhamer *et al.*, 1989; Bertsch *et al.,* 1999; Talvinen *et al.,* 2001), sepsis and glomerulonephritis (Scholz-Pedretti *et al.,* 2002).

The mechanism of $sPLA_2$-IIA activation and its functions during inflammation are still under active investigation. $sPLA_2$-IIA has low functional capability on the surface of intact cells because of its weak binding capacity to the external leaflet of the plasma membrane (interfacial binding). However, in activated cells with perturbed membranes, which occur during inflammation, $sPLA_2$-IIA can be sorted into the caveolin-rich vesicular and perinuclear compartment membranes (Golgi, endoplasmic reticulum, and nuclear envelope) through binding to GPI-anchored glypican, a glycosylphosphatidylinositol (GPI)-anchored heparan sulfate proteoglycan (HSPG) (Murakami *et al*, 2002). It is tempting to speculate that glypican binding and sorting of $sPLA_2$-IIA into specific subcellular compartments can contribute to more efficient AA release and to the cyclooxygenase (COX-2)-dependent prostaglandin E_2 (PGE_2) biosynthesis. On the other hand, more recent studies do not support this hypothesis, as it was demonstrated that AA release by

exogenously added $sPLA_2$-IIA does not require HSPG glypican and that this enzyme is inefficient at liberating AA from mammalian cells. There is some evidence that the action of $cPLA_2$ is a prerequisite for $sPLA_2$ function in cells and that $cPLA_2$ is responsible for most of the AA release in stimulated cells (Mounier *et al.,* 2004). In addition, further studies have shown that exogenous addition of $sPLA_2$-IIA to mammalian cells leads to activation of mitogen activated protein kinase (MAPK) and protein kinase C (PKC), which then phosphorylates and thus activates $cPLA_2\alpha$ (Huwiler *et al.,* 1997; Han *et al.,* 2003).

1.4.2 Phospholipases in regulation of FAs metabolism

Although PLAs are commonly involved in the digestion of nutrients and the formation of bioactive molecules involving FAs and their metabolites, there are no studies about regulation of FA oxidation and CPT-I by sPLAs.

FAs or their metabolites released by sPLAs under inflammatory conditions may activate CPT-Iα gene transcription and subsequently FA oxidation via activation of nuclar receptors of the steroid-thyroid superfamily, the peroxisome-proliferator-activated receptor (PPAR) (Clarke *et al.,* 2003). It is known that diverse FAs bind and activate all three PPAR isoforms, PPARα, PPARδ/β and PPARγ , with varying specificities (Corton *et al.,* 2000; Lee *et al.,* 2003). Ligand-activated PPARs recognise PPAR response elements (PPRE) upon heterodimerisation with the retinoic X receptor (RXR). FAs with chain lengths under 16 and over 22 carbons only weakly activate PPARs, if at all. PPARα is mainly expressed in liver and kidney, exhibiting strong binding affinity for both saturated and unsaturated FAs. Although the CPT Iα gene promoter possess a PPRE, where is located between 2859 and 2846 base pairs upstream of the start codon, the contribution of PPAR in mediating effects of LCFAs on CPT-Iα regulation remains controversial and the molecular mechanisms by which FAs may regulate this gene transcription are still unclear. In rat liver, for example, LCFA induced CPT-Iα gene transcription through a PPARα-independent mechanism owing to a sequence located in the first intron of the gene (Louet *et al.,* 2001[a,b]). By contrast, it has been shown that the LCFA-dependent upregulation of mHMG-CoA synthase expression, whese gene promoter also includes PPRE in the 5' upstream region, is clearly mediated by PPARα.

Another possible mechanism of CPT-Iα activation by sPLAs may include the MAPK signalling pathway. MAPK are known to phosphorylate, and thereby activate

many transcripton factors (for instance, nuclear factors) which may play a regulatory role in CPT-Iα expression. Also, the reciprocal connection and cross-regulation between $cPLA_2$s and $sPLA_2$ followed by an additive increase in FAs level involves PKC and MAPK. Thus, a MAPK-mediated activation of PLA_2s and subsequent increase in FFAs can significantly contribute to CPT-Iα regulation.

1.5 Rat mesangial cells

The glomerulus is part of the nephron, the smallest functional unit of the kidney, and is made by four cell types: endothelial cells outlining the glomerular capillaries, mesangial cells functioning as pericytes, visceral glomerular epithelial cells, named podocytes, and parietal glomerular epithelial cells (Remuzzi and Bertami, 1998). Glomerular mesangial cells are crucial for processes maintaining the glomerular filtration rate. On the other hand, these cells are critically important target and effector cells in the pathogenesis of renal diseases (Sterzel *et al.,* 1992, Pfeilschifter, 1986; Pfeilschifter, 1994).

As active part of the inflammatory response to glomerular injury, mesangial cells become self-competent to synthesis bioactive molecules like eicosanoids, nitric oxide, growth factors and inflammatory chemokines and cytokines. Reactive oxygen species have been thought to be produced by mesangial cells in response to hyperglycemia and increased levels of prostaglandins are characteristics of diabetic and other glomerulopathies. The role of mesangial cells in the generation of eicosanoids and propagation of inflammation has been extensively studied by several groups using mesangial cells derived from rats. However, the understanding of functions including lipid digestion and metabolism in various physiological and pathological conditions is complex and has been difficult to clarify.

1.6 Primary culture of rat hepatocytes

Primary culture of hepatocytes can easily be established from suspensions of hepatocytes as demonstrated by Bissell *et al.* (1973). Since then, this model has been used in several pharmacological and toxicological studies. The system of primary culture of hepatocytes is also suitable for metabolic studies, since surviving for several days keeps the DNA content stable, maintaining specific and unspecific enzyme activities at a reasonable level and being able to perform metabolic

functions, which are specific for the liver (Page, 1979; Dich *et al.,* 1988; Van Auken *et al.,* 1996; Blumberg *et al.,* 1998).

As the liver plays pivotal roles in a large number of metabolic and immune processes, this model has attracted numerous investigations from many laboratories. It has been shown that primary hepatocytes of different species have the capacity to produce an acute-phase response on treatment with inflammatory mediators such as the cytokines IL-6, IL-1 and TNFα (Bauer *et al.,* 1991). Using primary rat hepatocytes, many groups have examined the effects of interferons, cytokines, and lipopolysacchride on the release of NO via induction of iNOS (Geller *et al.,* 1993; Sewer and Morgan, 1997), or AA metabolites. Such reactions in the liver are believed to be a critical for energy metabolism in endotoxin shock (Kitano *et al.,* 2002) or hepatocellular dysfunction (Wang *et al.,* 1993; Prabha *et al.,* 1991). Thus, the primary culture of hepatocytes can be efficiently used in the investigation of the potential roles of many factors and enzymes, which are important for liver function and survival.

1.7 Aim of this thesis

This thesis focuses on the regulation of CPT-Iα as an important fatty acid oxidation enzyme, in rat glomerular mesangial and liver cells as model systems.
The main aim was to investigate CPT-Iα regulation under inflammatory conditions in particular, I wanted to analyse whether inflammatory agents or conditions such as nitric oxide, cytokines, exogenous $sPLA_2$s or hypoxia have an effect on CPT-Iα expression. Moreover, I performed preliminary studies on the regulation of mHMG-CoA synthase, which is another key enzyme of ketogenesis.
From the results of these studies, I would propose that under different inflammatory conditions, changes in CPT-Iα expression enable the cell to adapt, in order to withstand these adverse conditions.

II

Materials and Methods

2.1 Materials

2.1.1 Chemicals

Acrylamide/bisacrylamide-solutions	Roth, Karlsruhe
Agar	Gibco Life Technologies, Eggenstein
Agarose	Biozym, Oldendorf
Ammoniumpersulfate	Sigma Biochemicals, Deisenhofen
Ampicillin	Sigma Biochemicals, Deisenhofen
Aprotinin	Roche Biochemicals, Mannheim
Boric acid	Merk, Darmstadt
Bovine serum albumin, fatty acid free	Sigma Biochemicals, Deisenhofen;
5-Bromo-4-chloro-3-indolyl-β-D-galactoside	Roth, Karlsruhe
Bromphenolblue	Serva
Cell culture media	Gibco Life Technologies, Eggenstein
Cesium chloride	Merck, Darmstadt
Cobalt Chloride	Sigma Biochemicals, Deisenhofen
Coommassie-Brillant-Blue G250	Sigma Biochemicals, Deisenhofen
cycloheximide	Sigma Biochemicals, Deisenhofen
Deferoxamine Mesylate	Sigma Biochemicals, Deisenhofen
DETA-NONOate	Alexis Biochemicals, Grünberg
Diethylpyrocarbonate (DEPC)	Sigma Biochemicals, Deisenhofen
Dithiothreitol (DTT)	Sigma Biochemicals, Deisenhofen
Dulbecco´s modified Eagles medium	Gibco, Berlin
Dimethylsulphoxide (DMSO)	Merck, Darmstadt
EDTA	Sigma Biochemicals, Deisenhofen
EGTA	Sigma Biochemicals, Deisenhofen
Ethrane (enfluran)	Abbott, Wiesbaden
Ethidium bromide	Sigma Biochemicals, Deisenhofen
Fetal calf serum (FCS)	Gibco Life Technologies, Eggenstein
Glycerol	Roth, Karlsruhe

HEPES	Roth, Karlsruhe
Isopropylthiogalactopyranoside (IPTG)	Roth, Karlsruhe
Leupeptin	Roche Biochemicals, Mannheim
Loading Day Solution 6x	MBI Fermentas
Magnesium sulfate heptahydrate	Sigma Biochemicals, Deisenhofen
β-Mercaptoethanol	Sigma Biochemicals, Deisenhofen
Molecular weight markers (DNA) 1 kb/100 bp	MBI Fermentas, St. Leon-Rot
Molecular weight markers (protein)	Amersham Pharmacia, Braunschweig;
Nicotinamide	Sigma Biochemicals, Deisenhofen
NZY BROTH	Gibco, Berlin
Nucleotide triphosphates	Applied Biosystems Applera, Weiterstadt
1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one (ODQ)	Alexis Biochemicals, Lausen
Oligonucleotides	Roth, Karlsrue Gibco Life Technologies, Karlsruhe
PD 98059	Calbiochem,
Penicillin/Streptomycin	Gibco, Berlin
Peptone	Gibco Life Technologies, Eggenstein
Phenol/Chloroform	Roth, Karlsruhe
Phenylmethylsulfulfonylfluoride (PMSF)	Roth, Karlsruhe
Phosphate buffered saline	Gibco, Berlin
PIPES	Sigma Biochemicals, Deisenhofen
Ponceau S	Sigma Biochemicals, Deisenhofen
Potassium acetate	Merck, Darmstadt
Potassium phosphate monobasic	Sigma Biochemicals, Deisenhofen
Potassium chloride	Roth, Karlsruhe
Roti Load1	Roth, Karlsruhe
Sodium Dodecylsulfate (SDS)	Merck, Darmstadt
SNAP	Alexis, Grünberg
Sodium acetate	Merk, Darmstadt
Sodium bicarbonate	Sigma Biochemicals, Deisenhofen
Sodium butyrate	Sigma Biochemicals, Deisenhofen
Sodium chloride	Merck, Darmstadt
Sodium citrate	Merck, Darmstadt
Sodium fluoride	Sigma Biochemicals, Deisenhofen
Sodium orthovanadate	Sigma Biochemicals, Deisenhofen
Sodium phosphate dibasic dodecahydrate	Sigma Biochemicals, Deisenhofen

Sulfanilamide	Sigma Biochemicals, Deisenhofen
Template suppression reagent (TSR)	Applied Biosystems Applera, Weiterstadt
Tetramethylethylendiamine (TEMED)	Sigma Biochemicals, Deisenhofen
TRI REAGENT	Sigma Biochemicals, Deisenhofen
Triton X-100	Sigma Biochemicals, Deisenhofen
Trypan blue	Gibco Life Technologies, Eggenstein
Trypsin/EDTA	Gibco, Berlin
Trypsin Inhibitor	Serva
Tryptone	Gibco Life Technologies, Eggenstein
Tween 20	Sigma Biochemicals, Deisenhofen
Ultroser G	BioSepra, Ciphergen
U0126	Calbiochem, Schwalbach
WY 14.643	Sigma Biochemicals, Deisenhofen
3-(5'-Hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1)	Alexis Biochemicals, Lausen
Yeast extract	Gibco Life Technologies, Eggenstein
Xgal	Roth, Karlsruhe

Acetone, chloroform, ethanol, ether, methanol, isopropanol, acids and all other, not listed chemicals were supplied from Merck (Darmstadt), Roth (Karlsruhe) or Sigma Biochemicals (Deisenhofen).

Other materials and kits

Deoxynucleoside Triphosphate Set	Roche Biochemicals, Mannheim
ECL Detection Kit/Films	Amersham Pharmacia, Freiburg
Effectene Transfection Reagent	Qiagen, Hilden
Endofree Plasmid Maxi Kit (Qiagen-tip 500)	Qiagen, Hilden
Dual-Luciferase Reporter Assay System	Promega, Mannheim
Immobilon-P Transfer Membrane	Millipore, Eschborn
Kodak BioMax MR-1 Scientific Imaging Film	Kodak-Industrie, Chalon-sur-Saone
NucleoBond PC 100 Kit (AX 100)	Macherey & Nagel, Düren
NucleoSpin Extract 2 in 1	Macherey & Nagel, Düren
Penicillin/Streptomycin (10.000 U/ml /10.000 µg/ml)	Gibco, Berlin
Phosphate buffered saline	Gibco, Berlin
Stericup MILLIPORE	Millipore Carporation, Bedford
SuperScript II, Rnase H, Reverse Transcriptase	Invitrogen,

TOPO TA Cloning Kit	Invitrogen, Groningen (Netherlands)
Transwell-Clear inserts 6-well polystyrene-plates	Costar, Bodenheim
QIAGEN filter Plasmid Midi/Maxi Kit	Qiagen, Hilden
QIAGEN Plasmid Mini Kit	Qiagen, Hilden

2.1.2 Media, buffers and solutions

2.1.2.1 Immunoblot-analysis

4x Laemmli-buffer	125 mM 10 % 50 mM 40 % 0.01 %	Tris/HCl pH 6.8 (w/v) SDS Dithiothreitol (v/v) Glycerol (w/v) Bromphenol blue
10x PAGE	250 mM 1 % 520 mM	Tris (w/v) SDS Glycin
TBS Tris-buffered saline 10xsolution	50 M 150 M	Tris pH 8.0 NaCl pH 7.4
Transfer buffer	25 mM 192 mM 20 %	Tris Glycine pH 8.3 (v/v) Methanol
Triton-X100 lysis-buffer (supplemented with protease inhibitor's mix)	20 mM 1 mM 1 mM 2 mM 1 %	Tris/HCl pH 7.6 EDTA EGTA DTT Triton X-100
Protease inhibitor's mix	2 µg/ml 10 µg/ml 2 µg/ml 1 mM 2 µg/ml 1 mM 1 mM	Aprotinin Leupeptin Pepstatin A PMSF Trypsin inhibitor $NaVO_4$ NaF
Protein molecular weight marker (MBI Fermentas)	5 µl	per well

Coomassie staining solution	2.5 g/l	Coomassie Brilliant Blue R 250
	40 %	methanol
	10 %	glacial acetic acid
	50 %	water
Destaining solution	40 %	methanol
	10 %	glacial acetic acid
	50 %	water

2.1.2.2 Buffers and solutions for cell culture

Media for mesangial cell culture

RPMI medium		RPMI (Gibco)
	10 %	FCS
	5 ng/ml	Insulin
	5 ml	Penicillin/Streptomycin
	5 ml	Trypsin/EDTA
DMEM medium		DMEM (Gibco)
	0.1 mg/ml	Fatty acid free BSA
	5 ml	Penicillin/Streptomycin
	5 ml	Trypsin/EDTA

Buffers and media for primary culture of rat hepatocytes

Ultroser G solution (must be used immediately)	For 500 ml Dulbecco's MEM (Gibco) The flask content (BioSepra) was solubilised by gentle shaking in 20 ml DMEM and put back to the rest of the medium under sterile conditions
10 X Buffer concentrate	For final volume of 1.2 litre : 98.16 g NaCl 4.4 g KCl 2.4 g $MgSO_4$ x7 H_2O 11.4 g Na_2HPO_4 X 12 H_2O 0.64 g KH_2PO_4 Volume adjusted to 1200 ml with H_2O

EDTA perfusion buffer	For final volume of 1 litre : 100 ml 10 X Buffer concentrate (see above) 750 mg EDTA 2.1 g $NaHCO_3$ 200 U/ml Penicilin 200 µg/ml Streptomycin Volume adjusted to 1 litre with H_2O pH adjusted to 7.4 with 1M HCl sterilised by filtrating (Stericup MILLIPORE)
Collagenase perfusion buffer A	For final volume of 1 litre : 100 ml 10 X Buffer concentrate 0.5 g $CaCl_2$ Volume adjusted to 1 litre with H_2O pH adjusted to 7.4 with 1 M HCl sterilised by filtrating (Stericup MILLIPORE)
Collagenase perfusion buffer (for a cell isolation from one rat liver; must be used immediately)	For final volume of 0.2 litre: 200 mg collagenase H 20 ml collagenase perfusion buffer A sterilised by filtrating using sterilising membrane of 0.22 µm pore diameter. Volume adjusted to 200 ml with Collagenase perfusion buffer A 4 ml Penicillin/Streptomycin
DMEM medium for cell isolation	Dulbecco's MEM (Gibco) 500 ml: 10 ml Penicillin/ Streptomycin
DMEM medium for cell incubation	Dulbecco's MEM (Gibco) 500 ml: 4 % Ultroser G (20 ml Ultroser solution) 5 ml Penicillin/ Streptomycin 1 mmol/l NAD

2.1.2.3 Media for bacteria culture and agar plates

LB medium (sterilised)	21 g NZY BROTH Volume adjusted to 1 litre with H_2O
SOC medium	Invitrogen, Groningen (Netherlands)
2xYT Medium	in 900ml of H_2O dissolved: 16 gr bacto-tryptone 10 gr bacto-yeast extract 5 gr NaCL pH adjusted to 7.0 with 1M HCL Volume adjusted to 1 litre with H_2O
Agar plates	<u>For final volume of 100 ml:</u> 0.7 g NZY BROTH 1.5 % Agar sterilised by autoclaving
$CaCl_2$ Solution	60 mM $CaCl_2$ 15 % glycerol 10 mM PIPES sterilised by autoclaving

2.1.2.4 Additional buffers and solutions

Ampicillin	50 mg/ml stock solution sterilised by filtrating
10 x PBS (pH 7.4)	1.3 M NaCl 30 mM NaH_2PO_4 70 mM Na_2HPO_4
TAE (Tris/acetate/EDTA) electrophoresis buffer	50 x stock solution, 1 litter 2 M Tris 1 M Acetic acid 50 mM EDTA

TBE (Tris/borate/EDTA) electrophoresis buffer	10x stock solution, 1 litter 10 g Tris base (890 mM) 55 g Boric acid (890 mM) 40 ml 0.5 M EDTA , pH 8.0
TE-buffer	10 mM Tris (pH 8.0) 1 mM EDTA (pH 8.0)

2.1.2.5 DEPC-treatment

Solutions for RNA based methods were treated with diethyl pyrocarbonate (DEPC) to inactivate RN ases. DEPC-treated water was obtained by adding 1 ml DEPC per liter of distilled H_2O. After mixing in an overnight step at RT, the solution was autoclaved.

2.1.3 Enzymes

Collagenase H	Roche Diagnostics, Mannheim
Turbo *Pfu/Pfu*-DNA Polymerase	Stratagene, Heidelberg
Proteinase K	Roche Biochemicals, Mannheim
Restriction Enzymes	NEB, Frankfurt a.M.; MBI-Fermentas, St. Leon-Rot
Reverse Transcriptase	Invitrogen, Karlsruhe
Taq-DNA polymerase	Applied Biosystems Applera, Weiterstadt

2.1.3.1 sPLA$_2$'s used for treatment

Abbreviation	*Type*	*Source*	*Supplier*
WT-IB	Wild-type porcine sPLA$_2$-IB	Porcine pancreas	Sigma
IB-H48Q	Catalytically inactive porcine sPLA$_2$-IB mutant	Recombinant	provided by Dr. M. J. W. Janssen (Literaturstelle angeben: Beck *et al* ., 2003)
hIIA	Wild-type human sPLA$_2$-IIA	Recombinant	provided by Prof. Tibes, Roche Diagnostics, Germany

hIIA-H48N	Mutant human $sPLA_2$-IIA	Recombinant	provided by Dr. D.C. Wilton
Rat-IIA	Rat $sPLA_2$-IIA	Recombinant	provided by Dr. G. Lambeau
Bee	Bee venom $sPLA_2$	Bee venom	Sigma
Naja	Snake venom $sPLA_2$	*N. mossambica mossambica*	Sigma
OS1	Snake venom $sPLA_2$	*O. scutellatus sc.*	provided by Dr. G. Lambeau
OS2	Snake venom $sPLA_2$	*O. scutellatus sc.*	provided by Dr. G. Lambeau

2.1.3.2 Pretreatment of enzymes

Proteinase K

The lyophilized enzyme (10 mg/ml) was dissolved in distilled H_2O, incubated for 30 min at 37°C and aliquoted. The aliquots were stored at –20°C until use.

RNase A

RNase A was dissolved to a final concentration of 10 mg/ml in RNase-buffer [Tris/HCl (10 mM, pH 7.5), NaCl (15 mM)]. The enzyme solution was incubated for 30 min at 95°C and cooled to room temperature overnight. Aliquots were stored at –20°C until use.

2.1.4 Antibodies and antiserum

Anti-*a*-actin (mouse monoclonal)	Sigma Aldrich Fine chemicals
Anti-β–tubulin (D-10) IgG (mouse, monoclonal)	Santa Cruz Biotechnologies, Heidelberg
Anti-mouse IgG (horseraddish-peroxidase coupled)	Santa Cruz Biotechnologies, Heidelberg
Anti-rabbit IgG (horseraddish-peroxidase coupled)	Santa Cruz Biotechnologies, Heidelberg
Anti-CPT-Iα IgG (rabbit, polyclonal)	provided by Prof. Pfeilschifter
Anti-rat $sPLA_2$-IIA	provided by Prof. Van den Bosch

2.1.5 Proteins

Collagen from calf skin	Sigma, Deisenhofen
IL-1β	Cell Concepts, Umkirch, Germany
TNFα	Knoll AG, Ludwigshafen, Germany

2.1.6 Plasmids

2.1.6.1 Vectors

pCR II TOPO	Invitrogen, Groningen (Netherlands)
pGL3-Basic Vector	Promega, Mannheim

2.1.6.2 Constructs of CPT-Iα promoter luciferase vectors

pGL3 + rat CPT-Iα Prom Frag 1	Nucleotides -4495/+1240	provided by Dr. Edwards A. Park
pGL3 + rat CPT-Iα Prom Frag 2	Nucleotides -4495/+19	Park , *at al.,* 1998; Jansen M, *at al.*, 2000)
PGL3 + rat CPT-Iα Prom Frag 3	Nucleotides -210/+19	see 2.2.4

2.1.7 Bacterial strains

		Genotype
***E.coli* XL-1 blue**	Stratagene, Heidelberg	*supE44 hsdR17 recA1 endA1 gyrA46thi relA1 lac⁻ F`[proAB⁺ lacI^q lacZΔM15 Tn10(tet^r)]*
TOP 10	Invitrogen, Groningen (Netherlands)	*F⁻ mcrA Δ(mrr-hsdRMS-mcrBC)Φ80lacZ ΔM15 ΔlacX74 recA1 deoR araD139 Δ(araleu)7697 galU galK rpsL (Str^R) endA nupG*

2.1.8 Cell culture

primary rat liver cells	preparation see 2.2.2.2
Rat Mesangial Cell (rMC), Eukaryotic cell line	preparation of primary cells from rat kidney by Prof. Pfeilschifter, *pharmazentrum frankfurt*

2.1.9 Oligonucleotides

2.1.9.1 Cloning of CPT-Iα Promoter-Fragment 3

Fragment	**Forward Primer (Sac I)**	**Reverse Primer**
PGL3 + rat CPT-Iα Prom Frag 3	**CGAGC**TCACAAGGTAA GGAATGC	pGL3 Basic rev primer

2.1.9.2 Semiquantitative PCR

	Fragment size (bp)	**Sequence 5' ⇒ 3'**
GAPDH for		ACC ACA GTC CAT GCC ATC AC
GAPDH rev	452	TCC ACC ACC CTG TTG CTG TA
CPT-Iα for		ATG ACG GCT ATG GTG TCT CC
CPT-Iα rev	124	GTG AGG CCA AAC AAC AAG GTG AT
mHMG-CoAs for		CCT CTG GAG AAG CTG GTG TC
mHMG-CoAs rev	154	GTA CCA AGT GCC TGG GAA GA

2.1.9.3 Sequencing primer

	Sequence 5' ⇒ 3'
pGL3 Basic for	CTA GCA AAA TAG GCT GTC CC
pGL3 Basic rev	CTT TAT GTT TTT GGC GTC TTC CA

2.1.10 Laboratory equipment

ABI-Prism 310 Genetic Analyser	Applied Biosystems Applera, Weiterstadt
Recirculating recirculating apparatus	Gilson ABIMED
Minipuls 3	
Gel dryer 583	Bio-Rad, München
GeneAmp 2400/9600 Thermocycler	Applied Biosystems Applera, Weiterstadt
Gene Quant II	Amersham Pharmacia, Braunschweig
Herasafe clean bench	Heraeus, Hanau
Hyperprocessor	Amersham Pharmacia, Braunschweig
Incubator Heraeus BBD 6220	Heraeus, Hanau
AutoLumat LB953	Berthold, Pforzheim
Microplate reader Benchmark	Bio-Rad, München
PhosphoImager BAS 1500	Raytest, Straubenhardt

2.1.11 Computer software

DNA/Protein homology search	BLAST search (National Center of Biotechnology, USA; URL: *http://www.ncbi.nlm.nih.gov);*
Graphic processing	Corel Draw 8.0/10.0
Presentations	Powerpoint 2000
Statistical analysis	Sigma Plot 4.0 (Students T-Test) Microsoft Excel 2000
Text processing	Microsoft Word 2000

Programs belonging to special devices are mentioned separately in the appropriate sections.

2.2 Methods

2.2.1 Microbiologic methods

2.2.1.1 Bacterial culture

The *E. coli* XL1-blue strain (Stratagene) was used for amplification of plasmid DNA. The strain was either grown in liquid LB (**L**auria-**B**ertani) or SOC medium (2.1.2). For selection, the media contained ampicillin (50 µg/ml). Agar plates were generated with LB-ampicillin medium supplemented with agar (15 g/l). For long-term preservation of transformed bacteria, cells were mixed with sterile glycerol [30% (v/v)] and stored at –80°C.

2.2.1.2 Competent bacteria for transformation

To yield high transformation efficiencies from plasmid DNA in bacteria, cells have to be pretreated chemically. LB-Medium 250 ml was inoculated with 200 µl of an overnight bacterial *E. coli* culture and grown at 37°C until the suspension reached an optical density of 0.5 ($OD_{600\ nm}$). The bacterial growth was stopped by incubating the suspension for 5-10 min on ice. Bacterial cells were concentrated by centrifugation (15 min, 4300 rpm, 4°C). The cellular pellet was subsequently resuspended in 80 ml of ice-cold $CaCl_2$ solution, mixed and incubated on ice for 10 min. The bacterial suspension was centrifuged again (15 min, 4300 rpm, 4°C), subsequently the pellet was gently resuspended in 20 ml of ice-cold $CaCl_2$ solution and incubated on ice for additional 20 min. Thereafter, aliquots of competent bacteria were frozen immediately in liquid nitrogen and stored at –80°C.

2.2.1.3 Transformation

A competent bacteria suspension (100 µl) was thawed on ice and incubated with 5 µl of plasmid or DNA solution. The bacteria/DNA mixture remained on ice for additional 30 min followed by an incubation at 42°C for 1 min. The bacteria were chilled on ice again for 2 min, before 300 µl of SOC-medium was added and for initial expression of the plasmid, bacteria were incubated for 1 h at 37°C on a shaker. The plasmid encodes an ampicillin-resistance gene. Therefore, bacterial cells, which take up the plasmid genome and become ampicillin resistant. 20 - 200 µl of this transformation solution was spotted in the centre of prewarmed agar plates (37°C) containing ampicillin.

To enable a blue/white screening for recombinant clones (pCR TOPO), the agar plate was supplemented with 50 µl X-Gal (2% in DMSO) and 50 µl IPTG (0.1 M in distilled H_2O). The plates were incubated overnight at 37°C.

2.2.2 Cell biological methods

2.2.2.1 Cultivation of rat mesangial cells

Rat mesangial cells were grown in RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES and 10% heat-inactivated FCS (GIBCO-BRL, Eggenstein, Germany).

Mesangial cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 (Heraeus BBD 6220 incubator). For passaging, cells were washed twice in phosphate buffered saline (PBS), subsequently trypsinised (Trypsin, EDTA) and diluted in a 1:20 ratio with RPMI. After trypsinisation, cells were concentrated (5 min at 1100 rpm, Heraeus Megafuge 1.0, rotor 75750F) and diluted in RPMI medium. Thereafter, mesangial cells were seeded into culture plates (Greiner) and maintained in RPMI medium.

For long-term storage, the cells were treated as follows: after trypsinisation, cells were concentrated, diluted in freezing medium (growth medium supplemented with 10% DMSO) and stored in cryotubes (Nunc). The cryotubes were cooled down slowly overnight to – 70°C and finally stored in liquid nitrogen.

For stimulation, mesangial cells were stimulated in DMEM, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and bovine serum albumine (BSA), instead of RPMI medium.

2.2.2.2 Isolation and primary culture of adult rat hepatocytes

Prefasted adult male Sprague-Dawley rats with 250-300 g body weight were used for isolation of primary rat hepatocytes. The animals were fed standard rat laboratory diet, had

access to water and were kept in a temperature- and light-controlled room. Isolated hepatocytes were prepared according to a EDTA/collagenase protocol (Seglen, 1976). This method is widely used experimentally for the isolation of many different cell types from animal organs and tissues. Briefly, the rats were anaesthesised with enfluran. After cannulation of the portal vein, perfusion was carried out with a recirculating perfusion apparatus as follows.

First, the liver was perfused with ice-cold EDTA buffer (2.1.2.2.2) at a rate of 30 ml/min for 5 min. Then, the cannulated rat liver was taken out and placed in sterilised glass on ice and the perfusion was continued for another 5 min. The next perfusion steps were performed in an oxygen saturated environment by gassing with 100% O_2. The cannulated liver was transferred into a new sterilised glass at RT and perfused at a maximum rate of 37 ml/min for 10 min with the same EDTA buffer prewarmed at 37°C. These steps were required for the dissolving of the desmosome (cellular junction) by withdrawing Ca^{2+} from tissue via EDTA chelation of this ion.

Finally, the liver was perfused with collagenase buffer at 37°C at a rate of 20 ml/min for another 15 min. The Ca^{2+} activated collagenase digested the extracellular matrix and thereby caused release of cells from the tissue.

After the liver was disconnected from perfusion apparatus and transferred to Petri dishes, the cells were separated by gentle scraping with a spatula. The cellular suspension was filtered through a gauze, washed with 300 ml DMEM + 2% Pen/Strep buffer, and intact hepatocytes were separated from other cells such as fibroblasts and astrocytes, by low speed centrifugation (500 rpm, Heraeus Megafuge 1.0, rotor 75750F) at RT for 3 min. The pellets were washed in 200 ml DMEM + 2% Pen/Strep buffer, centrifuged at 1500 rpm for 10 min at RT, and the cell pellet was resuspended in DMEM incubation medium (2.1.2.2.2) to a concentration of 5 X 10^5 cells/ml. Hepatocytes were placed in six well plates (3 ml/well) and incubated for 16 hr. Experiments were performed thereafter.

2.2.2.3 Reporter gene assays

The regulation of gene expression was examined using a reporter-gene-assay. For this purpose, DNA sequences carrying putative regulatory elements were cloned in front of a reporter gene, in this case the gene encoded the luciferase enzyme. Newly generated constructs were transferred into the eukaryotic target cells by liposomal transfection. Thereafter, transfected cells were stimulated with the compounds to be tested. After transfection and stimulation, cells were harvested to measure the amount of synthesized reporter gene products. To assess the influence of different agents on CPT-Iα gene regulation, reporter gene expression in stimulated cells and unstimulated cells were compared. Each transfection was conducted threefold and repeated four to six times. No reporter gene products were synthesized when cells were transfected with empty vector.

.2.2.4 Transfection of mesangial cells with luciferase constructs

Confluent mesangial cell cultures were trypsinized and cultured in fresh medium. The cells were seeded in 2 ml RPMI medium per well onto 6-well-culture-plates and incubated for 18 - 24 h under standard conditions. The cells should have reached a confluency of about 60 - 80%. After the cells were washed with PBS, 1.6 ml DMEM/0.01 % BSA was added per well and cells were incubated at 37°C while Effectene Transfection Reagent (Qiagen, Hilden) was prepared. Although this preparation was done according to the manufacturer's instructions, some transfection conditions had to be optimized for the mesangial cell line. The volume of all medium used here is given per well of 6-well plate. Meanwhile, 400 ng of Plasmid DNA (CPT-Iα promoter luciferase expression construct, dissolved in TE, pH 7.4) plus 40 ng Renila-luciferase-DNA were diluted with 100 µl of DNA-condensation buffer (EC Buffer). After adding 3.2 µl Enhancer, the DNA mixture was thoroughly mixed for 1 min and incubated for 5 min at RT. Afterwards, 10 µl Effectene was supplemented to the DNA-Enhancer solution, mixed by pipetting and incubated for 10 min at RT. The transfection complex was diluted in 600 µl DMEM medium, and 713 µl of this solution was immediately added drop-wise onto the cells.

Cells were washed with PBS after transfection (16 h) and maintained in 1.5 ml of fresh DMEM. Subsequently, mesangial cells were either unstimulated (negative control) or stimulated with the substances to be tested.

Then, cells were washed with ice-cold PBS, lysed in 200 µl lysis buffer from the Dual-Luciferase Reporter Assay System (Promega), and scraped with a rubber policeman into 1.5-ml tubes. The cell lysates were subjected to a freeze/thaw cycle to complete lysis of cells. After short centrifugation, the assays for firefly luciferase activity and Renilla luciferase activity (transfection control) were performed sequentially using a luminometer (Autolumat; Berthold, Wildbad, Germany). Values for the CPT-Iα promoter activity were divided by those obtained for Renilla-luciferase activity.

2.2.2.5 Luciferase assay

The luciferase of the firefly (*Photinus pyralis*) catalyses the oxidation of luciferin. During this reaction, photons are released at a wavelength of 562 nm. A quantitative measurement of the emitted light was performed by employing a luminometer (AutoLumat LB953, Berthold). Cells were transiently transfected with a luciferase-construct, stimulated and harvested in 1x Reporter-Lysisbuffer (Promega) as described by the manufacturer. The solutions needed for measurement were prewarmed at RT and 20 µl of cell-extract were pipetted in a polystyrene-tube. The reaction was started in the Luminometer by injection of 100 µl Luciferase-Assay-Reagent (Promega) (20 mM Tricin, 1.07 mM $(MgCO_3)Mg(OH)_2$ x 5 H_20, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 µM Coenzym A, 470 µM Luciferin,

530 µM ATP pH 7.8) followed by injection of Renilla-Assay Reagent (Promega). The emitted light was measured for 10 s with a Photomultiplier and expressed in Relative Light Units short (RLUs) by the luminometer. Values for the CPT-Iα promoter activity were divided by those obtained for Renilla liciferase activity. Values obtained with treated cells were expressed as x-fold increase in luciferase activity compared to control.

2.2.3 Measurement of cell parameters

2.2.3.1 Nitric oxide synthase activity: Griess assay

Nitrite (NO_2^-) is a stable NO oxidation product, which can be evaluated as a direct readout for nitric oxide synthase activity using the Griess-assay as described (Mühl *et al.*, 1997). The lowest detection limit for nitrite in this assay is 1.5 µM.

Cell culture supernatants were cleared by centrifugation (5 min at 400 g). 200 µl of supernatants were mixed with an equal volume of ready-to-use Griess Reagent (Roche, Mannheim). After 5 min at room temperature, the absorbance was measured at 540 nm with a reference wavelength at 690 nm.

2.2.3. 2 Viability of the primary rat liver cell culture

The rat primary liver cell number and viability of the suspension after their isolation (2.2.2.2) was assessed by trypan blue exclusion. Trypan blue (50 µL) was mixed with 50 µl cell suspension and the number of viable cells, i.e. those cells which were not stained with trypan blue, were determined microscopically. Primary cell cultures which had a viability of more than 80% were used for the experiments.

2.2.4 Molecular biology methods

2.2.4.1 Reverse transcriptase reaction (RT)

The enzyme SuperScript^{tm}II RNase H$^-$ Reverse Transcriptase is purified from *E.coli* containing the *pol* gene of Moloney Murine Leukemia Virus. The enzyme synthesizes a first-strand complementary DNA (cDNA) strand using RNA as a template.

First, an Oligo dT primer mRNA is hybridised to a poly (A) tail of isolated mRNA. Then, in the next reaction the reverse transcriptase extends this primer to yield cDNA. Finally, the RNase H-treatment removed the RNA strand.

Reverse transcriptase reaction:

1-5	µg	total RNA
1	µl	oligo (dT)

DEPC water was added to a total volume of 12 µl.

The mix was heated to 65°C for 5 min and then incubated on ice for 10 min.

To the oligo (dT) hybridisation mix, the following components were added:

4 µl 5 X first-Standard Buffer

2 µl 0.1 M DTT

2 µl 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP and dTTP)

1 µl Reverse Transcriptase

The mix was incubated first for 50 min at 42°C and afterward for 15 min at 70°C. Aliquots of the cDNAs were stored at 4°C.

2 µl of the synthesised cDNA was used as a template for amplification in the Polymerase Chain Reaction (PCR).

2.2.4.2 Polymerase chain reaction (PCR)

This method enables the *in vitro* amplification of DNA fragments without time consuming cloning and identification steps. The method is based on the availability of heat-stable DNA polymerases which allow multiple denaturation of template DNA, annealing of driver sequences (primer) and synthesis of DNA by amplification steps within one tube.

Polymerase chain reaction:

2 µl RT-product (2.2.3.2) or

X µl Plasmid-DNA Template (50-100 ng)

5 µl 10x PCR-Buffer

1 µl 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP and dTTP)

1 µl Forward/Reverse Primer-Mix (10 µM)

0.5 µl *Taq*- Polymerase (1 U/µl)

Water was added to give a total volume of 50 µl

The reaction was performed in a thermocycler (GeneAmp 2400 or 9600, *PE* Biosystems). Conditions varied depending on the gene of interest, the type of polymerase used for amplification and the experimental set up. For **semi-quantitative gene analysis** *Taq* DNA polymerase which was originally isolated from thermophilic eubacterium *Thermus aquaticus* was used. This enzyme exhibits highest activity at 75°C and is stable against incubations at elevated temperatures (95°C). In general, 23-30 cycles of the following steps were carried out: 1 min 94°C, 1 min e.g. 60°C (primer-annealing) and 2 min 72°C (elongation). The amplification was completed by a final 7 min incubation step at 72°C. Depending on special conditions of template, primers and cell tipe, the protocol was altered accordingly (see table below). The samples were stored at 4°C and analyzed by gel electrophoresis.

Semi-quantitative gene analysis for	Annealing temperature	Cycle number
GAPDH	55°C	23
CPT-Iα	51°C	Mesangial cell: 28 Rat hepatocytes: 32
mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHMG-CoA synthase)	55°C	Mesangial cell: 30 Rat hepatocytes: 28

For amplification of plasmid-DNA, Template *taq* -polymerase was used. 30–35 cycles were sufficient to amplify genomic CPT-Iα promoter fragment 3 using the following PCR program: 1 min 94°C, 1 min 53°C and 2 min 72°C. Finally, amplification was completed by a 7 min incubation step at 72°C.

2.2.4.3 Cloning of PCR products in vector (pCR II TOPO) and luciferase vector (pGL3)

After amplification, PCR product (CPT-Iα promoter fragment 3) was cloned into the pCR II TOPO-Cloning Vector (Invitrogen), and then ligated to the target vector pGL3 (Promega). PCR insert in pCR II TOPO was restricted by type II endonucleases (Sac I/ Bgl II) and separated from these cloning vectors by gel electrophoresis and subsequent gel elution using a column gel extracting kit (NucleoSpin Extract 2 in 1 by Macherey & Nagel), followed by ligation into the prepared luciferase-vector pGL3. These luciferase constructs were used to transfect mesangial cells.

2.2.4.4 Preparation of plasmid DNA

Miniprep: Isolation of plasmid DNA was performed using the QIAGEN Plasmid Mini Kit according to the manufacturer's instruction.

Midiprep: Higher amounts of plasmid DNA (expected yields: 75 - 100 µg) were obtained using the NucleoBond PC 100 Kit (AX 100) as described by the manufacturer.

Maxiprep: To obtain endotoxin-free plasmid DNA for transfection experiments, we employed Endofree Plasmid Maxi Kit (Qiagen-tip 500) according to the manufacturer’s instruction.

2.2.4.5 RNA isolation from cultured cells

Total RNA isolation was performed with TRI-Reagent according to the protocol from the manufacturer (Sigma Biochemicals). All centrifugations were carried out at 13.000 rpm in

an Eppendorf centrifuge at 4°C. The cells were washed twice with PBS. The media and washing buffer were removed by a pipette tip attached to a vacuum pump. Subsequently, cells were lysed with 1 ml of TRI-Reagent. The lysate was transferred into an Eppendorf tube. After addition of 200 µl chloroform, the samples were mixed and stored at RT for 10 min. After centrifugation for 15 min, the upper phase was collected into a fresh tube. Precipitation of RNA with 700 µl of isopropanol was carried out overnight at -20°C. The precipitated RNA was pelleted by centrifugation for 15 min and then washed with cold 70% ethanol (30% DEPC-treated water), followed by centrifugation for 10 min. Subsequently, ethanol was carefully removed and the final RNA pellet was dried at 37°C and resuspended in a suitable volume of DEPC-treated water. After a 10 min incubation at 65°C, the amount of isolated RNA was quantified photometrically (2.2.4.6). 1-2 µg of isolated RNA was controlled for integrity by agarose gel electrophoresis (1%, 1x TBE buffer).

2.2.4.6 Quantification of nucleic acid concentrations

Concentrations of nucleic acids were determined photometrically at 260 nm (Gene Quant II, Amersham Pharmacia). The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimation of the purity of the nucleic acid preparation. Highly pure DNA or RNA are characterized by ratios between 1.8 and 2.0.
Low amounts of DNA were estimated by agarose gel electrophoresis (2.2.4.7) in comparison with a known standard.

2.2.4.7 Agarose gel electrophoresis of nucleic acids

Nucleic acids were separated by gel electrophoresis using agarose gels. The agarose concentration was dependent on the molecular weight of the analysed nucleic acids. For separation of DNA molecules from 0.5 to 2 kbp, usually 1% agarose gels (w/v) were employed. Smaller DNA fragments (100 bp – 500 bp) were separated in high density gels (1.5 - 2% agarose gels). Agarose (Roth/Gibco Life Siences) was dissolved in 1x TBE gel electrophoresis buffer. Ethidium bromide was added to a final concentration of 500 ng/µl. Ethidium bromide binds to DNA or RNA by intercalation between the bases and thus enables an ultraviolet fluorescence illumination of nucleic acids. The DNA/RNA samples were diluted with loading buffer [6x Loading Dye Solution] and loaded onto the gel. Electrophoresis was performed in 1x TBE buffer with a voltage of 10 V/cm electrode distance. DNA fragment sizes were estimated using molecular weight markers (MBI Fermentas).

2.2.4.8 DNA isolation from agarose gels

The use of the NucleoSpin Extract 2 in 1 Kit (Macherey & Nagel) enables a pure extraction of DNA fragments directly from agarose gels. The system is based on a silica membrane, which binds single and double stranded DNA. The DNA fragments of interest were cut from the gel with a razor blade and further processed according to the manufacturer's instructions.

2.2.4.9 Restriction

Type II endonucleases isolated from bacteria specifically bind palindromic sequences with a subsequent cleavage of the DNA molecule at their recognition site. This process generates either blunt-end fragments or overhanging cohesive ends, which allow the generation of recombinant DNA by enzymatic ligation. The standard approach for DNA digestion is subsequently listed. After incubation at the appropriate temperature, DNA cleavage was checked by agarose gel electrophoresis (2.2.4.7).

DNA Digestion:

X	µl	DNA (3 µg) target vector
1.0	µl	10x buffer
0.5	µl	restriction enzyme (10 U/µl)
X	µl	H_2O :10 µl Total volume

2.2.4.10 Ligation

Generation of covalent phosphodiester bonds between the 5´-phosphate and the 3´-OH of DNA fragments is catalyzed by T4-DNA ligase. The ligation reaction was performed with restricted or PCR-amplified DNA. The DNA was separated on an agarose gel. Subsequently, the DNA fragment of interest was isolated using a column gel extracting kit by Macherey & Nagel. The gel extracted fragment was added to the ligation reaction. The mixture was incubated overnight at 16°C. Afterwards, an aliquot of this reaction was transformed into competent bacteria as described in section 2.2.1.3.

DNA ligation:

X	µl	target vector DNA (70 ng)
X	µl	gel-extracted DNA fragment (130 ng)
2	µl	5x ligase buffer
0.5	µl	T4-DNA ligase (1U/µl)
X	µl	H_2O to total volume of 10 µl

2.2.4.11 DNA sequencing

DNA sequencing was performed using the ABI-Prism 310 Genetic Analyser (Applied Biosystems Applera) based on the dideoxynucleotide chain termination method [Sanger *et al.,* 1977]. In the termination labeling mix, the four dideoxy terminators (ddNTPs) were tagged with different fluorescent dyes. This technique allows the simultaneous sequencing of all four reactions (A, C, G, T) in one reaction tube. The probes were separated electrophoretically using a micro capillary. As each dye terminator emits light at a different wavelength when excited by laser light, all four colors corresponding to the four nucleotides can be detected and distinguished within a single run. Raw data were evaluated by the Abi Prism sequencing analysis software on a Power G3 Macintosh computer. The sequencing reaction, as listed in the table, was performed in a thermocycler (GeneAmp 2400, Applied Biosystems Applera) with 25 cycles of the following temperature steps: 96°C for 10 sec, 55°C for 5 sec, 60°C for 2 min. For the detection process, probes were prepared as described by the manufacturer.

DNA sequencing:

X	µl	DNA (250 ng plasmid DNA or 50 ng PCR derived DNA)
2	µg	sequencing premix
1	µl	primer (10 µM)
x	µl	H_2O 10 µl total volume

2.2.4.12 Cloning of CPT-Iα promoter fragment

Synthesis of the -210/+19 CPT-Iα promoter fragment was carried out by PCR (2.2.4.2) using pGL3+rat CPT-Iα prom Fragment 2 gifted by E. A. Park (Jansen, *at al.*, 2000) as template. In order to generate artificial restriction sites for directed ligation to the target vector pGL3, the oligonucleotides (2.1.10.1) were constructed. The forward primer was designed to produce a Sac I site, whereas the reverse primer was modified by generating a BglII site. The amplificated CPT-Iα promoter fragment (pGL3 + rat CPT-IαProm Frag3) contains a genomic region of 229 bp.

2.2.5 Biochemical Methods

2.2.5.1 Preparation of cell lysates

Cells were grown and stimulated as described above (2.2.2). For harvesting, cells were washed twice with ice-cold PBS. Last traces of PBS were removed by a pipette tip attached to a vacuum pump. The cells were treated with Triton X-100 lysis-buffer supplemented with protease inhibitors. To remove cellular debris, probes were centrifuged (13000 rpm, 2

min at 4°C). Finally the supernatants were stored at –80°C until use. Protein content was determined using the Roth Nanoquant Protein Assay.

Preparation of cell lysates from skeletal muscle cells

Skeletal muscle cell lysats were prepared from male Sprangue-Dawley rats. Hind leg muscle was removed and washed in ice-cold PBS buffer. Tissue was then placed immediately in 10 volumes of ice-cold Triton X-100 lysis buffer supplemented with protease inhibitor mix and homogenised by Hom. The homogenate was centrifuged at 10000 rpm for 5 min to remove cellular debris. The supernatant was filtered through two layers of gauze stored at -80° C.

2.2.5.2 Trichloroacetic acid (TCA) precipitation

This method was used to concentrate proteins from a defined volume of cell culture supernatant for Western blot analysis. Cell culture supernatants were cleared from cellular contaminations using a single centrifugation step (1000 rpm, 5 min at 4°C; Heraeus Megafuge 1.0, rotor 7570F). Then, 400 µl of 20 % trichloroacetic acid (TCA) were added to 1 ml of conditioned cell culture supernatant, mixed and incubated for 30 min on ice. TCA-precipitated proteins were concentrated by centrifugation (13000 rpm, 30 min, 4°C) and the protein pellets were lysed in 50 µl 2 x Laemmli-buffer. For sPLA$_2$-IIA detection, Laemmli buffer without ß-Mt-OH or DTT was used. After neutralization with 1-5 µl of 1 M Tris/HCl, pH 9.5, the samples were ready to use for gel electrophoresis in Western blot analysis.

2.2.5.3 Acetone precipitation

This method was necessary to concentrate protein from mesangial cells lysates with low amount of protein.

Lysates of the required protein amount (100-200 µl) were mixed with a threefold volume of ice-cold acetone and incubated overnight at 20°C. Samples with precipitated protein were then centrifuged (13000 rpm, 30 min, 4°C) and pellets were lysed in 40 µl of 2 x Laemmli-buffer.

2.2.5.4 Preparation of membrane fraction

Because of location of CPT-Iα in the mitochondrial outer membrane, this method was preferable to accumulate CPT-Iα protein, to diminish high background and achieve much greater sensitivity in Western blot. However, ß-tubulin from mesangial cells and also actin from liver cells were not detectable in this protein preparation for protein loading control.

Lysates of protein produced as described in 2.2.4.1 were the source for membrane fractions including mitochondria, and these were pelleted by centrifugation at 40000 rpm for

1 hr (4°C) at a concentration of 40-100 µg protein from liver cells and 100-200 µg protein from mesangial cells. The membrane pellet was finally resuspended in 20-50 µl of 2 x Laemmli-buffer. Protein of the cytosolic fraction was precipitated with acetone or TCA as described above and used for negative control.

2.2.5.5 Determination of protein concentration

The amount of protein in cellular lysates was determined using the Roth Nanoquant Protein Assay (Bradford method; Bradford, 1976). 20 µl of the samples (1:2 - 1:100 prediluted in Triton X-100 lysis-buffer) were pipetted in duplicate into appropriate wells of a 96-well ELISA plate. 20 µl of different BSA concentrations (10, 25, 50, 75, 100 and 150 µg/ml) were used in duplicate as standards. 180 µl of Roti Nanoquant (Roth, 1:4.5 diluted in distilled H_2O) were added to each well. After 10 min of incubation at RT, the optical density was measured at 595 nm and reference wavelength 450 nm using a microplate reader (Bio Rad). The absorption values were calculated using the Microplate Manager 4.0 software (Bio Rad).

2.2.5.6 Western blot analysis

The Western blot technique represents a sensitive method to detect specific polypeptides within a complex mixture of proteins. Proteins are separated electrophoretically and transferred to a polyvinylidene fluoride (PVDF) membrane, which is subsequently incubated with antibodies specific for the protein of interest. Finally, the bound antibody is recognised by a second anti-immunoglobulin which is coupled to horseraddish peroxidase or alkaline phosphatase. The detection limit of this method ranges between 1 and 5 ng of an average-sized protein using the ECL-Detection Kit by Amersham Pharmacia (Freiburg).

SDS gel electrophoresis

Electrophoretic separation of proteins was carried out in the discontinuous buffer system for SDS polyacrylamide gels as originally described by Laemmli [1970]. For detection of intracellular proteins, 100 µg of total protein from mesangial cell lysates and 40 µg from liver cell lysates were dissolved in 1 x Laemmli buffer (2.1.2.1). For detection of TCA-precipitated proteins in the cell culture supernatants, 1 ml of the supernatants was used. For loading of the protein samples on the gel, they were denatured by heating for 5 min at 95°C or for 30 min at 40°C in the presence of SDS. Mercaptoethanol (2-ME) or DTT was added during denaturation to reduce disulfide bonds. Importantly, for immunodetection with the monoclonal anti rat-sPLA$_2$-IIA antibody, the protein samples were treated with 2 x Laemmli buffer without any reducing reagents. A typical run was performed in 1 x PAGE

running buffer (2.1.2.1) at a constant current of 20 mA/cm^2, until the bromphenol blue (dye indicator) reached the bottom of the resolving gel.

The separating gel and stacking gel solutions were prepared as follows:

Resolving gel:	**Component volumes (ml) for 2 gels:**	
	10 % (60-120 kDa)	**15 % (< 60 kDa)**
H_2O	24	13.8
Acrylamide mix (30%)	20.1	36.15
Tris/HCl (1.5 M, pH 8.8)	15	15
SDS (10 %)	0.6	0.6
APS (10 %)	0.6	0.6
TEMED	0.03	0.03

Stacking gel :	**Component volumes (ml) for 2 gels :**
H_2O	18.2
Acrylamide mix (30%)	4
Tris/HCl (1.5 M, pH 6.8)	7.5
SDS (10%)	0.3
Ammonium persulfate (10%)	0.09
TEMED	0.03

The desired percentage of acrylamide in the gel depends on the molecular size of the protein to be separated. For the 88 kDa CPT-Iα, 10 % and for 15 kDa rat-sPLA$_2$-IIA, 15% running gels were used to achieve best separation of proteins. For the control of protein loading, immunodetection with 44 kDa α- actin or 52 kDa ß-tubulin was carried out. To remove any antibodies from the membrane, it was washed overnight in blocking-buffer at 4°C. Stripping of membrane was not necessary, because all proteins of interest were of different molecular sizes and well separated in the gels.

Coommassie-Brilliant-Blue staining after SDS-PAGE

The location of a protein in a gel and protein loading can be determined by Coommassie-Brilliant-Blue staining. Detection of the protein in a gel by Coommasie-Brilliant-Blue depends on nonspecific binding of the dye to the protein. The detection limit is 0.3-1 µg protein/ band.

All steps were carried out in volumes of 10-20 ml of the following solutions at RT with slow agitation on a shaker. The polyacrylamide gel was stained with Coommassie-Brilliant-Blue

solution for 2 hr, followed by destaining solution to obtain blue protein bands on a clear background.

Transfer to PVDF membrane

After gel electrophoresis, proteins were transferred to a PVDF membrane by electroblotting (Hoefer SemiPhor, Amersham Pharmacia, Freiburg). Prior to use, the PVDF membrane was activated in isopropanol for 15 s and subsequently rinsed in deionized water for additional 2 min. Six pieces of Blotting Paper (Sigma) were soaked in transfer buffer and positioned on the anode side of the transfer apparatus. The PVDF membrane was placed directly on the stack of blotting paper. The SDS gel containing the separated proteins was taken off the glass plates, rinsed shortly in transfer buffer, and placed on the top of the PVDF membrane. Finally, the gel was covered with six additional, transfer buffer-soaked blotting paper. Air bubbles were squeezed out by a roller apparatus. The upper electrode (cathode) was positioned on the top of the stack and a current of 0.8 mA/cm^2 (nearly 60 mA per mini gel) was applied. Transfer of proteins was carried out at RT and terminated after 90 min. After blotting, the membrane was checked by Ponceau S staining for correct electrophoretic transfer and equal loading. The successful protein transfer can also be controlled by staining the blotted SDS gel with Coomassie-Brilliant-Blue (2.5 mg/ml Coomassie-Brilliant-Blue G250, 45% Methanol, 45% H_2O and 10% acetic acid).

Immunodetection

After blotting, the PVDF membrane was directly soaked in a TBST-buffered BSA solution (1%) for 1 h at RT (or overnight at 4°C) to block non-specific binding sites. The membrane was subsequently exposed to primary antibodies, specific for the protein of interest and incubated overnight at 4°C or at RT.

Antibody	**Dilution**	**Block buffer**	
Anti-CPT-Iα	1:2000	TBST –2 % BSA	overnight at 4°C
Anti-rat-sPLA$_2$-IIA	1:100	PBS-1 % milk powder	overnight at 4°C
ß-tubulin	1:1000	TBST-1 % BSA	1 h at RT
Actin	1:1000	TBST- 1 % BSA	1 h at RT

The blot was washed four times for 10 min in 1x TBST. Specific binding of primary antibody was detected by incubation of the membrane with a secondary antibody coupled to horseraddish peroxidase diluted at 1:15.000-1:20.000 in 1x TBST for 1 h at RT. For

detection of the corresponding bands, the enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia) was used according to the manufacturer's instructions. The membrane was exposed to a special ECL film (Amersham Pharmacia) and developed using Hyperprocessor, Amersham Pharmacia. Developed films were scanned (GS 700 Imaging Densitometer, Bio Rad) and analyzed using the Molecular Analyst software from Bio Rad.

III

Results

3.1 Characterisation of CPT-Iα antibody

The liver isoform of CPT-Iα, with a molecular size of 88 kDa, is expressed in all cells except skeletal muscle cells and white adipocytes (Cook e*t al.,* 2001). Therefore, in this part of the thesis, the ability of anti-CPT-Iα antibodies to detect this isoenzyme in protein lysates of liver and mesangial cells in comparison to skeletal muscle cells was tested by Western blot analysis.

Protein lysates were prepared from the primary rat hepatocytes and mesangial cells after incubation in DMEM medium for 24 hr. For negative control, the tissue lysate from rat hind leg was prepared as described in Methods (2.2.5.1). Primary hepatocytes and skeletal muscle tissue were isolated from male Sprague-Dawley rats that had been starved for 24 hr (2.2.2.2). Cell lysates from primary hepatocytes containing 40 µg protein and from mesangial and skeletal muscle cells containing 100 µg protein were used for loading of SDS-PAGE (10 %). Subsequently, protein was transferred to PVDF membrane and Western blot analysis was performed as described in 2.2.5.6. As seen in Fig. 3.1 (A), CPT-Iα (88 kDa) was detectable on the Western blot by the antibody generated against this CPT isoenzyme in primary hepatocytes and mesangial cells. However, the required protein amount to detect CPT-Iα in mesangial cells was higher (100 µg) than that needed from liver cells (40 µg). This was expected as the liver is a metabolically more active organ than the kidney.

Also as expected, CPT-Iα was not detectable on the blot with protein samples of the skeletal muscle cells, as no bands corresponding to the size of 88 kDa were detectable.

Since CPT-Iα is integrated into the mitochondrial outer membrane (Fraser *et al.,* 1997), cytosolic and membrane fractions were prepared to increase the specificity and sensitivity of CPT-Iα detection.

Cytosolic and membrane fractions from cell lysates containing 100 µg protein from hepatocytes and 50 µg from mesangial cells were prepared as described in 2.2.5.4 and separated by SDS-PAGE (10 %) for Western blot analysis (Fig. 3.1 B)

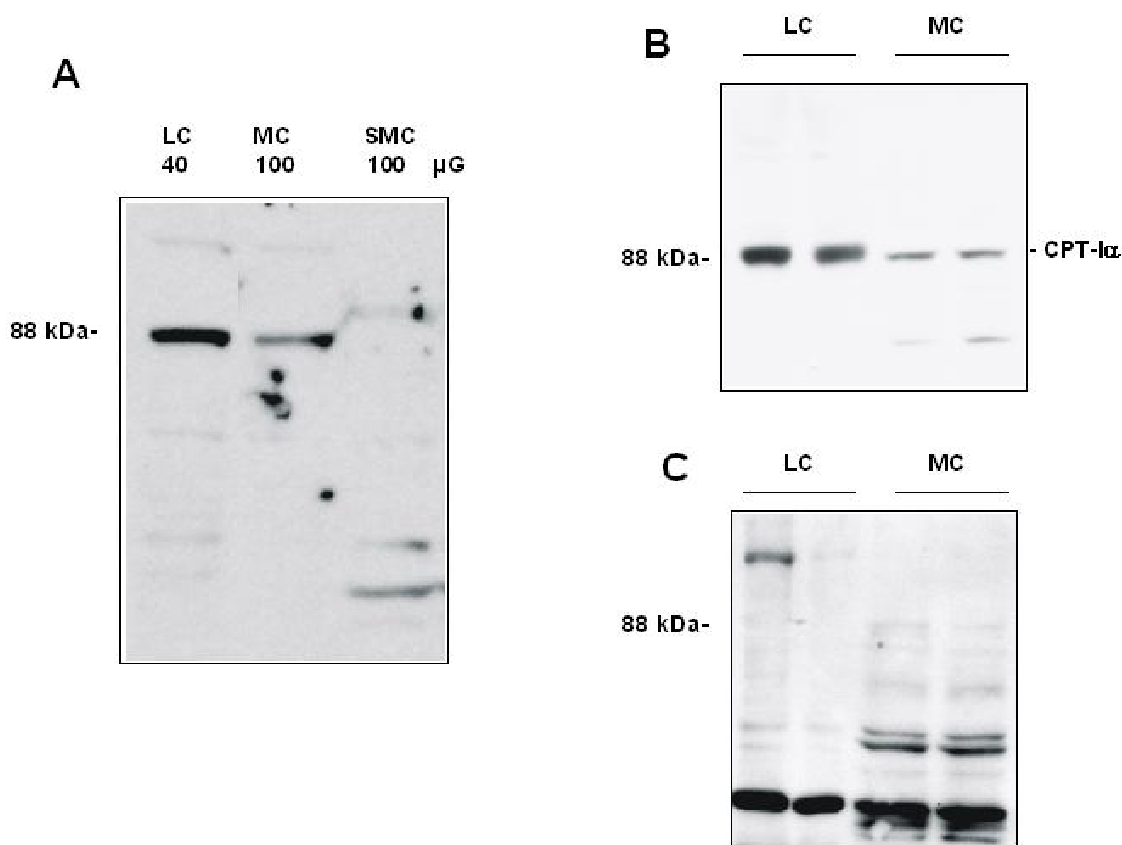


Figure 3.1 Characterisation of anti- CPT-Iα antibodies.

(A) Mesangial cell (MC) lysates were prepared from rat mesangial cell cultures after incubation in DMEM medium for 24 hr. Liver (LC) and skeletal muscle cell (SMC) lysates were prepared from isolated primary hepatocytes (2.2.2.2) and skeletal muscle tissue from male Sprague-Dawley rats starved for 24 hr. Thereafter, the cell lysates containing 40 µg protein from hepatocytes, and 100 µg from mesangial and skeletal muscle cells were taken for SDS-PAGE (10 %) and subsequent Western blot analysis as described under 2.2.5.6.

Cell lysates containing 50 µg protein from hepatocytes and 100 µg protein from mesangial cells were taken for preparation of membrane **(B)** and cytosolic fractions **(C)** as described under 2.2.5.4 and used for Western blot analysis.

As shown in Fig. 3.1 (B), the antibody was able to detect CPT-Iα only in the membrane fraction, whereas a band of 88 kDa size was not detectable in the blot of the cytosolic fraction (Fig. 3.1 C). This was in agreement with the known localisation of this protein in the mitochondrial membrane. Moreover, due to the separation of membrane protein from cytosolic protein, the clearance of unspecific bands on the blot detected by the anti-CPT-Iα antibody was markedly improved. Due to the time consuming isolation of membrane fraction, further Western blot analyses were performed mostly with total lysates. Despite improved detection of CPT-Iα in blots with membrane fraction, the blots with total protein lysates were more suitable for protein loading control with anti-actin or -ß-tubulin antibodies. However, the preparation of membrane fraction was useful for cells lysates containing small amounts of protein.

3.2 Stability of carnitine palmitoyltransferase-Iα protein

One important aim of this thesis was to study the regulation of CPT-Iα protein expression under proinflammatory conditions. Thus, it was important to know, how fast the turn-over of this protein is, due to de novo-protein biosynthesis, during the time course of the experiments. In order to investigate the stability of CPT-Iα protein in mesangial cells and primary hepatocytes, cells were treated with the known protein synthesis inhibitor cycloheximide, and the CPT-Iα protein level was monitored by Western blot analysis. The cells were maintained in DMEM medium and then mesangial cells were treated with 10 µM cycloheximide for time period ranging from 2 to 24 hr. For primary hepatocytes 100 µM cycloheximide was used for a period from 2 to 8 hr, because at later time points cycloheximide was toxic to the cells.

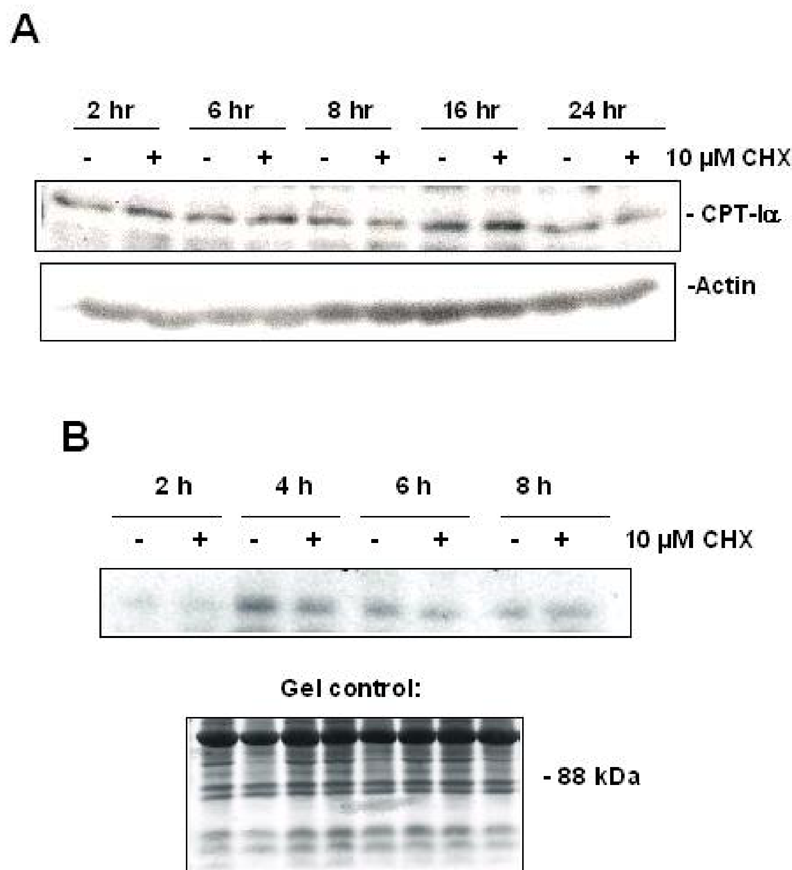


Fig 3. 2 The time course of protein expression of CPT-Iα in rat mesangial cells (A) and primary hepatocytes (B) in the absence or presence of cycloheximide.

(A) Rat mesangial cells were treated with 10 µM and **(B)** rat primary hepatocytes with 100 µM cycloheximide for the indicated time periods. Cell lysates containing 100 µg of protein from mesangial cells and 30 µg protein from hepatocytes were subjected to SDS-PAGE (10 %). Western blot analysis was performed using the CPT-Iα antibody at a dilution of 1:2000 and subsequently with an anti-actin antibody for protein loading control from mesangial cell lysates or by Coommassie blue staining of the gel from hepatocytes protein lysates.

CPT-Iα protein from mesangial cells and hepatocytes remained unchanged over the evaluated time period of 24 and 8 hr, respectively indicating that this protein, which is essential for basic metabolic functions, is very stable in both cell types at least during the time course of this study. This may indicate that even small increases in CPT-Iα protein amounts will have a significant effect on cellular lipid homeostasis. The purpose of the following studies was to search for conditions,

which would initiate an increase in CPT-Iα expression, and to investigate the regulatory mechanisms.

3.3 Regulation of the expression of CPT-Iα by nitric oxide (NO) in rat mesangial cells and primary hepatocytes

Nitric oxide (NO) has been identified as an universal intercellular messenger molecule regulating a variety of diverse cellular functions in many tissues. High amounts of NO produced by the inducible isoform of nitric oxide synthase (iNOS) can have beneficial microbicidal, antiviral, antiparasital, and antitumoral activity (Bogdan, 2001; MacMicking *et al.,* 1997). In contrast, excessive and uncontrolled production of NO may have damaging consequences and seems to be involved in the pathophysiology of several human diseases and in particular, of acute and chronic inflammation (Pfeilschifter *et al.,* 1995). Most tissue-derived cells are sensitive to the induction of iNOS when challenged with the proinflammatory cytokines, TNFα or IL-Iß. In particular in rat mesangial cells, IL-Iß is a strong stimulus for iNOS transcription (Pfeilschifter *et al.,* 1990; Beck *et al.,* 1998).
In a previous study (Gans 2003), it was shown that the NO donor DETA-NO induced CPT-Iα mRNA and protein expression in rat mesangial cells. Also, cytokines, like IL-Iß cause the production of NO by induction of iNOS in mesangial cells at the transcriptional level. In the present study, it was determined whether DETA-NO-induced CPT-Iα gene expression also occured at the transcriptional level. Moreover, the possible role of NO/cGMP pathway in the induction of CPT-Iα mRNA and protein expression in rat mesangial cells was elucidated.

Results:

3.3.1 Effect of nitric oxide on CPT-Iα promoter activity in rat mesangial cells

It has been previously demonstrated, that high levels of nitric oxide released from the NO-donors (SNAP or SNP) caused an increased CPT-Iα activation (Garcia-Villafranca *et al.,* 2002). Furthermore, it was shown more recently (Gans 2003) that DETA-NO upregulates CPT-Iα protein and mRNA expression. These studies were

continued, and the effect of DETA-NO on the CPT-Iα promoter activity in mesangial cells was performed to characterise the regulation of transcription.

Mesangial cells were transfected with plasmids containing the CPT-Iα promoter in length of -4495/+19 linked to the luciferase reporter gene as described in 2.2.2. Subsequently, cells were stimulated for 24 hr with 200 µM DETA-NO, 2 nM IL-Iß or 80 µM. These concentrations were shown to maximally stimulate CPT-Iα protein and mRNA expression at that time point (Gans 2003). Moreover, a previous study by Kunz *et al*. (1994) demonstrated that stimulation of mesangial cells for 24 hr with IL-1ß leads to increased levels of iNOS mRNA and nitrite formation. Thus, it was presumed that, similar to the NO donor, IL-1ß-induced NO formation may trigger CPT-Iα promoter activity.

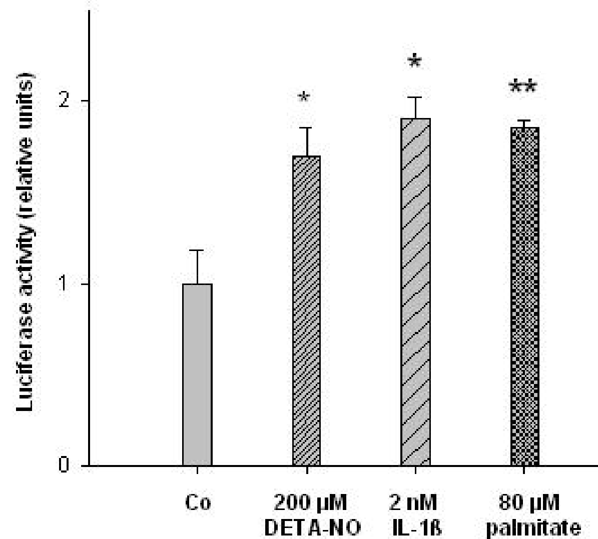


Figure 3.3 Effect of the DETA-NO and IL-1β in comparison to palmitate on CPT-Iα promoter activity.

Mesangial cells grown in six-well plates were transfected for 16 hr with 400 ng of the -4495/+19 promoter DNA construct plus 40 ng Renilla DNA (pRL-TK). After 24 h incubation with DETA-NO, 2 nM IL-1ß, or 80 µl palmitate, dual luciferase assays were performed as described in the Materials and Methods section (2.2.2.5). Values for luciferase were related to values for Renilla. */** Significant differences compared to vehicle as control (* $P < 0.1$; ** $P < 0.05$).

As shown in Fig. 3.3, DETA-NO and IL-1ß as well as palmitate stimulated a potentiation of CPT-Iα promoter activity by about twofold. In parallel, IL-1ß stimulated a significant increase in NO formation, measured as nitrite (40-fold) in comparison to control (date not shown).

3.3.2 Involvement of nitric oxide/cGMP signaling pathway on CPT-Iα expression in rat mesangial cells

The aim of the next study was to investigate the role of cGMP as a downstream second messenger of NO for the CPT-Iα protein and mRNA expression. It is well known that IL-1ß-induced NO generation activates the soluble guanylate cyclase (sGC) in rat mesangial cells (Pfeilschifter *et al.,* 1990). Therefore, in the next experiments cells were treated with ODQ, an inhibitor of sGC, or the sGC activator YC-1.

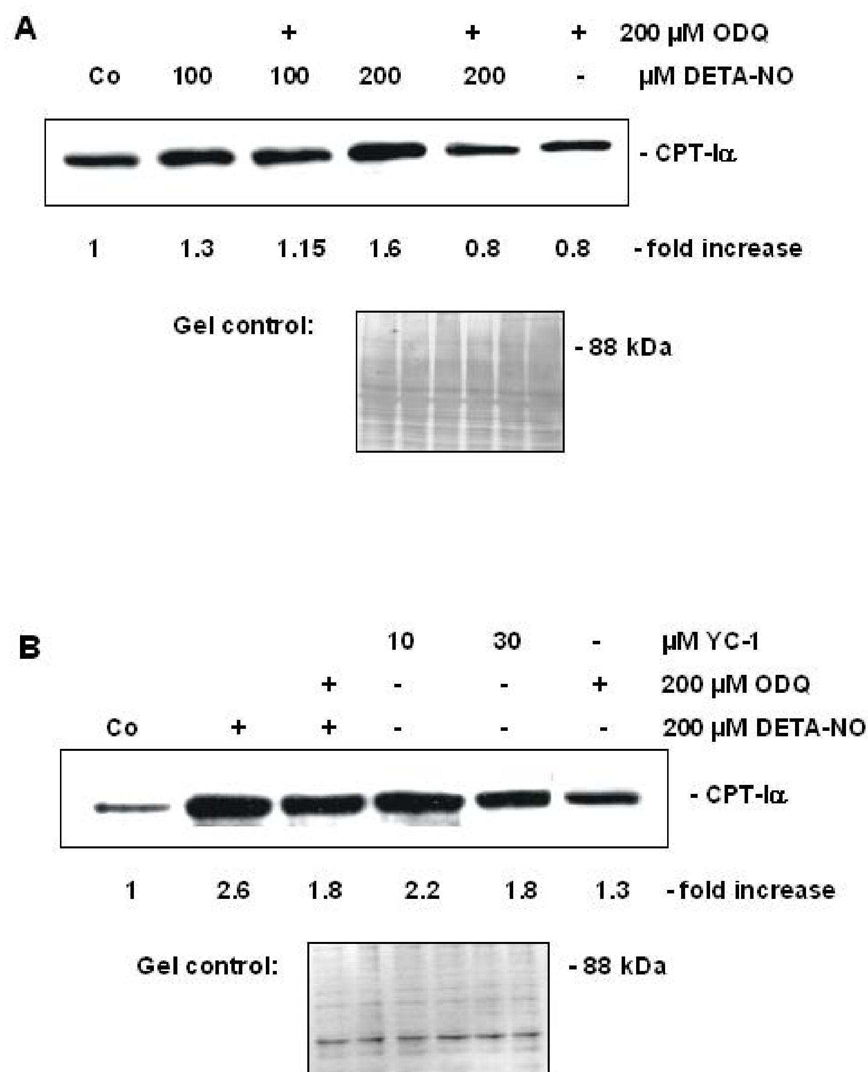


Figure 3.4 Effect of sGC activator YC-1 on CPT-Iα protein expression and the sGC inhibitor ODQ on DETA-NO-induced CPT-Iα protein expression.

Mesangial cells were treated for 24 hr with 100 and 200 µM DETA-NO in absence and presence of 200 µM ODQ **(A)** Mesangial cells were stimulated with 200 µM DETA-NO in absence and presence of 200 µM ODQ or **(B)** with YC-1 at concentrations of 10 and 30. Thereafter, cells were lysed and 100 µg protein of cell lysate was taken for SDS-PAGE (10%) and subsequent Western blot analysis with an antibody against CPT-Iα was performed. Protein loading was controlled by Coommassie-Brilliant-Blue staining.

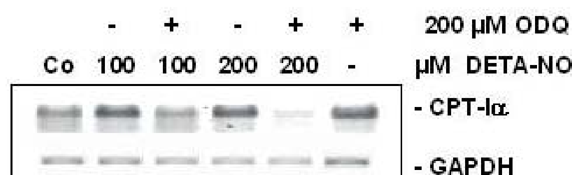


Figure 3.5 Effect of the sGC inhibitor ODQ on DETA-NO-induced CPT-Iα mRNA expression.

Mesangial cells were treated for 24 hr with 100 and 200 µM DETA-NO in the absence or presence of 200 µM ODQ. Total RNA was isolated and subjected to semiquantitative RT-PCR using specific primers for CPT-Iα and GAPDH as described under 2.2.4.

Treatment of mesangial cells with 100 and 200 µM DETA-NO for 24 hr resulted in a dose-dependent increase in CPT-Iα protein, which corresponds to the enhanced mRNA and promoter activity stimulated by this NO donor.
Incubation of the cells with the sGC inhibitor ODQ (200 µM) resulted in a marked decrease of CPT-Iα protein and mRNA back to control levels or even below (Fig. 3.4 A and 3.5). This suggests that the NO/cGMP pathway is crucial for a NO-mediated increase in CPT-Iα expression. An amplification of CPT-Iα protein expression was also evident when mesangial cells were stimulated with YC-1 as an NO-independent activator of sGC (Fig. 3.4 B). However, this stimulation was not dose-dependent, because 10 µM YC-1 caused a higher CPT-Iα protein expression (2.2-fold) than 30 µM YC-1. This could be due to toxic effects of YC-1 at concentration of 30 µM. In the some experiments, ODQ alone stimulated a weak increase in CPT-Iα expression. This suggests that a basal cGMP generation might be involved in the maintenance of constant CPT-Iα levels.

3.3.3 Effect of nitric oxide on CPT-Iα expression in primary rat hepatocytes

It was further investigated whether DETA-NO has an effect on CPT-Iα expression in primary rat hepatocytes. Therefore, hepatocytes were stimulated for 24 hr with different concentration of DETA-NO. Protein and mRNA levels of CPT-Iα were analysed by Western blot (Fig. 3.6 A) and RT-PCR (Fig. 3.6 B), respectively. The

secretion of rat-$sPLA_2$-IIA in supernatant after incubation was also analysed in Western blot for positive control of DETA-NO action and to check the responsiveness of hepatocytes to this NO donor (Fig. 3.6 C).

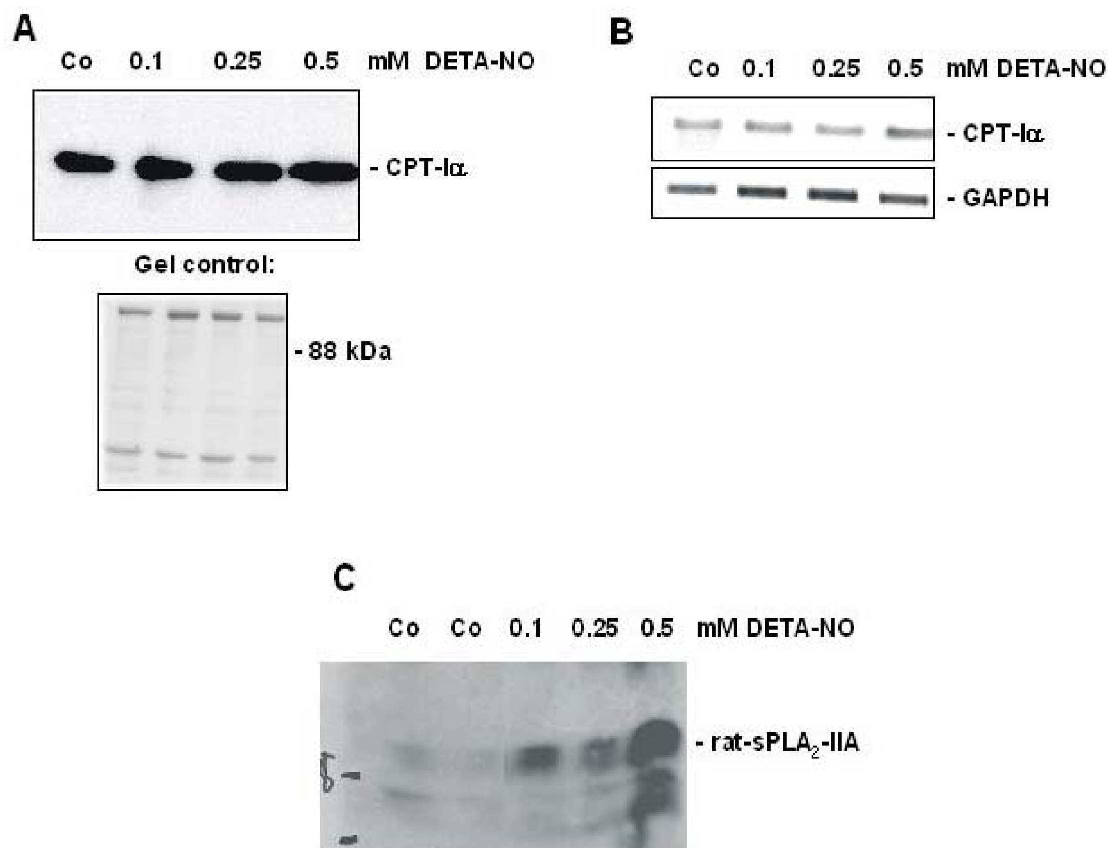


Figure 3. 6 Effect of DETA-NO on CPT- Iα protein (A) and mRNA (B) expression in primary rat hepatocytes.

(A) Primary hepatocytes were treated for 24 hr with DETA-NO at the indicated concentrations. Cell lysates containing 30 µg of protein were taken for isolation of protein from the membrane fraction as described in chapter 2.2.5.4 and subjected to SDS-PAGE (10%). Subsequently, protein was transferred for Western blot analysis with the anti-CPT-Iα antibody,and protein loading was controlled by Coommassie-Brilliant-Blue staining.

(B) Total RNA was isolated from hepatocytes after treatment for 24 hr with DETA-NO at the indicated concentration and subjected to RT-PCR using primers for CPT-Iα and GAPDH.

(C) Supernatants were taken for protein precipitation with TCA (2.2.5.2) and subjected to SDS-PAGE (15 %) and Western blot using anti-rat $sPLA_2$-IIA antibody as described in Methods 2.2.6.

As shown in the Fig 3.6, A and B, CPT-Iα protein and mRNA expression was not affected by DETA-NO treatment in hepatocytes in contrast to mesangial cells. On

the other hand, DETA-NO stimulation led to a clear induction of rat $sPLA_2$-IIA in dose-dependent manner (Fig. 3.6, C). These data indicate that rat hepatocytes did response to treatment with DETA-NO to produce an increase in rat -$sPLA_2$-IIA secretion, whereas CPT-Iα mRNA and protein expression was not altered.

To investigate a putative role of endogenously produced NO, hepatocytes were stimulated with different cytokines for different time points and at different concentrations. Also, under these conditions, neither a change in CPT-Iα expression nor in iNOS expression and nitrite formation were detectable by Western blot analysis, RT-PCR and Griess assay (data not shown).

3.4 Regulation of the expression of CPT-Iα under hypoxic condition

Hypoxia induces transcriptional regulation of a wide variety of genes encoding proteins necessary for cellular and physiological adaptation, including alterations in metabolic events and energy consumption. In this respect, HIF-Iα is an important regulatory factor for this adaptation during hypoxia. It has been shown earlier that mesangial cells and hepatocytes in culture respond to hypoxia with changes in proliferation, matrix production and secretion of signalling molecules (Sahai e*t al.,* 1997; Yuan *et. al.,* 2000; Göpfert e*t al.,* 1996).

However, the alterations of CPT-Iα in kidney and liver under hypoxia are poorly understood, and the observations in several studies are rather controversial. Moreover, nothing is known about the mechanisms of hypoxia-mediated effects on CPT-Iα in mesangial cells and hepatocytes.

In the present study 3 series of experiments were performed. Series 1 was to investigate the regulation of the CPT-Iα protein and mRNA expression under hypoxic conditions in rat mesangial cells. Series 2 was performed to examine hypoxic effects on the CPT-Iα expression in rat hepatocytes and to compare this with mesangial cells in order to delineate tissue specific differences of regulation. Series 3 was performed to investigate whether hypoxia regulates mHMG-CoA synthase, another important enzyme in lypolysis.

Results:

3.4.1 Regulation of CPT-Iα expression under hypoxic conditions in mesangial cells

First, the dose dependence of the hypoxia mimicing agent, $CoCl_2$, on CPT-Iα protein expression was investigated by Western blot experiments. Mesangial cells were exposed to medium containing 100 µM or 200 µM $CoCl_2$ for 16 and 24 hr (Fig. 3.7) and cell lysates containing 200 µg protein were taken for isolation of the membrane protein fraction.

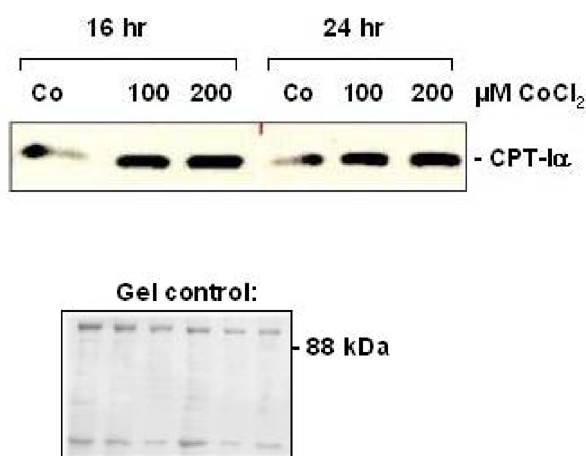


Figure 3.7 Western Blot analysis of CPT-Iα protein expression in mesangial cells treated with $CoCL_2$.

Mesangial cells were treated with 100 µM or 200 µM $CoCL_2$ for 16 and 24 hr. Subsequently, cells were lysed and 200 µg protein of cell lysates was used for membrane isolation as described in 2.2.5.4. The pellet of the membrane fraction was subjected to the SDS - PAGE (10 %) and subsequently, Western blot analysis was performed with an anti-CPT-Iα antibody. Coommassie-Brilliant-Blue staining of the gel was performed for protein loading control.

The data in Fig. 3.7 show that $CoCl_2$ stimulated an about 2- to 3-fold increase in CPT-Iα protein in mesangial cells after 16 hr, however, this was not dose-dependent and was not further increased during an incubation up to 24 hr.

The next experiments were performed to examine the effect of $CoCl_2$ on CPT-Iα mRNA expression, with respect to time and concentration of treatment. The mesangial cells were stimulated with 50, 150, 200, 400 mM $CoCl_2$ for 24 hr

(Fig. 3.8 A) and with 100 µM $CoCL_2$ for 2, 4 and 48 hr and changes in CPT-Iα mRNA expression were investigated by semiquantitative RT-PCR (Fig 3.8 B).

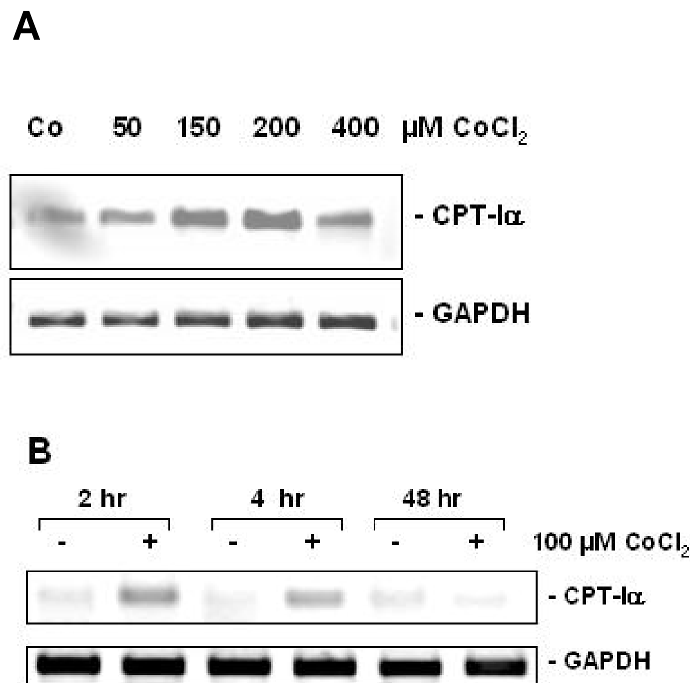


Figure 3. 8 Effect of $CoCl_2$ on CPT-Iα mRNA expression in rat mesangial cells.
Mesangial cells were treated for 24 hr with $CoCl_2$ at the indicated concentrations **(A)** and with 100 µM $CoCl_2$ for 2, 4 and 48 hr **(B)**. Total RNA was isolated and CPT-Iα mRNA expression was analysed by performing semiquantitative RT-PCR as described under 2.2.4.

As shown in Fig. 3.8 A and B, CPT-Iα mRNA levels were induced following $CoCl_2$ stimulation at the concentration range of 100 µM -200 µM. However, as shown in Fig. 3.7, the dose-dependency of CPT-Iα mRNA expression is not reflected by a dose-dependent increase in protein levels for yet unknown reasons.

Incubation of mesangial cells with 400 µM $CoCl_2$ did not induce a further increase in CPT-Iα expression, as this concentration was toxic to the cells. Moreover, it was observed that 48 hr of treatment with 100 µM $CoCl_2$ caused some downregulation of constitutive mRNA expression, presumably due to apoptosis (Kakinuma e*t al.,* 2001).

The aim of the next study was to determine, whether an incubation of mesangial cells in an hypoxic cell culture environment, generated by the infusion of a gas mixture containing 3% O_2 into the incubator, affects CPT-Iα mRNA and protein

expression. The effect of this hypoxic model was examined in comparison with $CoCl_2$ and desferrioxamine (DFO) treatment.

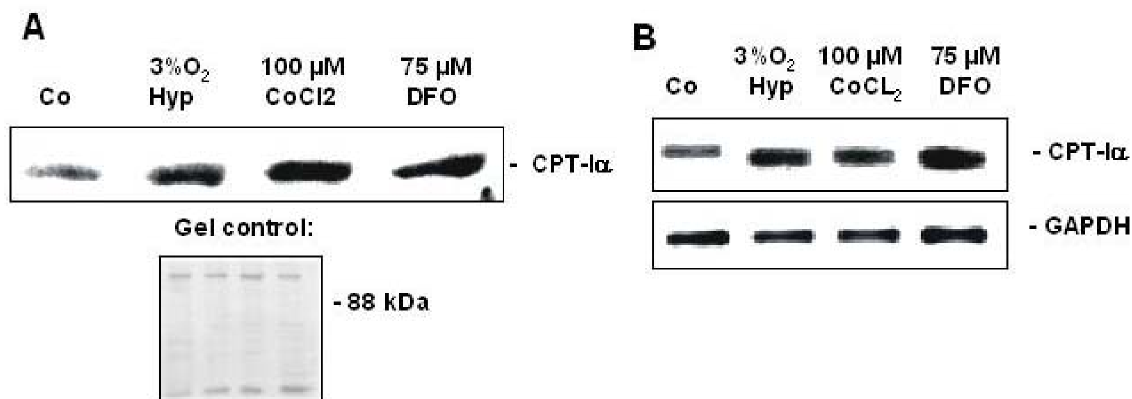


Figure 3. 9 Effect of hypoxia (3%), $CoCl_2$ and DFO on CPT-Iα protein and mRNA expression in rat mesangial cells.

Mesangial cells were incubated for 24 hr under hypoxic condition (3%) and with 100 µM $CoCl_2$ or 75 µM DFO under normoxic condition. Subsequently protein and total RNA was isolated for Western blot **(A)** and RT-PCR analysis **(B)**. Membrane fractions isolated from lysats containing 100 µg protein (2.2.5.4) were taken for SDS-PAGE (10 % acrlyamid) and subsequently Western blot with anti-CPT-Iα antiserum. Commassie-Brilliant-Blue staining of the gel were used for protein loading control.

The data in Fig. 3. 9 show that mesangial cells incubated for 24 hr during hypoxia (3% O_2) or with $CoCl_2$ (100 µM) and DFO (75 µM), during normoxia demonstrated an increase in CPT-Iα protein and mRNA expression.

3.4.2 Regulation of the expression of CPT-Iα under hypoxic conditions in primary rat hepatocytes

First, the $CoCl_2$ hypoxia model was used to characterise CPT-Iα mRNA and protein expression in rat primary hepatocytes. Western blot and RT-PCR analyses were performed with the membrane fraction of the protein and total RNA isolated from hepatocytes incubated with $CoCl_2$ at a concentration range between 100 and 400 µM.

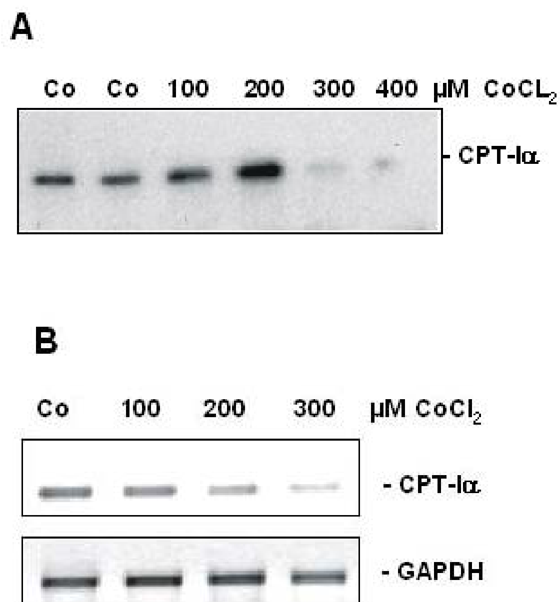


Figure 3. 10 Effect of $CoCl_2$ on CPT-Iα on protein (A) and mRNA (B) expression in rat primary hepatocytes.

Primary rat hepatocytes were stimulated for 24 hr for protein analysis and 16 hr for mRNA expression analysis with either vehicle (control) or the indicated concentrations of $CoCl_2$ under normoxic condition.

(A) Cell lysates containing 30 µg of protein were taken for isolation of the membrane fraction as described in chapter 2.2.5.4 and subjected to SDS-PAGE (10%). Subsequently, Western blot analysis was performed. **(B)** Total RNA was isolated from the hepatocytes and subjected to semiquantitative RT-PCR analysis.

As it is shown in Fig. 3.10 A, incubation of hepatocytes with 100 µM and 200 µM $CoCl_2$ increased CPT-Iα protein expression in a dose-dependent manner. After stimulation with higher concentration of $CoCl_2$ (300 µM and 400 µM) CPT-Iα protein levels fell under control levels, due to its toxic effect. An exposure of rat hepatocytes to 100 µM $CoCl_2$ did not cause detectable changes in CPT-Iα mRNA levels. Incubation with 200 µM and 300 µM $CoCl_2$ even decreased CPT-Iα mRNA levels, and stimulation with 400 µM $CoCl_2$ presumably caused degradation of RNA due to toxicity, which was estimated from the agarose gel electrophoresis of total RNA (2.2.4.7). Thus, it was not possible to analyse the effect of this concentration on CPT-Iα mRNA expression by RT-PCR.

Interestingly, the regulation of CPT-Iα by $CoCl_2$ in hepatocytes is in marked contrast to its effects in mesangial cells (see data in Fig. 3.7 and 3.8).

In the next study, the effect of hypoxia (1% O_2) in comparison to $CoCl_2$ and DFO on CPT-Iα mRNA expression was examined. Primary hepatocytes were incubated under hypoxic conditions of only 1% oxygen, or with $CoCl_2$ (100 μM) or DFO (75 μM) under normoxia for 2, 4 and 24 hr (Fig. 3.11).

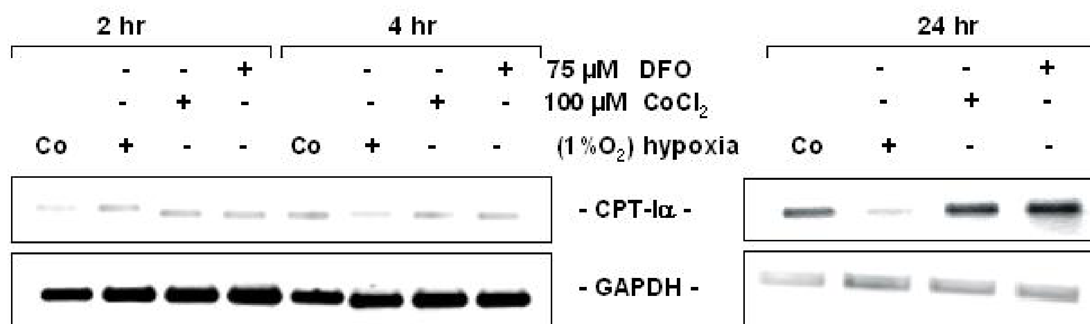


Figure 3.11 Effect of hypoxia (1%), $CoCl_2$ and DFO on CPT-Iα mRNA expression in rat hepatocytes.

Primary rat hepatocytes were incubated for 2, 4 and 24 hr under hypoxic condition 1% O_2) or with 100 μM $CoCl_2$ or 75 μM DFO under normoxic condition. Subsequently, total RNA was isolated and semiquantitative RT-PCR analysis was performed.

As shown in the Fig. 3.11, the CPT-Iα mRNA level began to decrease between 2 and 4 hr of hypoxic exposure and remained below control levels up to 24 hr. In contrast, after treatment of the hepatocytes with 100 μM $CoCl_2$, CPT-Iα mRNA levels remained unchanged. A slight induction of CPT-Iα mRNA was observed after 24 hr of treatment with 75 μM DFO. These results demonstrate marked differences between the hypoxic culture environment generated by 1 % O_2 and by the hypoxia-mimicking compounds $CoCl_2$ and DFO, which may be the result of affecting different regulatory pathways. Moreover, the oxidative fatty acid metabolism might be drastically reduced due to the lack of oxygen (1 % instead of 3 % oxygen in the cell culture environment).

3.5 Regulation of the mHMG-CoA synthase mRNA under hypoxic conditions in rat mesangial cells and primary hepatocytes

Results shown above indicated that different models of hypoxia cause changes in CPT-Iα protein and mRNA expression in rat mesangial and primary hepatocytes. The next experiments were performed to investigate whether expose of rat mesangial and primary hepatocytes to hypoxic conditions cause a similar alteration in expression of mHMG-CoA mRNA synthase, another enzyme important for FA oxidation rate.

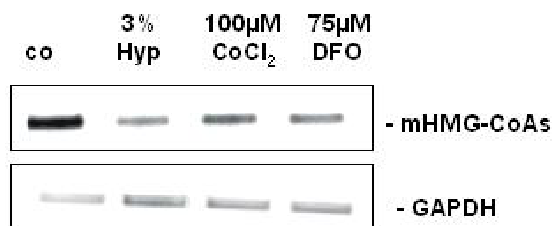


Fig. 3.12 Effect of hypoxia (3 %), $CoCl_2$ and DFO on mHMG-CoA synthase mRNA expression in rat mesangial cells.

Rat mesangial cells were incubated for 24 hr under hypoxic condition (3% O_2), or with 100 µM $CoCl_2$, or 75 µM DFO under normoxic condition. Subsequently, total RNA was isolated and subjected to semiquantitative RT-PCR analysis with specific primers for mHMG-CoA synthase as described under 2.2.4.2.

As is shown in the Fig. 3.12, mHMG-CoA synthase mRNA in rat mesangial cells was downregulated in response to hypoxia (3% O_2), $CoCl_2$ (100 µM), or DFO (75 µM) after 24 hr.

Next, mHMG-CoA synthase mRNA expression was examined in rat primary hepatocytes. For this propose, the cells were exposed to more pronounced hypoxia (1 % O_2), or treated with $CoCl_2$ (100 µM), or DFO (75 µM) under normoxic condition for 2, 4, or 24 hours.

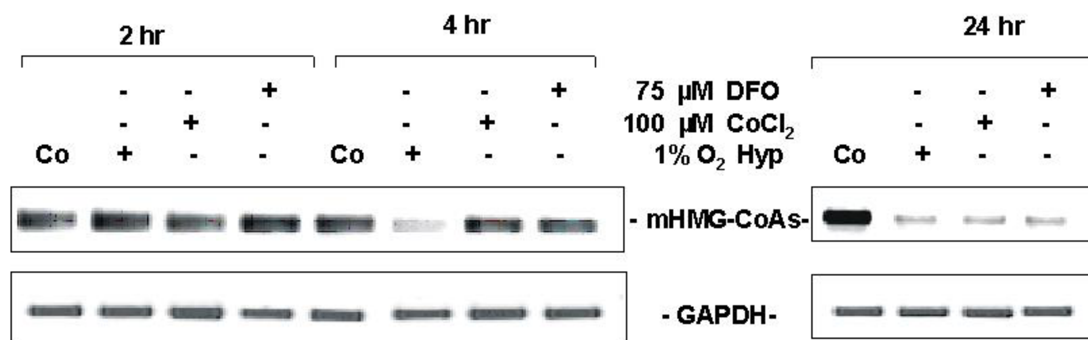


Fig. 3.13 Effect of hypoxia (1%), $CoCl_2$ and DFO on mHMG-CoA synthase mRNA expression in rat hepatocytes.

Primary rat hepatocytes were incubated for 2, 4 and 24 hr under hypoxic condition (1% O_2) and with 100 µM $CoCl_2$ or with 75 µM DFO under normoxic condition. Subsequently total RNA was isolated and subjected to RT-PCR analysis as described under 2.2.4.2.

Exposure of rat hepatocytes to a hypoxic environment of a gas mixture containing 1% O_2 or to 100 µM $CoCl_2$, or 75 µM DFO resulted in a decrease of mHMG-CoA synthase mRNA reaching lowest levels after 24 hr (Fig. 3.13). These results indicate that all three models of hypoxia cause a downregulation of mHMG-CoA synthase mRNA expression. The reduction induced by 1 % O_2 was obvious after only 4 hr, whereas $CoCl_2$ and DFO displayed their reducing effects after 24 hr.

3.6 Regulation of CPT-Iα expression by exogenous secreted phospholipase A_2-IIA in rat mesangial and primary rat hepatocytes

3.6.1 Effect of exogenously added human $sPLA_2$-IIA and TNFα on the CPT-Iα protein expression in rat mesangial cells

The elevated concentration of $sPLA_2$s under inflammatory conditions causes an increased release of FFAs and further proinflammatory lipid metabolites from glycerophospholipids such as lysophospholipids and eicosanoids, (Kudo and Murakami, 2002). As previous studies reported that FFAs upregulate CPT-Iα expression in different cell types (Clarke *et al.,* 2003; Assimacopoulos-Jeannet *et*

al., 1997), we have hypothesised a similar effect on CPT-Iα by FFAs, which were released via exogenously added $sPLA_2$s in mesangial and liver cells.

The dose dependence of exogenously added h-$sPLA_2$-IIA on CPT-Iα protein expression was investigated by Western blot analysis using a polyclonal antibody against rat CPT-Iα. Mesangial cells were stimulated for 24 hr with medium containing human recombinant $sPLA_2$-IIA (h-$sPLA_2$-IIA) at concentration of 0.1 µM and 0.3 µM. These concentrations were chosen, as previous studies demonstrated that thay produced optimal stimulation of prostaglandin synthesis and rat-$sPLA_2$-IIA secretion in these cells (Beck *et al.,* 2003). Subsequently, mesangial cells were harvested and 100 µg of cell lysate was used for Western blot analysis.

The treatment of mesangial cells with different concentrations of h-$sPLA_2$-IIA (0.1 µM and 0.3 µM) resulted in a dose-dependent increase in CPT-Iα protein levels as is shown in Fig. 3.14. Higher concentrations of this enzyme did not show a further increase in CPT-Iα protein amount (data not shown).

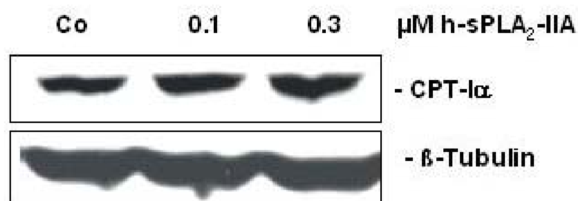


Fig. 3.14 Effect of h-$sPLA_2$-IIA on CPT-Iα protein expression.

Rat mesangial cells were cultured for 24 hr in DMEM medium containing 0.1 µM and 0.3 µM h-$sPLA_2$-IIA. 100 µg protein from total cell lysates were first subjected to SDS-PAGE (10 % acrylamide gel) and transferred to PVDF membrane. Western blot analysis was performed with an antibody against CPT-Iα and subsequently for protein loading control, with an anti-ß-tubulin antibody as described in 2.2.5.6.

Exogenously added $sPLA_2$s are known to amplify the cytokine-stimulated expression of $sPLA_2$-IIA in rat mesangial cells. This enhanced expression occurs at the mRNA and protein level and finally results in an increased secretion of the rat $sPLA_2$-IIA protein into the cell culture medium (Scholz-Pedretti *et al.,* 2002; Beck *et al.,* 2003). Therefore, the aim of the next experiment was to determine whether the CPT-Iα expression is potentiated after costimulation of h-$sPLA_2$-IIA with TNFα in comparison to treatment with h-$sPLA_2$-IIA alone.

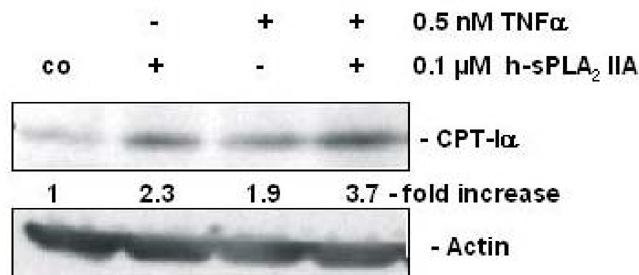


Fig. 3.15 Effect of h-sPLA$_2$-IIA and TNFα on CPT-Iα protein expression.

Mesangial cells were stimulated for 24 hr with 0.1 μM h-sPLA$_2$-IIA in the presence or absence of 0.5 nM TNFα. 30 μg protein from total cell lysates was taken for SDS -PAGE (10 %) Western blot analysis was performed with an anti-CPT-Iα antibody and subsequently with an anti-actin antibody for control of protein loading.

As shown in Fig. 3.15, costimulation of h-sPLA$_2$-IIA with TNFα elicits a 3.7-fold increase in CPT-Iα protein amount compared to an about 2-fold increase after treatment with h-sPLA$_2$-IIA and TNFα as single agents.

3.6.2 Effect of cycloheximide on h-sPLA$_2$-IIA or TNFα induction of CPT-Iα protein expression in rat mesangial cells.

To investigate whether de novo protein biosynthesis is involved in the increased expression of CPT-Iα by h-sPLA$_2$-IIA and TNFα, mesangial cells were incubated for different time points with 0.5 nM TNFα and 0.1 μM h-sPLA$_2$-IIA in presence or absence of the protein synthesis inhibitor cycloheximide (CHX). The cells were then lysed and CPT-Iα protein expression was analysed by Western blotting.

Fig. 3. 16 Effect of cycloheximid (CHX) on upregulation of CPT-Iα protein expression after treatment with h-sPLA$_2$-IIA and TNFα.

Mesangial cells were treated for 24 hr with h-sPLA$_2$-IIA (0.1 µM) or TNFα (0.5 nM) in the absence or presence of 10 µM CHX, or with 10 µM CHX alone. The cells were lysed and 100 µg protein of total cell lysates was taken for SDS -PAGE (10 %). Western blot analysis was performed with an anti-CPT-Iα antibody and subsequently with an anti-ß-tabulin antibody for control of protein loading.

Western blot analysis showed an increase in CPT-Iα protein level 24 hr after incubation with h-sPLA$_2$-IIA and TNFα (Fig. 3.16). In the presence of CHX, the increase in CPT-Iα protein expression was diminished indicating that the enhanced protein expression is indeed dependent on de novo protein biosynthesis. However, the reduction in CPT-Iα protein level did not reach the level of the control group. Furthermore, treatment with CHX alone in this experiment induced CPT-Iα protein expression. This effect could be explained by an inhibition of protein synthesis by CHX resulting in a reduced level of the enzymes which are responsible for CPT-Iα protein degradation.

3.6.3 Regulation of CPT-Iα expression by exogenous secreted phospholipase A$_2$-IIA and TNFα in rat primary hepatocytes

It was further investigated as to whether the up-regulation of CPT-Iα by exogenous sPLA2s as well as TNF-α occurs in rat primary hepatocytes. For this purpose, freshly isolated rat primary hepatocytes were stimulated for 24 hr with 0.1 µM h-sPLA$_2$-IIA in the presence or absence of 0.5 nM TNFα. Thereafter, the cells were lysed, and CPT-Iα protein level was analysed by Western blotting.

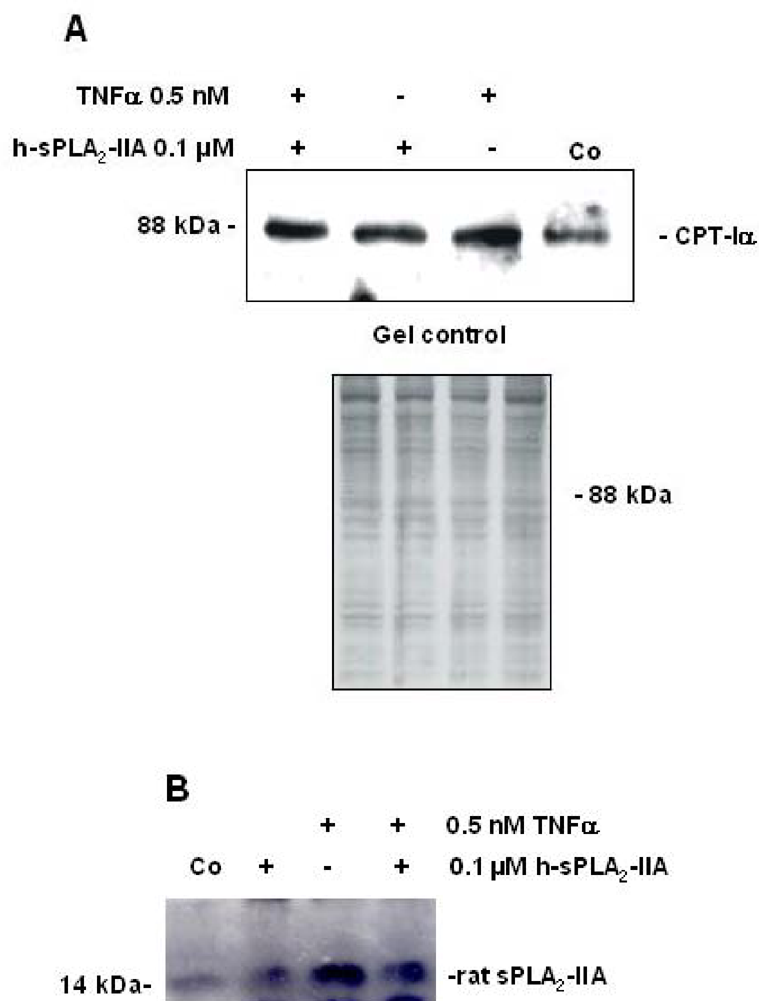


Fig. 3. 17 Effect of h-sPLA$_2$-IIA and TNFα on CPT-Iα (A) and endogenous rat-sPLA$_2$-IIA (B) protein expression.

Hepatocytes were treated for 24 hr with h-sPLA$_2$-IIA (0.1 µM) in the absence or presence of TNFα (0.5 nM).

(A) 100 µg of hepatocytes- protein lysates were subjected to SDS-PAGE (10%) and transfected to PVDF membranes. Western blot analysis was performed with anti-CPT-Iα antiserum. Coomassie staining was used for protein loading control.

(B) 1 ml cell culture supernatants were taken for r- sPLA$_2$-IIA precipitation as described under 2.2.5.2 and protein samples were subjected to SDS-PAGE (15%) and transfected to PVDF membranes. Western blot analysis was performed with anti r-s PLA$_2$-IIA antibodies as described under 2.2.5.6.

Fig. 3.17(A) shows that h-sPLA$_2$-IIA and TNFα alone triggered an increase in CPT-Iα protein expression. In contrast to the observation in rat mesangial cells, the combination of h-sPLA$_2$-IIA and TNFα did not result in a considerable potentiation of the stimulation CPT-Iα protein expression.

From the same experiment, rat-sPLA$_2$-hIIA protein secretion into the cell culture supernatants was analysed by Western blotting and used as a positive control. It was shown earlier that sPLA$_2$-IIA is constitutively expressed in rat liver (Kudo and Murakami, 2002). Additionally, rat hepatocytes show increased plasma group II PLA$_2$ activity, detectable under proinflammatoric conditions (Bertsch and Fischer, 1999; Talvinen *et al.,* 2001). As expected, TNFα triggered a significant increase in rat-sPLA$_2$-IIA protein into the cell culture supernatants. Interestingly, in contrast to rat mesangial cells (Fig. 3.17. B), secreted rat-sPLA$_2$-IIA protein was also detectable in supernatants from untreated hepatocytes, confirming the observation by Kudo (2002). Stimulation with human sPLA$_2$-IIA alone caused a considerable increase in sPLA$_2$-IIA secretion, which was not observed in rat mesangial cells. It is important to note at this point that the antibody used to detect rat sPLA$_2$-IIA does not detect the human enzyme given exogenously and does not cross-react with other known sPLA$_2$ enzymes which might be released from the hepatocytes and mesangial cells (Beck *et al.,* 2003).

3.6.4 Effect of exogenous sPLA$_2$s (0.1µM) from different species on CPT-Iα mRNA expression in mesangial cells

It is known that in many cell types free long-chain-fatty acids rapidly upregulate CPT-Iα mRNA expression in a dose-dependent manner, which is accompanied by an increase in CPT-Iα activity (Chatelain, *et al.,* 1996; Louet, *et al.,* 2001 [a; b]).

Therefore, in addition the effects of exogenous sPLA$_2$s and TNFα as FFA-realising agents on the CPT-Iα mRNA level were investigated using semi-quantitative RT-PCR analysis.

Rat mesangial cells were treated for 24 hr with various sPLA$_2$s from mammalian and venom origins (2.1.4.1) at a concentration of 100 nM in comparison with TNFα. These enzymes were previously shown to stimulate endogenous rat-sPLA$_2$-IIA secretion via FFA and their metabolites in rat mesangial cells. The effect of a catalytically inactive mutant of porcine sPLA$_2$-IB containing a single amino acid mutation at position 48 (H48Q) was also investigated. This mutant is able to bind to

the rat $sPLA_2$ receptor and thereby activates expression and secretion of rat $sPLA_2$-IIA in medium culture by indirect mechanisms involving cytosolic phospholipase A_2 ($cPLA_2$) (Beck *et al.,* 2003).

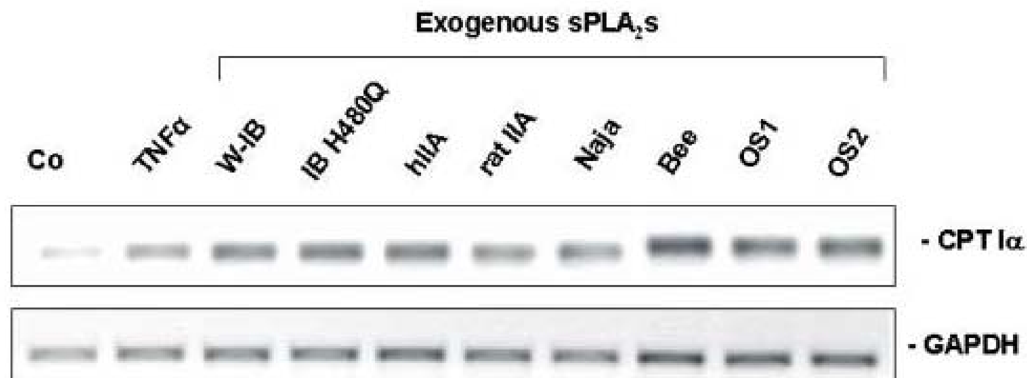


Fig. 3.18 Influence of exogenous added $sPLA_2$s and TNFα on CPT-Iα mRNA expression in rat mesangial cells.

Mesangial cells were treated for 24 hr with TNFα (0.5 nM) or with 0.1µM of human $sPLA_2$-IIA (hIIA), porcine $sPLA_2$-IB (IB), the catalytically inactive mutant $sPLA_2$-IB -H48Q (H48Q), rat $sPLA_2$-IIA (rat IIA), $sPLA_2$ from *N. mossambica mossambica* (Naja), $sPLA_2$-III from bee venom (Bee), and two snake venom $sPLA_2$s from Oxyuranus scutellatus sc. (OS1, OS2).
W, wild-type.
Total RNA was extracted from cell lysates, and RT-PCR was performed to detect CPT-Iα mRNA expression as described in 2.2.4.1.

All phospholipases, as well as, TNFα were able to enhance the mRNA expression of CPT-Iα in mesangial cells (Fig. 3.18). Furthermore, different experiments did not show much difference in regulation between enzymes studied. Interestingly, the catalytically inactive H48Q mutant of $sPLA_2$-IB was able potentiate the expression of rat CPT-Iα mRNA, suggesting an involvement of the $sPLA_2$ receptor.

3.6.5 Dose-response of CPT-Iα mRNA expression by exogenously added human $sPLA_2$-IIA and TNFα in mesangial cells

In the following experiment, the mRNA expression of CPT-Iα was analysed with two different concentrations of h-$sPLA_2$-IIA (0.1 µM and 1 µM) in the presence or absence of 0.5 nM TNFα (Fig. 3.19).

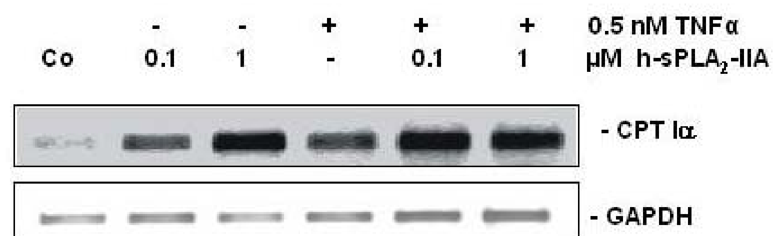


Fig. 3. 19 Effect of h-sPLA$_2$-IIA and TNFα on CPT-Iα mRNA expression.

Mesangial cells were treated for 24 hr with h-sPLA$_2$-IIA at the indicated concentrations in the presence or absence of 0.5 nM TNFα, or with TNFα alone. Total RNA was isolated and CPT-Iα mRNA expression was analysed by performing RT-PCR as described under 2.2.4.2.

As shown in Fig. 3.19, h-sPLA$_2$-IIA altered CPT-Iα mRNA level in rat mesangial cells in a dose-dependent manner. Treatment with TNFα, as a single effector, also markedly increased the CPT-Iα mRNA. Moreover, TNFα produced an additive effect on h-sPLA$_2$-IIA- induced accumulation of CPT-Iα mRNA. This potentiation corresponds to that observed for the CPT-Iα protein expression (see chapter 3.6.1).

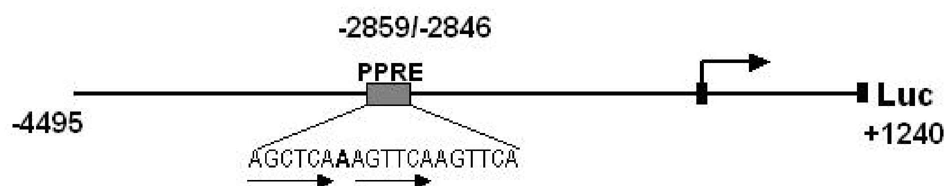
3.6.6 Effect of exogenous human sPLA$_2$-IIA and TNFα on the CPT-Iα promoter activity

To further investigate the molecular mechanisms involved in the regulation of CPT-Iα gene expression by h-sPLA$_2$-IIA and TNFα, transient transfections of plasmids containing the CPT-Iα promoter of different lengths (-4495/+1240, -4495/+19 or -210/+19) and linked to the luciferase reporter gene, were performed as described in 2.2.2.

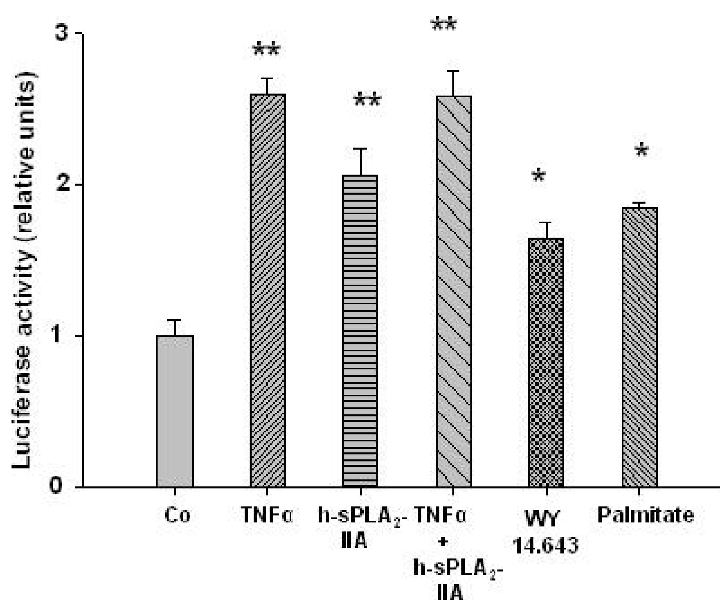
Mesangial cells transfected with the -4495/+1240 construct of the rat CPT-Iα promoter fused to a luciferase reporter gene (Park *et al.,* 1998; Jansen M, *et al.,* 2000) responded to treatment with h-sPLA$_2$-IIA (0.1 µM) and TNFα (0.5 nM) with a marked increase in luciferase activity (Fig. 3.20 A). However, the costimulation of sPLA$_2$-hIIA with TNFα did not show a potentiating effect. Similar results were obtained from transfection of mesangial cells with the -4495/+19 construct (Fig. 3.20 B). No effect was seen in mesangial cells transfected with the -210/+19 construct (Data not shown).

One possible mechanism, by which $sPLA_2$s may act on CPT-Iα expression, is the action of released lipid metabolites such as free FAs and prostaglandins via peroxisome proliferator-activated receptors PPARs. As natural ligands of PPAR, fatty acids may activate CPT-Iα gene expression by recognition of the peroxisome proliferator responsive element (PPRE) exhibited in the proximal CPT-Iα promoter (-2859/-2846). To characterise the functionality of PPRE on the CPT-Iα gene, mesangial cells were transfected with the -4495/+1240 promoter construct containing PPRE and the first intron. Then, cells were stimulated with 50 µM of the PPAR-α activator Wy14.643 and 100 µM of palmitate. As shown in Fig. 3. 20 A, both Wy14.643 and palmitate stimulated CPT-Iα promoter activity.

Interestingly, the CPT-Iα promoter activity in mesangial cells transfected with the -4495/+19 promoter construct containing PPRE but without the first intron was also stimulated with WY14.643 and palmitate (Fig. 3. 20 B).



A



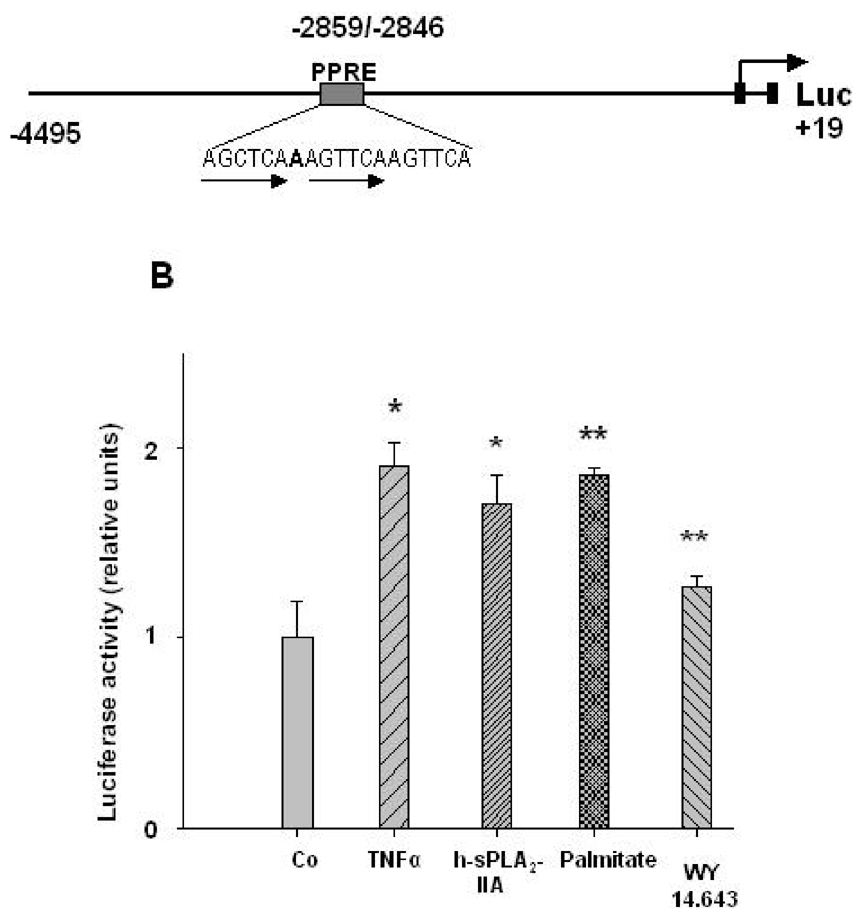


Fig. 3. 20 Effects of the human sPLA$_2$-IIA, TNFα as well as WY 14.643 and palmitate on the CPT Iα promoter activity (-4495/+1240 (A) or -4495/+19 (B) constructs).

Mesangial cells grown in six-well plates were transfected overnight with 400 ng of -4495/+1240 (**A**) or -4495/+19 (**B**) CPT-Iα promoter DNA linked to luciferase plus 40 ng Renilla-luciferase DNA (pRL-TK). After 24 h incubation with h-PLA$_2$-IIA (100 µM) in the absence or presence of TNFα (0.5nM), TNFα (0.5nM) as single agent, WY14.643 (50 µM) and palmitate (100 µM), dual luciferase assays were performed as described in the Materials and Methods section 2.2.2. Values for luciferase were related to values for Renilla.

TNFα and h-sPLA$_2$-IIα alone significantly (~2-fold) stimulated luciferase activity, whereas the addition of both effectors did not result in a potentiation of luciferase activity. These results suggested that TNFα and h-sPLA$_2$-IIA increase CPT-Iα expression at the promoter level, which corresponds to the effects obtained at the protein and mRNA level.

3.6.7 Human $sPLA_2$- and TNFα-induced upregulation of CPT-Iα protein expression may involve mitogen-activated protein kinase (MAPK)-pathway in mesangial cells

To further elucidate the signal transduction pathways by which $sPLA_2$-IIA and TNFα increase CPT-Iα expression, the role of mitogen-activated kinase ERK-1/2 (MAPK) was tested. MAPKs are known to play an important role in activation of $cPLA_2$ through phosphorylation and targeted by specific low molecular mass inhibitors (English and Cobb, 2002). The $cPLA_2$ inhibition may lead to diminished formation of lipid mediators and $sPLA_2$-IIA in mesangial cells and may thus influence CPT-Iα expression. Mesangial cells were treated with 0.1 µM h-$sPLA_2$-IIA and 0.5 nM TNFα in the absence or presence of 1 µM U0126, which is a selective inhibitor of the MAPK kinase MEK, or in the presence or absence of 10 µM PD98059, a selective inhibitor of ERK-1/-2 (Favata *et al.,* 1998). These are typical inhibitory concentrations of both compounds.

As seen in Fig. 3. 21 A and B, the inhibition of the MAPK pathway caused a decrease in h-$sPLA_2$-IIA- and TNFα-induced CPT-Iα protein expression in mesangial cells. The 2.1-fold TNFα-induction of CPT-Iα protein expression was abolished to 1.7-fold with PD 98059 and to 1.5-fold with U0126, and 2.5-fold $sPLA_2$-IIA-induction was abolished to 1.8-fold with PD 98059 or U0126. These results demonstrated that the reduction of TNFα-mediated CPT-Iα protein increase, by inhibition of the MAPK pathway, does not occur to the level in the control group. This suggests that the constitutively expressed basal CPT-Iα protein seems to be rather stable and is not under the control of the MAPK pathway in mesangial cells.

Interestingly, both inhibitors alone caused an increase in CPT-Iα protein expression (U0126 weakly: 1.2-fold, and PD 98059 more pronounced: 1.8-fold).This could be a result of the long-term inhibition of the MAPK pathway for 24 h, which may affect further critical steps in protein biosynthesis in general.

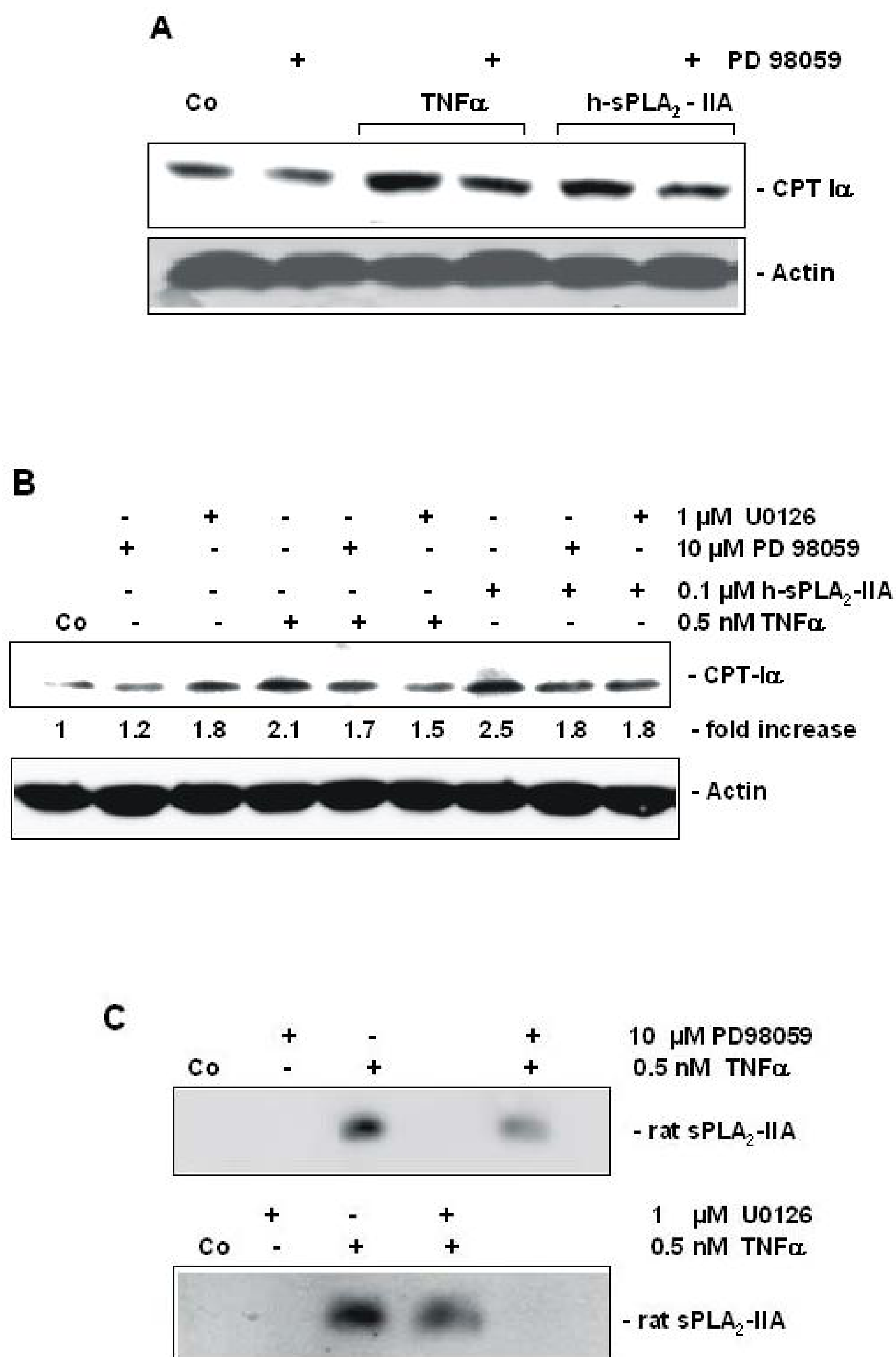


Fig. 3. 21 Effect of the MAPK inhibitors U0126 and PD98059 on h-sPLA$_2$-IIA and TNFα - induced CPT-Iα protein expression.

Mesangial cells were treated for 24 hr with h-sPLA$_2$-IIA (0.1 µM) and TNFα (0.5 nM) in the absence or presence of 10 µM PD98059 **(A)**. Mesangial cells were treated for 24 hr with sPLA$_2$-hIIA (0.1 µM) and TNFα (0.5 nM) in absence and presence of 10 µM PD98059 or 1

µM U0126 **(B)**. 1 ml of the cell culture supernatants were taken for rat $sPLA_2$-IIA protein detection by Western blot analysis as described under 2.2.5. **(C)**.
100 µg protein of the cell lysate were subjected to SDS-PAGE (10% PAGE), and Western blot analysis was performed with an antibody against CPT-Iα and subsequently with actin for protein loading control. Protein samples form supernatants were subjected to SDS-PAGE (15 %), and protein levels were detected with anti-rat- $sPLA_2$-IIA antibody by Western blotting.

Induction of the expression and secretion of $sPLA_2$-IIA in mesangial cells by cytokines has been extensively reviewed (Schalkwijk, *et al.,* 1991; Scholz, *et al.,* 1999; Scholz-Peretti, *et al.,* 2002). In this study the MAPK inhibitory effect of U0126 and PD98059 on TNFα-induced $sPLA_2$-IIA enzyme secretion was monitored. The cell culture supernatants of mesangial cells treated with both inhibitors were analysed for $sPLA_2$-IIA protein by Western blotting with an anti-rat $sPLA_2$-IIA antibody as described in 2.2.4.2.
As seen in Fig. 3. 21 C, rat $sPLA_2$-IIA protein level was detectable in the cell culture supernatants of TNFα-stimulated mesangial cells, as described in the previous studies, and was used here as positive control. A coincubation of mesangial cells with TNFα and the inhibitors resulted in a decrease of secreted rat $sPLA_2$-IIA protein level.
In summary, these results suggest that extracellular $sPLA_2$-IIA stimulates an enhanced expression of CPT-Iα, probably via endogenous lipid metabolites, which were either produced directly or generated via $cPLA_2$, and that this involves MAPK activation.

3.7 Regulation of mHMG-CoA synthase mRNA expression by exogenous $sPLA_2$s and TNFα in rat mesangial cells

Additionally to CPT-Iα, several authors suggest that mitochondrial 3-hydroxy 3-methylglutaryl coenzyme A synthase (mHMG-CoA synthase), the enzyme that catalyses formation of HMG-CoA in mitochondria, is another important site for control of ketogenesis (Ayte *et al.,* 1990). It has been shown that fatty acids induce the transcription of the gene for mHMG-CoA synthase both *in vivo* and in *vitro*
(Hegadt, 1999) and this induction is meditated by PPARα, which binds to PPRE located in the mHMG-CoA synthase promoter (Rodriguez *et al.,* 1994). With

thibackground, we investigated the role of exogenously added $sPLA_2$s as well as TNFα on mHMG-CoA synthase mRNA.

Results:

After stimulation of rat mesangial cells for 24 hr with TNFα or exogenous $sPLA_2$s from different species (see 2.1.4.1) in absence or presence of TNFα, the mHMG-CoA synthase mRNA expression was analysed by RT-PCR as described in 2.2.3. $sPLA_2$s stimulated a marked increase in mHMG-CoA synthase mRNA expression, only after costimulation of $sPLA_2$s with TNFα (Fig. 3.22). Neither TNFα or the different $sPLA_2$s alone had an inducing effect.

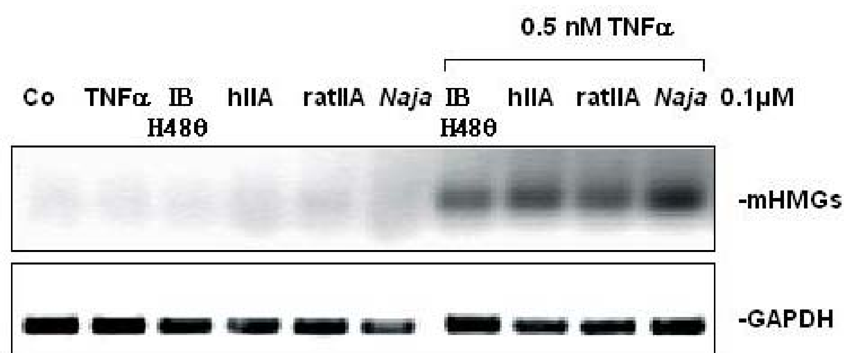


Fig. 3. 22 Effect of h-$sPLA_2$-IIA and TNFα on mHMG-CoAs mRNA expression.

Mesangial cells were treated for 24 hr with 0.5 nM TNFα and h-$sPLA_2$-IIA at the concentrations of 0.1 µM in the presence or absence of 0.5 nM TNFα. Total RNA was isolated and mHMG-CoA synthase mRNA expression was analysed by performing RT-PCR as described under 2.2.4.2.

While CPT-Iα mRNA expression was enhanced after stimulation with h-$sPLA_2$-IIA or TNFα as single agents compared to control cells, the mHMG-CoA synthase mRNA level was not altered under these conditions. On the other hand, mHMG-CoA synthase mRNA level was markedly increased by the costimulation of h-$sPLA_2$-IIA with TNFα. These preliminary results suggest that under pathophysiological conditions, cytokines act in concert with extracellular $sPLA_2$s to induce mHMG-CoA synthase as an important enzyme for the metabolism of surplus free fatty acids to ketone bodies.

The analysis of HMG-CoA synthase protein is not possible because of the lack of specific antibodies for this enzyme. In future studies, the analysis of ketone bodies will be performed as a read out for an increased enzyme expression.

IV

Discussion

4.1 Characterisation of CPT-Iα antibody

Of the two isoforms of CPT-I (CPT-Iα and CPT-Iß), only isoform CPT-Iα is expressed in most tissues including liver and kidney. CPT-Iα is not detectable in skeletal muscle or brown adipocytes, whereas CPT-Iß is the predominant isoform in these tissues. This knowledge, together with the fact that this enzyme is located on the mitochondrial inner membrane, was used in the present study to characterise the specificity of the available anti-CPT-Iα antiserum. The antiserum (provided by Prof. Pfeilschifter) was raised in rabbit against an epitope of liver isoform CPT-Iα, which is not expressed on the CPT-Iß isoform.

To confirm that the antiserum recognises CPT-Iα in rat mesangial cells and primary hepatocytes, Western blot analysis was performed on protein from cell lysates of these cells. As negative control, skeletal muscle cells were analysed. The specificity of the antiserum was proved by the following facts: 1) a major band of 88 kDa, which corresponds to the molecular mass of rat CPT-I (McGarry and Brown, 1997; Esser *et al.,* 1993), was observed in the blots; 2) antibody raised against rat liver isoform CPT-Iα recognises this protein in rat primary hepatocytes and mesangial cells but not in skeletal muscle, where only CPT-Iß isoform is expressed. Furthermore, much less protein from hepatocytes was needed for optimal detection by Western blotting (40 µg) in comparison to mesangial cells, where 100 µg protein was required. This suggests that mesangial cells express lower levels of the CPT-Iα than hepatocytes. These results are in accordance with the fact that the liver is the most important central metabolic site which provides a major source of energy from mitochondrial fatty acid oxidation and thus, much higher CPT-Iα protein level are required. Further evidence for the specificity of the CPT-Iα antiserum was a Western blot analysis of protein from the membrane fraction of the cell lysates. As

expected, the CPT-Iα was only detectable in the membrane fraction, which included mitochondrial membrane proteins.

4.2 Stability of CPT-Iα protein

There are few studies characterising the CPT-I mRNA or protein stability, this stability significantly influence the action of various agents on the protein level and subsequently on FA homeostasis in the cells. For instance, it was shown that the protein synthesis inhibitor cycloheximide (CHX) inhibits the effect of FAs on CPT-I mRNA while it has no effect on its constitutive expression as it was analysed in the ß-cell line INS-1 after 6 hr incubation (Assimacopoulos-Jeannet *et al.,* 1997). Thus, the CPT-I gene was characterised in INS-1 cells as an early-response gene whose induction is depended on the action of pre-existing factors and does not require *de novo* protein synthesis. Nothing is known about stability of CPT-Iα protein in rat mesangial cells and primary hepatocytes. In the present work, the incubation of mesangial cells for 2-24 hr and hepatocytes for 2-8 with CHX did not change CPT-Iα expression levels indicating a slow turn-over and/or a long half-life of the protein. Moreover, *de novo* protein synthesis is required for the observed upregulation after stimulation of these cells under inflammatory conditions (see Chapter 4.5) as the enhanced CPT-Iα level through sPLA$_2$-IIA was slightly decreased by the treatment with CXH in mesangial cells. The high stability of CPT-Iα protein in mesangial cells and hepatocytes might be very important in terms of FA homeostasis. This also indicates that the smallest changes in the CPT-Iα level, for example in inflammation, could lead to significant changes in metabolism, which in turn influence adaptation, survival or death of the cell.

4.3 Effect of NO on CPT-Iα expression

Among the many metabolic effects of NO, its role in modulation of FA metabolism has been demonstrated. The previous study by Gans, 2003 has shown that exogenously added NO donors such as DETA-NO were able to upregulate CPT-Iα mRNA and protein expression in mesangial cells. Most importantly, it was shown that endogenously produced NO, which appears in high amounts after IL-1ß-stimulated induction of iNOS (Kunz *et al*., 1994), is involved in an enhanced CPT-Iα

expression. The study presented here (also examined) the NO-mediated regulation of the CPT-Iα promoter activity in mesangial cells.

4.3.1 Regulation of the CPT-Iα promoter by NO

An approximately 2-fold increase in the CPT-Iα promoter activity was found after DETA-NO- and IL-1ß-stimulation of mesangial cells transfected with the CPT-Iα -4495/+19 promoter construct. In addition, the transfected mesangial cells were stimulated with palmitate as a positive control, which induced the activity of CPT-Iα -4495/+19 promoter construct 2-fold (For further discussions concerning palmitate action on CPT-Iα promoter see in "Effects of secretory Phospholipases A_2", Chapter 4.5). Similar activation was obtained after stimulation with DETA-NO, IL-1ß, or palmitate in mesangial cells transfected with -4495/+1240 containing the first intron (data not shown). These results suggested that (i) these two promoter constructs, without (-4495/+19) and with intron (-4495/+1240), might contain elements important for the NO-mediated promoter activation, and that (ii) the accumulation of CPT-Iα mRNA and protein levels might be the result of an increase in the transcription rate. Further studies including serial deletion of the promoter and/or site-directed mutations, are necessary to define regions within CPT-Iα promoter required for the gene induction.

The CPT-Iα promoter is TATA-less, and basal expression is driven by transcription factor Sp1 and nuclear factor NF-Y (Steffen *et al.,*1999). Thus, it is similar to the promoters of other acyl transferases and enzymes involved in fatty acid oxidation. For instance, the medium-chain acyl-CoA dehydrogenase promoter is TATA-less with Sp 1 playing a prominent role in basal expression. The characterisation of the CPT-Iα promoter and identifying elements that regulate gene expression by NO and cytokines might lead to an understanding of the regulation of other renal acyltransferases and FA metabolism under inflammatory conditions.
Moreover, to assess whether NO modifies CPT-Iα mRNA and protein stability, the half-life of CPT-Iα mRNA and protein should be determined after NO-donor or cytokine stimulation in the presence of the transcription inhibitor actinomycin D and inhibitor of protein synthesis CHX. To directly address whether changes in CPT-Iα expression induced by NO can result in a switch of substrate utilisation, the FFAs analysis and their oxidation rate in mesangial cells should be further investigated.

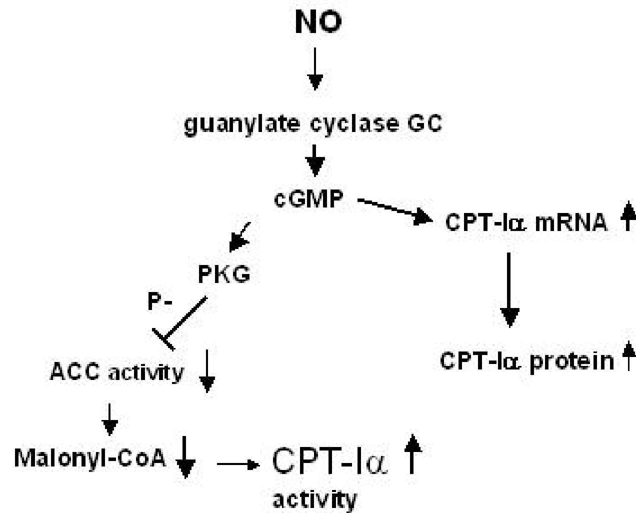
4.3.2 Role of cGMP in the regulation of CPT-Iα

It is well known that many of the biological actions of NO, but not all, are mediated by the direct activation of soluble guanylyl cyclase (sGC) and the consequent increase in intracellular cGMP levels (Lucas *at al.,* 2000). cGMP may modulate PKG, cGMP-gated ion channels, cGMP-regulated phosphodiesterases, and cyclic AMP-dependent protein kinases (Lucas *at al.,* 2000). These are considered to be early and rapid mechanisms of NO signalling. However, the late phases which include such intercellular changes as release of secretory PLA_2 (Rupprecht *et al.,* 1999) or regulation of lipid metabolites (Huwiler and Pfeilschifter, 2003) are mediated by alterations in gene expression (Pfeilschifter *et al.,* 2001a; Pfeilschifter *et al.,* 2001b).

Recently, it was shown that in rat hepatocytes NO donors inhibited acetyl-CoA carboxylase (ACC) activity, the rate-limiting enzyme in the fatty acid biosynthesis pathway, which catalyses the formation of a potent CPT-Iα inhibitor malonyl-CoA. This inhibiton occurs via phosphorylation of ACC through cGMP-dependent protein kinase. Due to ACC inhibition by NO donors an increase in CPT-Iα activity was detectable (Garcia-Villafranca *at al.,* 2002).

In the present study, the involvement of NO/cGMP signaling pathway was further investigated at the CPT-Iα mRNA and protein expression level. The treatment of mesangial cells with ODQ as an inhibitor of the sGC reduced the inducible effect of NO donor DETA-NO on CPT-Iα mRNA and protein expression. In addition, further experiments have shown that similar to DETA-NO, the sGC activator YC-1 was effective in induction of CPT-Iα protein level. These results show that the NO/cGMP pathway, at least in part, is crucial not only for the stimulation of CPT-Iα activity through inhibition of ACC and reduction of malonyl-CoA synthesis, but also for a direct induction of CPT-Iα mRNA and protein expression. Furthermore, similar to rat hepatocytes, in mesangial cells NO seems to mediate its action on CPT-Iα activity through the activation of sGC and production of intracellular cGMP. Thus, this combination might result in a dual control of CPT-Iα and fatty acid oxidation by increasing both the amount as well as the activity of the enzyme (Fig. 4.1). Both CPT-Iα induction and the downregulation of ACC and decrease in FA synthesis by

the NO/cGMP pathway, with a possible reduction in malonyl-CoA formation could participate in the stimulation of FA oxidation and presumably of ATP production.



Carcia-Villafranca J. *et al.,* (2003)

Figure 4.1 Schematic diagram of the involvement of NO/cGMP signalling pathway in the regulation of CPT-Iα activity, mRNA and protein expression.

These results open the possibility that also *in vivo,* the NO/cGMP pathway may play a role in the control of lipid metabolism in liver and kidney. This knowledge could lead to a better understanding of the regulation of hepatic and renal metabolism and the mechanisms involved in inflammatory diseases of the liver and kidney where NO is drastically increased.

4.3.3 Effect of proinflammatory cytokines on CPT-Iα expression in rat primary hepatocytes

In the present study, I investigated whether NO upregulates the CPT-Iα protein and mRNA levels in rat hepatocytes. For this propose, rat primary hepatocytes were stimulated with DETA-NO for 24 hr. It was observed that the expression of CPT-Iα protein and mRNA was not altered at the concentration range of 0.1-0.5 mM. The same results were obtained at higher concentration (up to 1mM) and for earlier time points. Moreover, the stimulation of hepatocytes with IL-1ß did not cause any changes neither in endogenous NO production through activation of iNOS nor in CPT-Iα expression (data not shown). In contrast, the release of rat-sPLA$_2$-IIA as a proinflammatoy enzyme was markedly elevated in supernatants from the

hepatocyte cell culture stimulated with DETA-NO in dose-dependent manner suggesting that hepatocytes responded to the treatment with the NO donor during the time course of 24 hr.

There are a number of studies carried out in primary rat hepatocytes, which have shown the role of cytokines in stimulation of iNOS and NO production. For instance, Sewer and Morgan (1997) demonstrated that iNOS mRNA and subsequently NO release was induced after 24 hr incubation with pro-inflammatory agents such as IL-1ß, TNFα and LPS. However, in this study the rat hepatocytes were cultured in medium and plating conditions different from those used in the present work (in Sewer and Morgan's experiments, the primary hepatocytes were cultured for 5-7 days with Waymouth's medium). Thus, the apparent discrepancies in endogenous NO induction through cytokine could be attributed to the culture conditions.

Furthermore, the regulation of CPT-Iα expression might require much longer stimulation time, and thus, experiments *in vivo* could be more suitable to study long-term regulatory effect of NO donors. The model in our study has its limitations as the survival time of hepatocytes as primary cell cultures was rather short. Hepatocytes loose their differentiated phenotype after 2 days of culture, and the cells tend to detach from the culture dishes. The reason for this degenerative course is uncertain, but three factors must principally be considered to be of importance, namely, the absence of other liver cells, the matrix used for plating the cells and the composition of the medium (Dich *et al.,*1988). Despite this, in the present study the primary hepatocyte cultures were suitable for investigation of short-term regulatory effects on CPT-Iα regulation.

4.4 Hypoxia

4.4.1 Regulation of CPT-Iα by hypoxia

Hypoxia induces transcriptional regulation of a wide variety of genes encoding proteins necessary for cellular and physiological adaptation, including alterations in metabolic events and energy consumption. For example, several genes encoding glycolytic enzymes are activated leading to a switch from ß-oxidation to glycolysis. While strong evidence exists that the hypoxia-induced activation of glycolytic enzymes is mediated by the HIF-Iα (Semenza *et al.,* 1994; Semenza *et al.*, 1996),

no information is available on the mechanisms involved in the modulation of FA metabolism and expression of lipolytic enzymes such as CPT-Iα or mHMG-CoA synthase.

In the present work, the effect of hypoxic conditions on CPT-Iα mRNA and protein expression in the rat mesangial cells and hepatocytes was investigated.
In the first series of experiments it was shown that the incubation of mesangial cells with $CoCl_2$, which mimics hypoxia, leads to an increase in CPT-Iα mRNA and protein expression. With 100 µM $CoCl_2$, the CPT-Iα mRNA level already started to increase after 2 hr and remained elevated up to 24 hr. The treatment for 24 hr with concentrations of $CoCl_2$ between 50 and 200 µM showed a dose-dependent induction. The incubation with higher concentration (400 µM) caused a slight decrease, and the incubation with 100 µM $CoCl_2$ for longer time (between 24 and 48 hr) resulted in a decrease in mRNA levels which could be explained by the toxicity and apoptotic effect of $CoCl_2$ (Kakinuma, e*t al.,* 2001; Araya *et al.,* 2002). In contrast to the mRNA level, CPT-Iα protein accumulated in a manner independent of the concentrations used.

An even more greater difference between effects of $CoCl_2$ on protein and mRNA level was seen in the next series of experiments performed with rat primary hepatocytes. Here, the treatment with 200 µl $CoCl_2$ caused a significant induction of CPT-Iα protein, while CPT-Iα mRNA level decreased. The reason for this discrepancy between mRNA and protein expression is unknown. One explanation might be that in renal cells of hypoxic rats, swelling, rupture of inner and outer membranes, and leakage of mitochondrial matrix are induced (Benitez-Bribiesca *et al*., 2000). Since CPT-Iα is located in the mitochondrial outer membrane, the isolation and detection of CPT-Iα protein by Western blot analysis may be complicated because of these ultrastructural changes of mitochondria which may also have occurred in the experiments with rat mesangial cells and hepatocytes.

While exposure to hypoxia led to the clear accumulation of CPT-Iα protein in both the mesangial cells and hepatocytes, the upregulation of detectable CPT-Iα mRNA in the mesangial cells, was not seen in the hepatocytes. This difference in mRNA expression between mesangial cells and hepatocytes might mirror the tissue

specificity described in an earlier study, which demonstrates that regulatory factors of CPT-Iα expression such as development, hormones and diet have different effects in liver and heart (Cook *et al.,* 2001).

These results showed that CPT-Iα in mesangial cells and hepatocytes is affected by the hypoxia mimicking $CoCl_2$. However, the treatment with $CoCl_2$ triggers not only hypoxia and activation of HIF-Iα or HIF-2α, but also other physiologically significant signalling systems. For instance, $CoCl_2$ increases expression of heat shock proteins hsp 65 and hsp 72 in keratinocytes (Nordlind, 2002). Furthermore, $CoCl_2$ induces apoptosis via mitochondrial pathway thereby releasing cytochrome c and formation of highly reactive oxygen radicals, which may contribute to mitochondrial damage (Clyne *et al.,* 2001; Araya *et al.,* 2002). Therefore, the treatment with $CoCl_2$ has many other effects, which are not related to hypoxia but may even dominate the hypoxia effects.

Based on this information, in the next series of experiments cell culture incubation was carried out using a gas mixture containing low percentage of oxygen (1% or 3%). These experiments were done in comparison to the treatment with $CoCl_2$ and the iron chelator DFO. The results show that the CPT-Iα mRNA and protein levels were increased in response to hypoxia (3% O_2) as well as to $CoCl_2$, or DFO in the mesangial cells. This result suggests that similar mechanisms are involved in these three models of hypoxia-mediated activation of CPT-Iα expression in the mesangial cells.

Because many initial observations showed that hypoxia results in inhibition of FA oxidation, the further investigation should be performed to determine the relationship between the CPT-Iα expression and the rate of ß-oxidation under hypoxic condition. Despite of the influence of hypoxia on CPT-Iα expression, the rates of ß-oxidation during hypoxia might be affected by several steps involved in fatty oxidation including the cellular uptake, activation of FA or enzymes of the ß-oxidation spiral.

By using hepatocyte culture conditions with a lower percentage of oxygen (1%) the clear downregulation of CPT-Iα mRNA expression was achieved which is consistent with observations of previous studies showing a decrease in FA-oxidation and a switch to glycolysis as source of energy during hypoxic condition

(Huss, *et al.,* 2001). The downregulation of CPT-Iα mRNA level through exposure to more pronounced hypoxia (1% O_2) and, on the other hand, induction in response to $CoCl_2$ and DFO suggest that different regulatory mechanisms of both forms of hypoxia might be responsible for the differences in CPT-Iα expression in hepatocytes.

Moreover, these preliminary experiments show that alterations in the oxygen content of the gas mixture (1%-3% O_2) may trigger different effects on CPT-Iα level. Therefore, further studies are needed to explore the hypoxia-mediated effects on CPT-Iα expression in relation to the oxygen content.

Several mechanisms could be responsible for the regulation of CPT-Iα under hypoxic condition. A previous study has shown that in cardiac myocytes, hypoxia suppresses up-regulation of CPT-Iß in response to LCFA via PAPRα/RXR pathway, where the nuclear protein level of RXRα is reduced and thereby, the LCFA-induced transcription of CPT-Iß gene is decreased (Huss *et al.,* 2001). It could be predicted that signalling through RXR-dependent pathways would similarly affect CPT-Iα in mesangial or liver cells, as in these cells PPARα also plays a critical role in the regulation of gene expression (Scholz-Pedretti *et al.,* 2002, Lawrence *et al.,* 2001; Eberhardt *et al.,* 2000; Eberhardt *et al.,* 2002; Hsu *et al.,* 2001). Another mechanism that might regulate CPT-Iα is the HIF-dependent transcriptional change in gene expression. Computer analysis of the promoter sequence of CPT-Iα gene reveals a HRE consensus sequence (ACGTGC) located at -6245/-6239 bp upstream the start site of transcription, which could be able to bind HIF-Iα or HIF-2. To investigate the possible role of the putative HRE and PPRE in regulating CPT-Iα gene expression in mesangail cells and hepatocytes, further transfection experiments under hypoxic conditions should be carried out using constructs with mutations in the PPRE and HRE sites.

Taken together, the results show the complexity of CPT-Iα regulation during hypoxia, and leaving many open questions.

4.4.2 Regulation of mHMG-CoA synthase by hypoxia

In the next series of experiments, the expression of mHMG-CoA synthase mRNA was investigated in mesangial cells and hepatocytes under hypoxic conditions. As shown in Fig. 3.13, the exposure of mesangial cells to 3% O_2, $CoCl_2$ or DFO leads to a reduction of mHMG-CoA synthase mRNA expression. A similar decrease was observed in hepatocytes using 1% O_2, $CoCl_2$ or DFO. Interestingly, in hepatocytes 1% oxygen decreased mHMG-CoA synthase mRNA at an earlier time point (after 4 hr incubation) as compared to the chemical compounds. It was reported that allocation of acetyl-CoA to either ketogenesis or the Krebs cycle (a universal metabolic pathway in which the acetyl group of acetyl-CoA is oxidized to two CO_2, and four pairs of electrons are transferred to coenzymes) is controlled by the mitochondrial ATP concentration. Furthermore, an insufficient oxygen supply and ATP production reduce the level of ketone bodies in patients suffering from respiratory dysfunction (Tsubokawa *et al.,* 1998). Thus, the reason for the rapid response to hypoxia might be explained by a fast depletion of ATP production, which is strongly regulated by oxygen supply. Moreover, it is likely that a decrease in mitochondrial ATP production rate after exposure of the cells to $CoCl_2$ or DFO might be secondary to the changes in the activity and/or expression of factors in oxygen sensing (e.g. HIF-Iα). This might explain the delayed effect of the chemical compounds (after 24 hr) in comparison to environmental hypoxia (1% oxygen).

The observation of inhibitory effect of hypoxia on mHMG-CoA synthase mRNA expression suggests that ketone body building might also be decreased in mesangial cells and hepatocytes. To explore this hypothesis, the ketone body content in mesangial cells and hepatocytes exposed to hypoxia should be analysed in future studies. Moreover, nothing is known about the regulatory mechanism of hypoxia-mediated downregulation of mHMG-CoA synthase protein expression as there are currently no antibodies available for this protein.

4.5 Effects of secretory Phospholipases A_2

sPLA$_2$s, as extracellar and circulating enzymes, play an important role in direct or indirect stimulation of the release of arachidonic acid and other free FAs and prostaglandins under inflammatory conditions (Schalkwijk *et al.,* 1991; Pfeilschifter *et al.,* 1993; Pfeilschifter *et al.,* 1997) thereby providing a rapid as well as sustained

increase in substrate for the CPT-Iα. Moreover, it was speculated that a drastic increase in $sPLA_2$-released free FAs has an effect on CPT-Iα expression, as it is known from earlier studies that free FAs regulate the transcription of CPT-Iα in rat hepatocytes and another cell types (McGarry and Foster, 1980; Chatelain *et al.,* 1996).
Therefore, the effects of extracellular $sPLA_2$s on the CPT-Iα expression were investigated.

Furthermore, several diseases are associated with high levels of extracellular $sPLA_2$ which itself can induce significant increases in proinflammatory cytokines such as TNFα, IL-1 and IL-6 (Granata *et al.,* 2003) and chemokines (Beck GCh *et al.,* 2003). Moreover, initial observations demonstrated that TNFα and IL-1ß are potent inducers of $sPLA_2$ mRNA expression (Pfeilschifter *et al.*, 1997; Couturier *et al.*, 1999; Scholz-Pedretti *et al.,* 2002; Beck *et al.,* 2003). Together, cytokines and $sPLA_2$ potentiate each others synthesis, thereby creating an amplification loop for the progression of inflammatory responses. The role of extracellular $sPLA_2$s on CPT-Iα expression was completely unknown and was studied in the present work in rat mesangial cells and primary hepatocytes.

It was shown that in mesangial cells, exogenously added h-$sPLA_2$-IIA as well as TNFα enhanced CPT-Iα protein expression which was also potentiated by co-treatment with both agents. However, in rat primary hepatocytes the h-$sPLA_2$-IIA-stimulated increase in CPT-Iα protein expression was no longer potentiated by TNFα. This difference in regulation of CPT-Iα protein expression between mesangial cells and hepatocytes might be tissue specific.

Furthermore, it was investigated as to whether the $sPLA_2$ or TNF-α-induced increase in CPT-Iα was derived from de novo protein biosynthesis. The coincubation of TNFα or h-$sPLA_2$-IIA with the protein synthesis inhibitor CHX resulted in a slight decrease of CPT-Iα protein level in comparison with incubation with TNFα or h-$sPLA_2$-IIA alone, suggesting that TNFα or h-$sPLA_2$-IIA stimulate CPT-Iα protein synthesis. Interestingly, in this experiment CHX alone induced CPT-Iα protein expression. One could speculate that CHX inhibits the protein synthesis

of a regulatory protein working as a repressor of CPT-Iα. Its disappearance may then lead to an increased synthesis of CPT-Iα protein.

The stimulatory effect of h-sPLA$_2$-IIA as well as of further exogenously added sPLA$_2$s and TNFα was also demonstrated in mesangial cells at mRNA level. Induction of CPT-Iα mRNA expression with these sPLA$_2$s was potentiated by costimulation with TNFα, which correlates with the increases in CPT-Iα protein.

The mode of sPLA$_2$s action on CPT-Iα expression is not clear. One possibility may be an activation of PPARα via the released lipid metabolites. As natural ligands of PPARα (Lee *et al.,* 2003, Corton *et al.,* 2000), fatty acids and eicosanoids may activate CPT-Iα gene expression by recognition of PPRE exhibited in the proximal CPT-Iα promoter (-2859/-2846) (Louet *et al.,* 2001[a; b]). Thus, in mesangial cells transfected with two different CPT-Iα promoter constructs (-4495/+1240 containing first intron, or -4495/+19) containing PPRE, exogenous sPLA$_2$s and TNFα as well as palmitate and the PPARα activator WY14.643 increase the promoter activities up to 2-fold, whereas the -210/+19 promoter construct was not activated. The -210/+19 promoter construct does not include PPRE but contains binding sites for other transcription factors such as CCAAT box element, NF-Y and Sp1 sites, which as shown in a previous study, regulates basal transcription of the CPT-Iα promoter (Steffen *et al.,* 1999).

In contrast to the results at protein and mRNA level, the costimulation of h-sPLA$_2$-IIA with TNFα has no potentiating effect on the promoter activity, suggesting that either both compounds use the same transcription factors to activate CPT-Iα transcription, or that the additive effect of their combination at the protein level may occur through a post-transcriptional mechanism.

To support the hypothesis that PPARα might be involved in the induction of CPT-Iα gene expression by FAs released through exogenously added sPLA$_2$s and also by rat sPLA$_2$s endogenously produced after cytokine treatment, further studies have to be done. First, FAs, which are specifically released by sPLA$_2$s and cytokines in mesangial cells and hepatocytes and which might be active in the regulation of gene transcription, should be characterised. Next, it must to be confirmed that these

FAs could act as PPAR ligands. Therefore, rat CPT-Iα promoter construct containing mutated PPAR binding sites should be cloned and the effects of exogenously added $sPLA_2$s and TNFα on the promoter activity should be analysed in the absence or presence of the functional PPAR binding sites.

Until recently, it was generally thought that FA regulation of gene transcription was a simple PPAR-mediated process. However, recent publications show that various sensors can relay the transcriptional effects of FAs either through direct binding to DNA (PPAR, liver x receptor LXR, hepatic nuclear factor 4 HNF-4α) or via modulations in the abundance of other transcription factors (sterol regulatory element binding protein SREBP, carbohydrate responsive element binding protein ChREBP) in an indirect manner. The list of potential transcription factors involved in FA-mediated gene expression is probably not yet complete (Pégorier *et al.,* 2004). For example, it was shown that a DNA sequence responsible for FA-induced CPT-Iα gene expression in rat hepatocytes was located in the first intron of the gene and analysis of the intronic sequence reveals no consensus sequences, suggesting that other transcription factors could transduce the FA signals to DNA (Louet *et al.,* 2001). In contrast in this study, luciferase activity in mesangial cells transfected with -4495/+19 promoter construct, which did not include the first intron, was stimulated with palmitate (as well as $sPLA_2$s and TNFα) in the similar manner as it was seen with cells transfected with the promoter construct containing first intron. This discrepancy with previous studies could be due to the tissue specificity of the FAs in the regulation of gene expression.

Exogenously added $sPLA_2$ and cytokines, such as IL-1ß and TNFα, enhance the activity of cytosolic PLA_2 ($cPLA_2$). $cPLA_2$ mainly regulates AA release from membrane phospholipids (Pfeilschifter J, 1989; Pfeilschifter J, 1994), which is the rate-limiting step in the formation of prostaglandins. Lipid metabolites produced by the action of $cPLA_2$ can activate $sPLA_2$ gene expression and its secretion and thus, potentiate the release of a broad spectrum of free fatty acids under inflammatory conditions (Beck *et al.,* 2003). This feed back regulation between exogenously added and cytosolic phospholipases and thereby potentiated release of FAs might be associated with amplification of $sPLA_2$ and cytokine effects on CPT-Iα activation.

The mechanism of such cross-talk between PLA_2s remains poorly understood. But there is evidence that activation of $cPLA_2$ through $sPLA_2$s and cytokines occur, at least partly, by a phosphorylation of $cPLA_2$ through mitogen-activated protein kinase (MAPK) (Huwiler e*t al.,* 1997; Mounier e*t al.,* 2004). Thus, the regulation of CPT-Iα expression by stimulation with $sPLA_2$s and cytokines may also involve the activation of the MAPK pathway (Fig 4.2). Indeed in rat mesangial cells, MAPK inhibitors PD 98059 and U0126 were able to block the enhancing effect of h-$sPLA_2$–IIA and TNFα on CPT-Iα protein expression. Moreover, in the same experiment the TNFα-induced secretion of endogenously expressed rat-$sPLA_2$-IIA was significantly blunted by PD 98059 and U0126 in mesangial cells. The results of this study suggested that activation of CPT-Iα by $sPLA_2$ and TNFα might involve the MAPK pathway, however, further investigation of this pathway in the regulation of FA metabolism are necessary. A number of conditions such as inflammation which evokes metabolic stress are known to activate MAPK and the outcome of studies with MAPK inhibitors, which prevent the action of $cPLA_2$ and $sPLA_2$s on metabolic process, might be of significant interest.

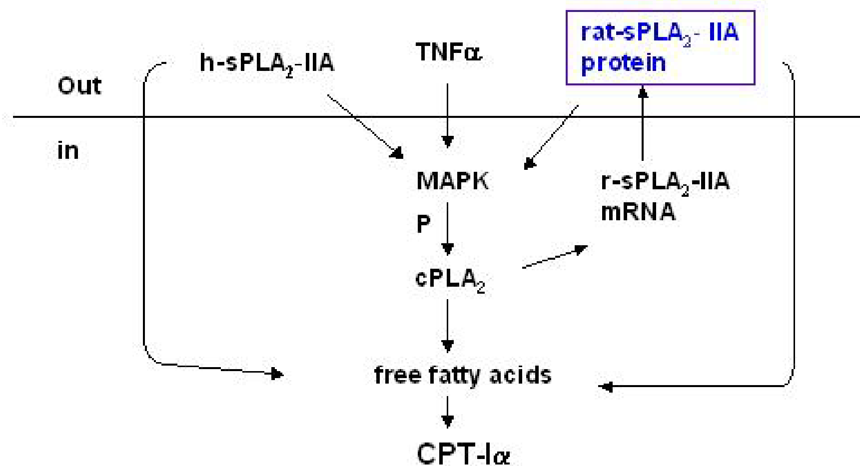


Figure 4.2 Schematic diagram of the contribution of the MAPK signalling pathway to the regulation of CPT-Iα expression by $sPLA_2$s and TNF.

It is well known that ketone body synthesis and activation of mHMG-CoA synthase expression, which catalyses rate-limiting steps of ketogenesis pathway, is increased by stimulation with FAs via PPRAα (Rodriguez *et al.,* 1994). Therefore, it was investigated whether enhanced FAs levels produced by $sPLA_2$s and TNFα

affects the mRNA expression of mHMG-CoA synthase. The results show that after treatment of mesangial cells with these agonists, the mRNA expression of mHMG-CoA synthase displayed a different pattern in comparison to CPT-Iα (3.22). The mRNA mHMG-CoA synthase was dramatically upregulated through various exogenous $sPLA_2$s only by costimulation with TNFα, suggesting that regulation of this protein requires not only $sPLA_2$ activity but also TNF-α-activated signalling pathways. To study, whether the increase in mHMG synthase mRNA level is accompanied by increased gene transcription, protein expression and enzymatic activity, further studies at the promoter and protein level or determination of ketone body formation are required.

The control of ketogenesis by substrate availability (acetyl-CoA) after entry of FAs into mitochondria, which is regulated by CPT-Iα, has been extensively reviewed (McGarry and Braun 1997; Hegardt, 1999). Therefore, the increase in the mHMG-CoA synthase mRNA level may be, in part, secondary to increased CPT-Iα expression. It might be speculated that the enhanced CPT-Iα gene and protein expression induced by $sPLA_2$s and TNFα contribute to ß-oxidation and production of acetyl-CoA as a substrate for mHMG-CoA synthase and thus, stimulate the ketogenic pathway. To prove this hypothesis it would be necessary to study the effect of specific CPT-Iα inhibitors such as etomoxir (Lopaschuk *et al.,* 1988)

The data indicate that under inflammatory conditions, extracellular $sPLA_2$ activity might be a potent regulator of lipid and energy homeostasis because $sPLA_2$:
a) produces increased amounts of free FAs and their metabolites (as it is known from previous studies); b) enhances CPT-Iα expression and thereby increases the transport of FAs to the mitochondria for oxidation; c) activates the mHMG-CoA synthase mRNA expression and presumably ketogenesis. In addition, it would be beneficial to support these data with measurements of FAs metabolites and high energy phosphates (ATP, ADP) or by evaluating the rate of fatty acid oxidation in mesangial cells and primary hepatocytes.

4.6 Clinic relevance

Regulation of the gene expression of CPT-Iα or mHMG-CoA synthase which are involved in lipid metabolism is of significant physiological and clinical interest and should be considered in drug development and therapeutic practise in the treatment of diseases such as hyperlipidemia, atherosclerosis, and obesity. It is well known that an inhibition of the carnitine palmitoyltransferase system and consequently, the transport of long-chain fatty acids across mitochondrial membranes by certain medication or pathology (e.g. ischemia/hypoxia, inflammation) may lead to dramatic changes in energy homeostasis and thus culminate in life threatening conditions. For example, the therapy with adriamycin (ADR), which inhibits the CPT system, leads to ADR toxicity manifesting in cardiomyopathy as a result of globally impairment of fatty acid oxidation (Yoon *et al.,* 2003;). Furthermore, determination of CPT-Iα as the rate-limiting step of ketogenensis (in addition to mHMG-CoA synthase) led to the observation that inhibition of this enzyme may cause a significant downregulation of mHMG-CoA synthase and depression of ketone body production, a major substrate for extrahepatic organs such as the brain. The nervous system has one of the highest rates of oxygen metabolism in the body and lacks tissue oxygen stores, depending on a continuous exogenous supply of oxygen from the liver (Blazquez *et al*., 1999).

Fatty acids have been identified as one of the main energy substrates used by the mammalian kidney. Mitochondrial fatty acid ß-oxidation, which allows high yields of ATP production, plays an essential role in supporting kidney reabsorptive functions, as evidenced by the effects of mitochondrial fatty acid utilisation blockers, which induce marked impairments in proximal tubule reabsorption (Wirthensohn and Guder, 1983). Also, the activation of CPT-Iα in liver might be of critical importance because of an increased FA oxidation and ATP production, which is necessary for proteins used in killing of pathogens and repair mechanisms in inflammatory renal and hepatic diseases. Thus, modulation of the expression of CPT-Iα might have an important role in preservation of kidney and liver during inflammation.

The next important question concerns involvement of CPT-Iα and mHMG-CoA synthase in FAs degradation. Elevated level of free FAs through induction of $sPLA_2$s during inflammation (Murakumi and Kudo 2004) might potentiate

pathological process. Some diseases such as atherosclerosis, diabetes or certain forms of cancer are associated with the enhanced amount of the FAs which secondarily contribute to their effects on phospholipid composition of membrane or on the expression of specific genes.

For instance, it was shown that a chronically elevated level of free FA can cause apoptosis of pancreatic ß-cells as a result of the formation of ceramides, which induce nitric oxide-dependent cell death (Cnop *et al.,* 2001; Schimabukuro *et al.,* 1998). Furthermore, recent studies show that alcohol-mediated accumulation of free FAs potentiate liver injury and fatty liver (Fischner *et al.,* 2004). Thus, from the effects of $sPLA_2$s on CPT-Iα and mHMG-CoA synthase in renal mesangial cells and hepatocytes, it might be concluded that a decrease in fatty acids protects liver and kidney from lipid accumulation and further pathological consequences.

Further studies will help to delineate whether PPARα and MAPK might play an regulatory role in triggering the fatty acid utilisation by CPT-Iα under inflammatory conditions in organs such as kidney or liver which strongly depend on FAs metabolism to ensure their energy homeostasis. In this respect, the identification of effects of the PPAR and MAPK activators and/or specific inhibitors of these target molecules may lead to a better understanding of the complete role of PPARs and MAPKs pathway in the control of lipid metabolism (Guan and Breyer, 2001; Englisch and Cobb, 2002).

V

Summary

Energy substrate utilisation rates are regulated during development and in response to physiological and pathophysiological stimuli. Injury and inflammation result in marked changes in whole body lipid metabolism, including free fatty acid oxidation (Show *et al.,* 1989). The regulation of fatty acid oxidation is most dependent on the enzyme carnitine palmitoyl transferase-I (CPT-I), which exists in two isoforms, hepatic CPT-Iα and muscle CPT-Iß isoform, and which catalyses the initial reaction in the mitochondrial import of long-chain fatty acids. This is a tightly regulated step in the cellular fatty acid utilisation pathway to provide ATP. In concert with mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHMG-CoA synthase), CPT-Iα is also responsible for the production of ketone bodies, the alternative fuel for extrahepatic organs.

The aim of this thesis was to investigate the role of CPT-Iα under different inflammatory conditions, using pharmacologically active agents like nitric oxide donors, hypoxia-mimicking substances and exogenous phospholipases A_2.

This project is divided into four parts. The first part of the experimental work was devoted to the determination of the optimal Western blot conditions using a polyclonal antibody against CPT-Iα in rat mesangial and primary liver cells. The stability and concentration of CPT-Iα protein in these two cell cultures was also characterised.

In a previous thesis performed by Dr. Annette Gans (2003), a stimulating effect of nitric oxide on the CPT-Iα protein and mRNA expression in rat mesangial cells was described. Thus, in the second part of the work, based on this thesis, studies were carried out to investigate the effect of NO on the CPT-Iα promoter activity.

Moreover, a possible involvement of NO/cyclic GMP pathway on the regulation of CPT-Iα expression was explored.

The data show that NO is able to increase luciferase activity of -4495/+19 CPT-Iα promoter construct in mesangial cells correlating with its activation at protein and mRNA level obtained in previous study.

The NO-induced upregulation is reversed by the soluble guanylate cyclase sGC inhibitor, ODQ, at protein and mRNA level. Moreover, the sGC activator YC-1 was able to increase CPT-Iα protein level. These results suggest that in rat mesangial cells the cGMP pathway is possibly involved in the induction of CPT-Iα mRNA and protein expression through NO.

The third part of this project includes the investigation of the effect of hypoxic conditions on CPT-Iα and mHMG-CoA synthase in rat mesangial and primary rat liver cells.

Two models of hypoxic culture conditions were used in this study:

1) Exposure of the cells to the transition metal Co^{2+} or to the iron chelator desferrioxamine during normoxia.

2) Culturing cells in an air-tight chamber, which was infused by a gas mixture containing 3% or 1% O_2.

Hypoxia mimic Co^{2+} or desferrioxamine as well as 3% O_2 hypoxia were able to upregulate CPT-Iα protein and mRNA expression in rat mesangial cells. In rat hepatocytes Co^{2+} induced CPT-Iα at protein level and did not cause changes at mRNA level, whereas the expose to more severe hypoxia (1% O_2) markedly decreased and treatment with desferrioxamine increased CPT-Iα mRNA. Furthermore, Co^{2+} and desferrioxamine and 3% O_2 hypoxia were also able to decrease mHMG-CoA synthase mRNA level in rat hepatocytes. These results suggest that in regulation of CPT-Iα and mHMG-CoA in rat mesangial cells and hepatocytes by the above mentioned models of hypoxic culture conditions different regulatory pathways are affected.

The fourth part of the thesis was devoted to the effect of exogenously added secreted phospholipases A_2 (sPLA$_2$s) on CPT-Iα expression at the protein, mRNA and promoter level. This project was stimulated by a recent observation that sPLA$_2$s, when released under inflammatory conditions into the extracellular space, cleave fatty acids from glycero-phospholipids, which then may trigger an

upregulation of CPT-Iα expression. In addition, this part of the project includes preliminary studies on the molecular mechanisms of $sPLA_2$s on mHMG-CoA synthase expression.

The treatment with exogenously added $sPLA_2$s and TNFα, which are known to amplify expression of endogenous $sPLA_2$-IIA, led to induction of CPT-Iα at protein, mRNA and prompter level in mesangial cells and to protein level in hepatocytes.

This effect was reduced by co-treatment of mesangial cells with mitogen-activated protein kinase (MAPK) inhibitors PD 98059 and U0126, thus suggesting that $sPLA_2$s-mediated induction of CPT-Iα might, at least partly, involve MAPK pathway.

Moreover, the also mHMG-CoA synthase mRNA level was markedly enhanced by the costimulation of h-$sPLA_2$-IIA with TNFα.

In summary, this work demonstrated that the CPT-Iα is a carefully regulated protein that plays a critical role in response to inflammatory condition.

VI

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VII

Appendix

7.1 Abbreviations

AA	arachidonic acid
AMPK	AMP-activated protein kinase
BSA	bovine serum albumin
CHX	cycloheximide
CPT-I	carnitine palmitoyltransferase I
$cPLA_2$	cytosolic phospholipase A_2
DFO	disferrioxamine
FA	fatty acid
HIF-1α	hypoxia-inducible factor -1α
HMG-CoAs	3-hydroxy-3-methylglutaryl-CoA synthase
HRE	hypoxia-responsive element
HSPG	heparan sulfate proteoglycan
IL	interleukin
iNOS	inducible NO synthase
kbp	kilobase pair(s)
LCFA	long-chain fatty acid
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinese
mHMG-CoAs	mitochndrial mHMG-CoAs
NAD	nicotinamide
NF-κB	nuclear factor-κB
NF-Y	nuclear factor-Y
NO	nitric oxide
PAF	platelet-activating factor

PBS	phosphate-buffered saline
PIPES	piperazine-N,N'-bis(2-hydroxypropanesulfonic acid)
PKC	protein kinase C
PP	peroxisome proliferator
PPARα	peroxisome proliferator-activated receptor α
PPRE	PPAR response element
rMC	rat Mesangial cell
RT	room temperature (20-25°C)
RXR	retinoic X receptor
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sGC	soluble guanylyl cyclase
sPLA$_2$	secreted phospholipase A$_2$
TBS	Tris-buffered saline
TBST	Tris-buffered saline (TBS) supplemented with 0,05%Tween 20
TNFα	tumor necrosis factor-α
VEGF	vascular endothelial growth factor
WAT	white adipose tissue

7.2 Poster presentation

Eschenröder N., Gebhard B., Beck K.-B., Böhles H., Pfeilschifter J. and Kaszkin M.
Enhanced expression of carnitipalmitoyl transferase Ia (CPT-Iα) in rat mesangial cells by exogenous secreted phospholipase A2-IIA.
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7.4 Deutsche Zusammenfassung

Fettsäuren erfühlen vielfältige Funktionen im Organismus. Sie sind Brennstoffmoleküle, intrazelluläre Signalmoleküle und sie stellen wichtige Bestandteile biologischer Membranen dar.

Der Abbau von Fettsäuren findet im Mitochondrium statt. Da langkettige Fettsäuren nicht ohne weiteres die Mitochondrienmembran überwinden können, benötigen sie einen Transportmechanismus, das so genannte Carnitin-Palmitoyltransferase-System. Zu diesem System gehören die Carnitin-Palmitoyltransferase-(CPT)-I an der Aussenseite, und die CPT-II an der Innenseite der Mitochondrienmembran, welche beide maßgeblich am Transport der Acylgruppe der Fettsäuren in Form von Acyl-CoA in das Innere des Mitochondriums beteiligt sind. CPT-I ist bei diesem Transport der geschwindigkeitbestimmende Schritt und existiert in zwei Isoformen, eine vom hepatischen (CPT-Iα) und einem vom muskulären Typ (CPT-Iß).

Ziel dieser Arbeit war die Untersuchung der CPT-Iα in kultivierten Mesangiumzellen der Rattenniere und primären Hepatocyten der Rattenleber unter Bedingungen, die an der Entstehung und Progression entzündlicher Prozesse in diesen Organen beteiligt sind:

1) Hypoxie,

2) Stickstoffmonoxid (NO),

3) extrazelluläre sekretorische Phospholipasen A_2 ($sPLA_2$).

Die Wahl der o.g. Bedingungen wurde aus folgenden Gründe gewählt:

Viele Entzündungsreaktionen zeichnen sich durch eine massive Produktion von NO und einer erhöhten Sekretion von $sPLA_2$ sowie durch eine lokale Durchblutungsstörung des Gewebes aus, was zu einer Sauerstoff-Unterversorgung der Zellen führt. Die CPT-Iα ist zudem mit einem Schlüsselenzym der Ketogenese, der mitochondrialen Hydroxymethylglutaryl-CoA-Syntase (mHG-CoA-Syntase) gekoppelt. Daher wurde die Wirkung von Hypoxie und exogenen $sPLA_2$s auch auf die mHG-CoA-Syntase Expression untersucht.

Diese Arbeit ist in vier Teile gegliedert.

1.) Zunächst wurden optimale Bedingungen erarbeitet, die mit einem spezifischen Antiserum mittels Western-Blot-Analyse erfolgreich zur Detektion des CPT-Iα Proteins in den verwendeten Zellsystemen führten. Zudem wurde mit Hilfe des Proteinsynthese-Inhibitors Cycloheximid keine Abnahme der CPT-Iα

Proteinmengen festgestellt. Unter unstimulierten Bedingungen scheint dieses Protein demnach einem langsamen ‚turn-over' zu unterliegen.

2.) In einer früheren Arbeit in Mesangiumzellen wurde gezeigt, dass durch DETA-NO- und Interleukin 1ß (IL-1ß)-Stimulation freigesetztes NO einen stimulierenden Effekt auf die CPT-Iα-Protein und -mRNA Expression aufweist.

In der vorliegenden Arbeit wurde nun der NO-stimulierende Effekt auf den CPT-Iα–Promotor untersucht, um die Regulation der Transkription dieses Enzyms zu untersuchen. Es wurde festgestellt, dass sowohl DETA-NO als auch IL-1ß eine signifikante Steigerung der Promotor-Aktivität des -4495/+19 CPT-Iα Promotorkonstrukts induzieren, die mit dem Anstieg der mRNA- und Proteinexpression korreliert. Bezogen auf frühere Untersuchungen in Ratten-Hepatocyten zur Beteiligung der cytosolische Guanylatcyclase (cGC) und cyclischem Guanosinmonophosphat (sGMP) an der CPT-Iα Aktivität, wurde nun auch in Mesangiumzellen die Rolle des NO/cGMP-Signal-Weges in der Regulation des CPT-Iα untersucht. Es zeigte sich, dass die NO-induzierte CPT-Iα Expression durch den sGC-Inhibitor ODQ gehemmt werden konnte. Zudem induzierte YC-1 (ein sGC–Aktivator) die CPT-Iα Proteinexpression. Daraus konnte gefolgert werden, dass in Mesangiumzellen das unter proinflammatorischen Bedingungen gebildete NO die Expression der CPT-Iα über die Aktivierung der cGMP-vermittelten Signaltransduktion reguliert.

3.) Zur Untersuchung des Einflusses von Hypoxie auf die CPT-Iα und mHMG-CoA Synthase-Expression in Rattenmesangiumzellen und -hepatocyten wurden zwei verschiedene Hypoxie-Modelle gewählt:

a) Stimulation der Zellen mit $CoCl_2$ oder mit dem Fe^{2+}-Chelator Desferrioxamin unter normoxischen Bedienungen oder

b) Kultivierung der Zellen in einer luftdichten Kammer, die mit einer 3%igen oder 1%igen O_2-Mischung begast wird.

In Measangiumzellen wurde die CPT-Iα Protein- und mRNA- Expression durch $CoCl_2$, Desferrioxamin und 3% O_{2-} vermittelte Hypoxie verstärkt. In Gegensatz dazu erhöht $CoCl_2$ in den Hepatocyten nur die CPT-Iα Proteinmenge, während die mRNA-Expression unbeeinflusst blieb. Dagegen wurde in den Hepatocyten die CPT-Iα mRNA-Expression durch Desferrioxamin verstärkt und durch 1% O_2 Hypoxia gehemmt. Solche unterschiedliche Auswirkungen von zwei hypoxischen Modellsystemen auf die CPT-Iα Protein- und mRNA-Expression in

Mesangiumzellen und Hepatocyten könnten durch zellspezifische Regulationsmechanismen erklärt werden. Weiterhin wurde in beiden Zellsystemen die mRNA-Expression der mHMG-CoA Synthase unter allen eingesetzten hypoxischen Bedingungen reduziert. Dies bedeutet, dass die Ketogenese O_2-abhängig abläuft und unter Hypoxie herunter reguliert wird.

4.) Der letzte Teil der vorliegenden Arbeit befasst sich mit der Regulation der CPT-Iα Expression durch exogene $sPLA_2$s in Mesangiumzellen und Hepatozyten. Auf Grund früherer Arbeiten war bekant, dass bei entzündlichen Prozessen $sPLA_2$s in großen Mengen in Extrazellularräume und Körperflüssigkeiten sezerniert werden, um dort ein breites Spektrum von Fettsäuren aus Phospholipiden freizusetzen. Die Arbeitshypothese war, dass die auf diesem Weg freigesetzten Fettsäuren eine Hochregulation der CPT-Iα induzieren könnten. Es konnte gezeigt werden, dass es in Mesangiumzellen, die mit rekombinanten oder gereinigten $sPLA_2$s exogen behandelt wurden, zu einer Erhöhung der CPT-Iα Expression auf Protein-, mRNA- und Promotor-Ebene kommt. In Hepatocyten konnte ebenfalls eine verstärkte CPT-Iα–Proteinexpression festgestellt werden. Ein synergistischer Effekt mit TNF-α konnte nur auf Protein- und mRNA-Ebene, nicht jedoch auf Promotorebene beobachtet werden, was auf einen gemeinsamen Mechanismus der Promotor-Aktivierung durch $sPLA_2$s und TNF-α schließen lässt. Wurden die Zellen gleichzeitig mit den Mitogen-aktivierten-Protein-Kinase (MAPK)-Inhibitoren PD 98059 and U0126 behandelt, wurde diese Hochregulation der CPT-Iα gehemmt. Daraus kann gefolgert werden, dass der $sPLA_2$-induzierte Effekt auf die CPT-Iα -Expression durch die bereits bekannte $sPLA_2$-induzierte Aktivierung der MAPK vermittelt wird. In Mesangiumzellen wurde weiterhin gezeigt, dass $sPLA_2$s in Koinkubation mit TNF-α die mRNA Expression der mHMG-CoA Synthase stark induziert .

In dieser Arbeit konnte also gezeigt werden, dass unter den gewählten proinflammatorischen Bedingungen die CPT-Iα sowohl auf der Ebene der Genexpression als auch auf Proteinebene reguliert wird. Auch die an die CPT-Iα gekoppelte mHMG-CoA Synthase wird in ihrer mRNA-Expression moduliert, sodass Effekte auf die Ketogenese zu vermuten sind. Zukünftige Arbeiten könnten zeigen, ob CPT-Iα als ein mögliches Target für die Entwicklung neuer Therapie-Strategien zur Verbesserung der Energiebilanz und damit auch der Überlebungsrate bei entzündlichen Erkrankungen dienen kann.

7.5 Curriculum vitae

Angaben zur Person:

Name:	Nina Eschenröder
Geburtsdatum und Ort:	09.08.1965, in Alma-Ata, Kasachstan.
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Ausbildungsdaten und Promotion:

Schule:	1972-1982	Staatliche Schule in Wolskij, Rußland.
Studium 1:	1982-1987	Pädagogische Hochschule, Wolgograd, Rußland. Abschluss Lehrerin für Chemie und Biologie.
Studium 2:	1995-2001	Johann Wolfgang Goethe-Universität in Frankfurt am Main. Diplombiochemikerin.
Promotion:	2001-2003	An der Uniklinikum in Frankfurt am Main. Klinik für Allgemein Pädiatrie.

Berufliche Tätigkeiten:

	1987-1991	Gymnasium in Wolskij, Rußland. Lehrerin für Chemie und Biologie.
	1991-1992	Übersiedlung in die Bundesrepublik Deutschland, Deutschkurs.
	1993-1994	Eisfabrik "Frostdog" in Ortenberg, Hessen. Aushilfskraft im Labor.

Frankfurt am Main, März 2006

Nina Eschenröder

Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin zur Promotionsprüfung eingereichte Arbeit mit dem Titel
Regulation of carnitine palmitoyltransferase Ia under inflammatory conditions in rat mesangial and primary liver cells
In der Klinik für Allgemeine Pädiatrie I, Johann Wolfgang Goethe-Universität Frankfurt am Main.
Unter Leitung vom Herren Professor Dr. H. Böhles
Mit Unterstützung durch Herren Dr. Gebhardt, Zentrum der Kinderheilkunde, Herren Dr. Beck, Frau PD Dr. Kaszkin, Zentrum der Pharmakologie.

ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.

Ich habe bisher an keiner in- oder ausländischen Medizinischen Fakultät ein Gesuch um Zulassung zur Promotion eingereicht noch die vorlegende Arbeit als Dissertation vorgelegt.

Frankfurt am Main, März 2006

Nina Eschenröder