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"Investigations on the effects of peroxisome proliferator-activated receptors and of nuclear factor kappa B on novel organic cation transporter 2 and carnitine uptake in bovine kidney cells"

Dissertation

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LIST OF ABBREVIATIONS

AF-2	Activation fuction 2
ANOVA	Analysis of variance
BBD	γ-Butyrobetainedioxygenase
CACT	Carnitine-acylcarnitine translocase
cDNA	Complementary deoxyribonucleic acid
CoA	Coenzyme A
CPT	Carnitine-palmitoyltransferase
IKK	IκB kinase
IL	Interleukin
IL-1R	Interleukin-1 receptor
IκB	Inhibitor of kappa B
LBD	Ligand-binding domain
LPS	Lipopolysaccharides
MDBK	Madin-Darby bovine kidney
NCoR	Nuclear receptor corepressor
NEFA	Non-esterified fatty acids
NF-κB	Nuclear factor kappa B
OCTN	Novel organic cation transporter
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator response elements
TG	Triglycerides
TLR	Toll-like microbial pattern recognition receptor
TML	Trimethyllysine
TNFR	TNFα receptors
TNFα	Tumor necrosis factor-α
TRAF	TNFR-associated factor
TZD	Thiazolidinediones
VLDL	Very low-density lipoprotein

1 INTRODUCTION

High-producing dairy cows undergo a negative energy balance and inflammation in the transition period, which result in a physiological activation of peroxisome proliferator-activated receptors (PPARs) and nuclear factor kappa B (NF-κB) (Schlegel et al., 2012; Gessner et al., 2013). Many studies in non-ruminants have shown that PPARα regulates carnitine homeostasis, which is an essential cofactor for fatty acid β-oxidation (van Vlies et al., 2007; Eder and Ringseis, 2010). In addition, a limited number of studies have shown that NF-κB influences carnitine uptake. The present dissertation focuses on the role of PPARs and NF-κB in carnitine transport in cattle.

1.1 Biochemical function of carnitine

L-Carnitine (L-β-hydroxy-4-N-trimethylaminobutyric acid) is a water soluble metabolite with a number of indispensable roles in intermediary metabolism. Its most prominent function is to serve as an essential cofactor for mitochondrial fatty acid oxidation by transferring long-chain fatty acids as acylcarnitine esters across the inner mitochondrial membrane (McGarry et al., 1997). Long-chain fatty acids are trans-esterified to long-chain acylcarnitine catalyzed by carnitine-palmitoyltransferase-1 (CPT-1) at the outer membrane of the mitochondria. The long-chain acylcarnitine crosses the outer membrane and is transported over the inner membrane of the mitochondria by carnitine-acylcarnitine translocase (CACT), which is a specific carrier and is located in the inner membrane of the mitochondria. CPT-2 catalyzes the trans-esterification of the long-chain acylcarnitine back into long-chain acyl-coenzyme A (acyl-CoA) and releases carnitine. Long-chain acyl-CoA is an activated substrate inside the matrix for β-oxidation. The intramitochondrial carnitine is able to react with acetyl-CoA, which is the product of β-oxidation via carnitine-acetyltransterase, and form acetylcarnitine. Again, acylcarnitine can leave the mitochondria via CACT, waiting for another round of transport (Vaz and Wanders, 2002). Additional roles of carnitine include transfer of products of peroxisomal β-oxidation to the mitochondria for oxidation in the citrate cycle, modulation of the acyl-CoA/CoA ratio, as well as storage of energy in the form of acetylcarnitine (Vaz and Wanders, 2002). When the substrate oxidation exceeds the energy demand, the acetyl and acyl groups will be transported out of the mitochondria via carnitine-acetyltransterase and CACT. High ratio of acetyl-CoA/CoA in the mitochondria inhibits the pyruvate dehydrogenase complex, which is a key enzyme for the citric acid cycle. The carnitine shuttle system is able to convert the accumulating acyl-CoA back to acylcarnitines and transport

them out of mitochondria, therefore stimulates the pyruvate dehydrogenase complex activity and increases the glucose oxidation (Ramsay and Zammit, 2004).

So far, two kinds of carnitine deficiency have been well defined, namely primary or secondary carnitine deficiency syndromes. Primary carnitine deficiency is an autosomal recessive disorder characterized by mutations of an organic cation/carnitine transporter, leading to a decrease of intracellular carnitine concentration that impairs fatty acid oxidation (Wang et al., 1999). Primary carnitine deficiency is not associated with another identifiable systemic illness such as amino acid and glucose oxidation defects that might deplete carnitine stores of the tissue (Pons and Darryl, 1995). Secondary carnitine deficiency manifests in a low level of carnitine in plasma and associates with a large number of metabolic disorders. The most characteristic cause of secondary carnitine deficiency is the failure of oxidation of acyl-CoA intermediates in the mitochondria (Pons and Darryl, 1995).

1.2 Carnitine biosynthesis and transport

In mammals carnitine is derived from dietary sources as well as endogenous biosynthesis. Carnitine biosynthesis involves a complex series of reactions. Lysine provides the carbon backbone of carnitine. In protein peptide linkages it undergoes methylation of the ε-amino group to yield N6-trimethyllysine (TML), which is released upon protein degradation. The released TML further undergoes four enzymatic steps in the reaction to carnitine by the action of TML dioxygenase, 3-hydroxy-N6-trimethyllysine aldolase, 4-N-trimethylaminobutyraldehyde dehydrogenase and γ-butyrobetainedioxygenase (BBD) (Strijbis et al., 2010). In humans and rodents, the genes encoding TML dioxygenase and 4-N-trimethylaminobutyraldehyde dehydrogenase have been reported to be widely expressed in the tissues of skeletal muscle, heart and brain, but the highest activity is found in the liver and kidney (Vaz and Wanders, 2002; Ringseis et al., 2009). However, BBD activity is found predominantly in the liver of all mammals and also in the kidney in some species such as humans, cats, hamsters, rabbits or Rhesus monkeys (Vaz and Wanders, 2002). The tissues which lack BBD or have a very low activity of BBD are highly dependent on active carnitine uptake from the blood. Delivery of carnitine and carnitine precursor butyrobetaine is carried by novel organic cation transporters (OCTN). This transport system is involved in the intestinal absorption and renal tubular reabsorption of carnitine. Taken together, carnitine distribution and homeostasis is maintained by dietary intake, a modest rate of endogenous

synthesis and efficient tubular reabsorption of carnitine by the kidney (Eder and Ringseis, 2010).

It has been reported that more than 95% of carnitine from the urine is filtered and reabsorbed in the kidney (Tamai et al., 2000, Glube et al., 2007). OCTNs are responsible for delivery of carnitine and carnitine precursor butyrobetaine from plasma into cells. OCTNs belong to the solute carrier 22A family and localize on the apical membrane of cells. Three OCTN have been identified so far, OCTN1, OCTN2 and OCTN3 (Tamai et al., 1997, 1998, 2000). OCTN2 is the most pivotal carnitine transporter due to its wide expression in tissues and its high binding affinity to carnitine (Koch et al., 2007; Luci et al., 2008). The structure of OCTN2 is characterized by nine short loops and two large hydrophilic loops connecting twelve transmembrane segments. The substrate-binding sites are composed of three subsites: two subsites contain recognition pockets for carboxyl groups and ammonium ion of the substrate, respectively, and one subsite is specific for the Na^+ site (Pochini et al., 2013). Thus, the structure of OCTN2 demonstrates that this transport system has well-defined substrate specificity for carnitine and its derivatives, whose transport is dependent on Na^+, but not Li^+ or K^+ (Tamai et al., 2001; Tamai et al., 1998).

1.3 The general characteristics and functions of PPARs

PPARs are ligand-activated transcription factors. They belong to the nuclear receptor superfamily, which controls the transcription of sets of genes encoding proteins in metabolic pathways and contributes to the complex fine-tuning of gene activity required for mammals to adapt to changing conditions. The term PPAR originally came from the observation that a group of agents such as WY-14,643 could increase peroxisome numbers in rodent liver tissue (Issemann and Green, 1990). PPARs consist of three isoforms PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3). The modular structure of PPARs is similarly organized as other nuclear receptors, with an N-terminal A/B domain, a DNA-binding domain, a hinge region, a ligand-binding domain (LBD), and a C-terminal domain. There is a ligand-independent transactivation domain at the extreme N-terminal region, which is a key determinant of isotype-selective gene expression and function (Hummasti and Tontonoz, 2006). The DNA-binding domain is a highly conserved domain with two zinc fingers, which are small protein structural motifs that coordinate with zinc ions to stabilize the fold. The LBD, that contains the ligand-dependent activation function 2 (AF-2), is composed of 13 α-helices and a small 4-stranded β-sheet. Due to the large pocket, comprising the binding site,

various structural diverse, xenobiotic ligands are accepted by PPARs (Berger and Moller, 2002). PPARs regulate gene expression as heterodimers with retinoid-X-receptor, binding to a specific DNA sequence called peroxisome proliferator response elements (PPRE) in the promoter region of target genes. The consensus sequence of PPRE is a direct repeat of AGGTCA, separated by a single nucleotide spacer in the regulatory region (Desvergne and Wahli, 1999).

The tissue distribution of PPARs is broad, but isotype-characteristic, which at least partly accounts for the variety of functions. PPARα is abundantly expressed in tissues, which have high rates of fatty acid oxidation, such as liver, kidney, skeletal muscle, and myocardium (Bocos et al., 1995; Fruchart, 2009; Speeckaert et al., 2014). PPARα acts as a key regulator of energy homeostasis and lipid metabolism (Eder and Ringseis, 2010). PPARα target genes are involved in fatty acid uptake, fatty acid transport, mitochondrial fatty acid oxidation, gluconeogenesis and ketogenesis (Mandard et al., 2004; Cullingford, 2003). PPARβ/δ distribution in mammals is ubiquitous. Activation of PPARβ/δ mediates fatty acid oxidation in muscle and heart from rodents (Luquet et al., 2003; Cheng et al., 2003), and is involved in the healing of skin wounds, inflammatory responses and general fundamental cellular processes (Montagner et al., 2011; Contreras and Sordillo, 2011). PPARγ is abundant in all adipose tissues, where it promotes adipogenesis, differentiation, maintenance and lipid storage (Yu and Reddy, 2007). PPARγ is also associated with cellular energy homeostasis, glucose homeostasis and anti-inflammation (Berger and Moller, 2002).

1.4 The role of PPARs in the transcriptional regulation of OCTN2 in different species

It is well established that PPARα activation leads to an up-regulation of OCTN2 in rodents. The treatment of rats and its hepatoma cells with clofibrate, which is a synthetic PPARα activator, causes a strong elevation of the transcription level of OCTN2 in liver and hepatocytes (Luci et al., 2006). Subsequent studies with rats confirmed that PPARα activation also increases the mRNA content of OCTN2 in the small intestine (Ringseis et al., 2007). The transcriptional up-regulation of OCTN2 in liver and small intestine were associated with an elevation of the carnitine concentration in rat liver and an improvement of the absorption rate of carnitine in small intestine (Luci et al., 2006; Ringseis et al., 2008). The physiologic and nutritive activation of PPARα such as energy deprivation and oxidized fat administration have a similar effect on OCTN2 as clofibrate treatment (Luci et al., 2008; Koch et al., 2007). Studies have clearly demonstrated that treatment with PPARα agonist WY-14,643 caused an

up-regulation of OCTN2 in liver, kidney, skeletal muscle and small intestine of wild-type mice, but not of PPARα-null mice (van Vlies et al., 2007; Koch et al., 2008). Furthermore, the promoter region of rat and mouse OCTN2 both contain a functional PPRE, which is the direct evidence that OCTN2 is a target gene of PPARα in rodents (Maeda et al., 2008; Wen et al., 2009).

It is known that the findings in rodents cannot be directly applied to other species, because numerous studies have observed that the expression and activation level of PPARα in mice and rats are much higher and stronger than in other species like pigs and humans (Holden and Tugwood, 1999; Eder and Ringseis, 2010). However, the treatment of pigs with clofibrate, fasting or oxidized fat still induced an up-regulation of OCTN2 in tissues (Luci et al., 2007; Luci et al., 2007; Ringseis et al., 2009). In humans, so far there is no direct evidence that the activation of PPARα can regulate the transcriptional level of OCTN2. In contrast to the numerous studies in rodents and pigs, only few studies are available in the literature investigating the effect of PPARα activation on OCTN2 gene expression in ruminants. A recent study showed that mRNA level of OCTN2 in the liver of dairy cows was dramatically increased in the early lactation period, when PPARα activation may occur (Schlegel et al., 2012). To our knowledge, there is only one publication investigating the role of PPARγ on OCTN2 gene expression in the colon in human and mice (D'Argenio et al., 2010). In this study, colon OCTN2 gene expression is up-regulated by PPARγ agonist thiazolidinediones (TZD) and completely blocked by its antagonists (D'Argenio et al., 2010). There is no investigation on the role of PPARβ/δ in OCTN2 gene regulation.

1.5 NF-κB pathway in inflammation

NF-κB is a ubiquitous and inducible nuclear transcriptional activator. The term NF-κB derives from a DNA-binding protein that regulates the immunoglobulin kappa light-chain gene expression in murine B lymphocytes (Sen and Baltimore, 1986). Subsequent studies confirmed that NF-κB can be activated in most cell types in response to various stimuli, with a major role in inflammation (Lawrence, 2009; Hoesel and Schmid, 2013).

Inflammation is the process of innate immunity responding to different stresses such as physical, physiological and oxidative stress. Inflammation is associated with activation of the canonical NF-κB signaling pathway. In the cytoplasm, NF-κB exists as both a homodimer and a heterodimer of Rel-related proteins. The most common form of NF-κB is composed of RelA

and p50 subunits associated with inhibitor of kappa B (IκB) in the inactive form (Sen and Smale, 2010). On the one hand, when tissues are injured by infection, cytokines are rapidly released from the resident tissue, such as tumor necrosis factor-α (TNFα) and interleukin-1 (IL-1) representing the common cytokines. The TNFα receptors (TNFRs) and IL-1 receptors (IL-1R) on the surface of the membrane of cells can specifically bind these released TNFα and IL-1. The lipopolysaccharides (LPS) from bacteria can be identified by the Toll-like microbial pattern recognition receptors (TLRs), which belong to the IL-1R family. On the other hand, endogenous ligands including heat shock protein 70 (Vabulas et al., 2002) and nucleic acids (Barrat et al., 2005) may also trigger TLRs during tissue injury and certain disease states, which may promote inflammation in the absence of infection (Karin et al., 2006). TNFα, IL-1 and LPS use TNFRs signal transduction mechanisms to activate IκB kinase (IKK). In short, upon ligand-dependent activation of TNFRs and IL-1R/TLRs, TNFR-associated factors (TRAFs) are recruited to the intracellular domain of the TNFRs either via direct interaction or via the adaptor proteins such as TNFR associated death domain protein and IL-1 receptor-associated kinase. Instead of phosphorylase activities, TRAFs have ubiquitin activities, which act as ubiquitin ligase in cooperation with the ubiquitin conjugating enzyme 13 forming Lys 63-linked polyubiquitin chains, providing an activated TRAFs platform for the assembly of other signaling molecules including cellular inhibitor of apoptosis protein 1/2. The cellular inhibitor of apoptosis protein 1/2 can regulate the polyubiquitination of target proteins such as receptor interacting protein 1 kinase. Ubiquitinated receptor interacting protein 1 not only directly binds to IKKγ, but also helps TRAFs to recruit transforming growth factor-β-activated kinase complex, which can phosphorylate IKKβ, promoting IKK complex activation (Napetschnig and Wu, 2013; Zheng et al., 2011; Hoesel and Schmid, 2013). Subsequently, IKK leads to phosphorylation of IκB, and the activated IκB releases NF-κB allowing free NF-κB to translocate into the nucleus. Then, NF-κB binds to its consensus sequence, and initiates the transcription of target genes, which are mainly encoding pro-inflammatory cytokines and chemokines (Lawrence, 2009).

Besides the canonical NF-κB pathway, an alternative NF-κB pathway is activated by different classes of receptors including lymphotoxin β-receptor, CD40, B-cell activating factor, and receptor activator of NF-κB. They lead to activation of the NF-κB inducing kinase, which phosphorylates and activates IKKα. IKKα induces the activation of the RelB/p52 heterodimer, which is another form of NF-κB. The alternative NF-κB pathway is characterized by its

activation process, which is independent of both IKKβ and IKKγ (Malinin et al., 1997; Ghosh and Karin, 2002).

It is worth noting that NF-κB activation can have both pro- and anti-inflammatory roles. NF-κB can directly regulate the expression or activity of anti-inflammatory cytokines such as IL-10 (Tomczak et al., 2006) and induce leukocyte apoptosis during the resolution of inflammation, which is an essential mechanism that prevents prolonged inflammation (Lawrence et al., 2001). Furthermore, IKKβ exhibits anti-inflammatory ability in sepsis (Greten et al., 2007). Specific deletion of IKKβ in the model of streptococcal pneumonia results in the inhibition of neutrophil recruitment and bacterial clearance (Fong et al., 2008; Gordon and Taylor, 2005).

1.6 The occurrence of inflammation affects lipid metabolism in cattle

Activation of NF-κB is a typical phenomenon in dairy cows undergoing transition period (Gessner et al., 2013). Transition period is from 3 weeks before to 3 weeks after parturition (Drackley, 1999). This period has great importance for its association with lipid metabolic alteration and inflammation, which strongly affect the performance and health of dairy cows (Bertoni et al., 2008; Esposito et al., 2014). The energy demand in early lactation dramatically increases for the milk production, whereas the dry matter intake of dairy cows decreases as calving approaches. The high requirement of energy and insufficient food intake, that causes a negative energy balance, results in a massive mobilization of non-esterified fatty acids (NEFA) from adipose tissue. NEFA are transported and taken up into liver and other tissues for compensating the energy requirement (Bell, 1980; Drackley, 1999). One characteristic of NEFA is that they are well-established endogenous activators of PPARs (Xu et al., 1999; Bionaz et al., 2012; Kadegowda et al., 2009). Another characteristic of NEFA is that they have pro-inflammatory effects on the resident macrophages and act as ligands for TLR, which in turn leads to NF-κB activation (Lee et al., 2004; Lee et al., 2006). Besides endogenous ligands, infection caused by bacteria and virus in dairy cows also contributes to systemical activation of NF-κB (Ingvartsen and Moyes, 2013). Bradford and colleagues (2009) administered TNFα daily by the subcutaneous infection for 7 days in lactating dairy cows, which caused promotion of hepatic triglycerides (TG) accumulation and increase in the transcript abundance of genes involved in NEFA uptake. Carnitine has a major role in lipid metabolism that helps NEFA to enter the mitochondria for β-oxidation, which can decrease the accumulation of fatty acids in liver. Carnitine transporter OCTN2 is responsible for

maintaining carnitine homeostasis. Therefore, it is very interesting to investigate the response of OCTN2 and carnitine uptake to the activation of NF-κB.

2 AIMS AND HYPOTHESES

The overall aim of this thesis was to investigate the effect of PPARs (PPARα and PPARβ/δ) and NF-κB activation on carnitine transporter OCTN2 and OCTN2-mediate carnitine uptake in a commercially available bovine cell line, the Madin-Darby bovine kidney (MDBK) cell line. This cell line has recently shown to be a suitable model to study PPARα-dependent effects in bovine tissues (Bionaz et al. 2008).

In order to activate PPARα, PPARβ/δ and NF-κB respectively in bovine kidney cells, we chose WY-14,643, GW0742 and TNFα as inducers. WY-14,643 is an abbreviation of [4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid, which is a synthetic hypolipidemic drug (Willson and Wahli, 1997). WY-14,643 stimulates activation of PPARα via a traditional mechanism and a novel bipartite mechanism. The conventional mechanism is that WY-14,643 performs polar and hydrophobic contacts with the protein residues in LBD of PPARα, and forms a well recognized hydrogen-bonding network with several residues including Y464, which is located on the inner surface of the AF-2 helix, that is crucial for maintaining the protein active conformation and regulating the recruitment of coactivators (Xu et al., 1999; Berger and Moller, 2002). The novel bipartite mechanism has been recently indentified and besides the first WY-14,643 binding in LBD, as mentioned above, a second WY-14,643 binds to a secondary site called Ω-loop in LBD, which is now well ordered instead of poorly structured as usually. This second WY-14,643 is mainly stabilized by the non-polar interaction with residues contacting the fused heterocyclic rings and by salt bridges between the carboxylic group of the WY-14,643 and the Ω-loop (Bernardes et al., 2013). The interaction of WY-14,643 in the second site promotes a more subtle stabilization of AF-2, which is required for full PPARα activation. GW0742 is the name of [4-[[[2-[3-fluoro-4-(trifluoromethyl)phenyl]-4-methyl-5-thiazolyl]methyl]thio]-2-methyl phenoxy]-acetic acid, which is a synthetic small molecule agonist for PPARβ/δ (Sznaidman et al., 2003). GW0742 uses hydrophilic head group (the carboxylic group) and hydrophobic tail (the thiazole and the fluorine substituted phenyl ring) interacting with residues in LBD, which are responsible for activation of AF-2. The residues Val312 and Ile 328 in LBD are essential for PPARβ/δ selective GW0742 binding (Batista et al., 2012). TNFα belongs to cytokines, which are cell signaling molecules and are able to change many physiological systems for the wide expression of receptors in nearly all cell types. TNFα has been demonstrated to activate NF-κB via TNFR-TRAF-IKK pathway as mentioned in the introduction of the dissertation.

2.1 Study 1

Zhou X, Wen G, Ringseis R, Eder K (2014) Short communication: the pharmacological peroxisome proliferator-activated receptor α agonist WY-14,643 increases expression of novel organic cation transporter 2 and carnitine uptake in bovine kidney cells. Journal of Dairy Science. 97: 345-9. (Reproduction with permission of the publisher)

2.1.1 General aim: Studies in rodents demonstrated that PPARα is an important transcriptional regulator of the gene encoding carnitine transporter OCTN2. In contrast, it has not been known whether PPARα regulates OCTN2. In addition, the role of PPARα for carnitine transport in cattle is unclear, even though PPARα activation physiologically occurs in the liver of high-producing cows during early lactation. To address this issue, the aim of the present study was to investigate whether PPARα agonist WY-14,643 influences the transcription and protein levels of OCTN2 and carnitine uptake in the presence and absence of PPARα antagonist GW6471 in bovine kidney cells.

2.1.2 Specific hypothesis:

The following two hypotheses were tested in the present study:

(i) WY-14,643 increases the gene expression of OCTN2 in MDBK cells and co-treatment of MDBK cells with WY-14,643 and the PPARα antagonist GW6471 blocks the WY-14,643-induced increase of mRNA and protein levels of OCTN2.

(ii) WY-14,643 specifically stimulates Na^+-dependent carnitine uptake in MDBK cells, and WY-14,643-stimulated increase of L-carnitine uptake is blocked by treatment of cells with a PPARα antagonist GW6471.

2.2 Study 2

Zhou X, Ringseis R, Wen G, Eder K (2014) Carnitine transporter OCTN2 and carnitine uptake in bovine kidney cells are regulated by peroxisome proliferator-activated receptor β/δ. Acta Veterinaria Scandinavica. 56: 21.

2.2.1 General aim: PPARα has been shown to be a transcriptional regulator of the gene encoding the carnitine transporter OCTN2 in bovine kidney cells (**study 1**). It is currently unknown whether PPARβ/δ, another PPAR subtype, which has partially overlapping functions as PPARα and is known to share a large set of common target genes with PPARα,

also regulates OCTN2 and carnitine transport in cattle. To address this issue, the aim of the present study was to investigate whether PPARβ/δ agonist GW0742 influences the transcription and protein levels of OCTN2 and carnitine uptake in the presence and absence of PPARβ/δ antagonist GSK3787 in bovine kidney cells.

2.2.2 Specific hypothesis:

The following two hypotheses were tested in the present study:

(i) GW0742 increases the gene expression of OCTN2 in MDBK cells and co-treatment of MDBK cells with GW0742 and the PPARβ/δ antagonist GSK3787 blocks the GW0742-induced increase of mRNA and protein levels of OCTN2.

(ii) GW0742 specifically stimulates Na^+-dependent carnitine uptake in MDBK cells and GW0742-stimulated increase of L-carnitine uptake is blocked by treatment of cells with a PPARβ/δ antagonist GSK3787.

2.3 Study 3

(unpublished data) Zhou X, Ringseis R, Wen G, Eder K (2014) The nuclear factor kappa B inducer TNFα increases expression of novel organic cation transporter 2 and carnitine uptake in bovine kidney cells

2.3.1 General aim: Our previous researches have shown that OCTN2 and carnitine uptake are regulated by PPARs in bovine kidney cells (**study 1 and 2**). It is well-documented that inflammation is involved in infections and metabolic disease in dairy cows in early lactation (Bllou, 2012; Bradford et al., 2010; Bobe et al., 2004). Fujiya et al., (2011) have reported that OCTN2 level and carnitine uptake in human colonic epithelial cells are increased by proinflammatory cytokines TNFα. However, it is unclear whether inflammation is able to alter OCTN2 and carnitine uptake in bovine kidney cells. To address this issue, the aim of the present study was to investigate, whether TNFα can induce NF-κB activation and affect the gene expression of OCTN2 and carnitine uptake in the presence and absence of NF-κB inhibitor BAY 11-7085 in bovine kidney cells.

2.3.2 Specific hypothesis:

The following three hypotheses were tested in the present study:

(i) The optimized concentration of TNFα increases the transcription level of IL-6 and IL-1B, which are well-known NF-κB target genes. The optimized concentration of TNFα stimulates NF-κB transactivation in MDBK cells.

(ii) TNFα increases the gene expression of OCTN2 in MDBK cells, and co-treatment of MDBK cells with TNFα and the NF-κB inhibitor BAY 11-7085 blocks the TNFα-induced increase of mRNA and protein levels of OCTN2.

(iii) TNFα stimulates carnitine uptake in MDBK cells, and TNFα-stimulated increase of L-carnitine uptake is blocked by treatment of cells with a NF-κB inhibitor BAY 11-7085.

3 ORIGINAL WORKS

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Short communication: The pharmacological peroxisome proliferator-activated receptor α agonist WY-14,643 increases expression of novel organic cation transporter 2 and carnitine uptake in bovine kidney cells

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ABSTRACT

Recent studies in rodents demonstrated that peroxisome proliferator-activated receptor α (PPARα), a central regulator of energy homeostasis, is an important transcriptional regulator of the gene encoding the carnitine transporter novel organic cation transporter 2 (OCTN2). Less is known with regard to the regulation of OCTN2 by PPARα and its role for carnitine transport in cattle, even though PPARα activation physiologically occurs in the liver of high-producing cows during early lactation. To explore the role of PPARα for OCTN2 expression and carnitine transport in cattle, we studied the effect of the PPARα activator WY-14,643 on the expression of OCTN2 in the presence and absence of PPARα antagonists and on OCTN2-mediated carnitine transport in the Madin-Darby bovine kidney (MDBK) cell line. The results show that WY-14,643 increases mRNA and protein levels of OCTN2, whereas co-treatment of MDBK cells with WY-14,643 and the PPARα antagonist GW6471 blocks the WY-14,643-induced increase in mRNA and protein levels of OCTN2 in bovine cells. In addition, treatment of MDBK cells with WY-14,643 stimulates specifically Na^+-dependent carnitine uptake in MDBK cells, which is likely the consequence of the increased carnitine transport capacity of cells due to the elevated expression of OCTN2. In conclusion, our results indicate that OCTN2 expression and carnitine transport in cattle, as in rodents, are regulated by PPARα.
Key words: bovine kidney cells, novel organic cation transporter 2, peroxisome proliferator-activated receptor α

Short Communication

L-Carnitine is an essential compound with several indispensable roles in intermediary metabolism. Its most prominent function is to serve as an essential cofactor for mitochondrial FA oxidation by transferring long-chain FA as acylcarnitine esters across the inner mitochondrial membrane (McGarry and Brown, 1997). Carnitine in the body is derived from endogenous synthesis, which occurs mainly in the liver, and from the intestinal absorption of carnitine from the diet. Tissues that cannot provide carnitine via endogenous synthesis, such as skeletal muscle or myocardium, are dependent on carnitine uptake from the circulation, which occurs against a high concentration gradient. This active carnitine transport across the plasma membrane is mediated by the novel organic cation transporters (**OCTN**), which belong to the solute carrier 22A family (Lahjouji et al., 2001). The OCTN2 isoform, which is sodium dependent and high affinity, is considered the physiologically most important one due to its wide tissue expression (Tamai et al., 1998). The OCTN2-mediated carnitine transport is also responsible for the tubular reabsorption of carnitine in the kidney and is, therefore, fundamental for maintaining normal carnitine levels in serum (Lahjouji et al., 2004).

Recent studies in mice and rats convincingly demonstrated that peroxisome proliferator-activated receptor α (**PPARα**; encoded by *PPARA*), which is well known to act as a central regulator of lipid metabolism and energy homeostasis (Desvergne and Wahli, 1999), is an important transcriptional regulator of genes encoding OCTN2 and enzymes involved in carnitine biosynthesis (Ringseis et al., 2012). Gene transcription by PPARα is initiated when ligands, such as FA that are liberated from adipose tissue during energy deprivation and taken up into tissues during this state, or exogenous ligands such as fibrates (WY-14,643), bind to the ligand-binding domain of this transcription factor. In contrast to rodents, less is known with regard to the regulation of OCTN2 by PPARα and its role for carnitine transport

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in cattle. In cattle, PPARα activation physiologically occurs in the liver during early lactation because the negative energy balance associated with early lactation leads to the release of FA from adipose tissues, which are taken up into the liver and bind to and activate PPARα (Loor et al., 2005; Loor, 2010). Interestingly, we have found recently that OCTN2 and also genes involved in carnitine synthesis in the liver are upregulated and hepatic carnitine concentration is increased during early lactation in dairy cows (Schlegel et al., 2012), providing at least weak evidence that PPARα also regulates carnitine homeostasis in cattle. However, more convincing evidence is necessary to clearly establish a role for PPARα as a regulator of OCTN2 and carnitine transport in cattle, especially because it is well documented that the response to PPARα activators is different between rodents and other species (Richert et al., 1996). To provide this evidence, we studied the effect of a high-affinity ligand of PPARα (WY-14,643) on the expression of OCTN2 in the presence and absence of PPARα antagonists and on OCTN2-mediated carnitine transport in a commercially available bovine cell line, the Madin-Darby bovine kidney (**MDBK**) cell line. This cell line was recently demonstrated to be a suitable model to study PPARα-dependent effects in bovine tissues (Bionaz et al., 2008).

The MDBK cells, obtained from Cell Lines Service GmbH (Eppelheim, Germany), were cultivated in HyClone Minimum Essential Medium/Earle's Balanced Salt Solution (**MEM/EBSS**) medium supplemented with 10% fetal bovine serum and 0.05 mg/mL gentamicin (all from Invitrogen GmbH, Karlsruhe, Germany) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For experiments, cells were seeded out into 6-well plates at a density of 2.0×10^5 cells (for qPCR and Western blotting experiment) or 24-well plates at a density of 7×10^4 cells (for L-carnitine uptake studies) in HyClone MEM/EBSS complete medium. After reaching 80% confluence, MDBK cells were treated with 150 μ*M* WY-14,643 [dissolved in dimethyl sulfoxide (**DMSO**); both from Sigma-Aldrich Chemie GmbH, Steinheim, Germany] as a selective PPARα agonist in MEM/EBSS medium without fetal bovine serum but 5 mg/L of bovine insulin (Sigma-Aldrich Chemie GmbH) for 24 h. Cells treated with vehicle alone (DMSO) were used as control. Incubation media of control cells contained the same vehicle concentration of 0.1% (vol/vol). For experiments using a PPARα inhibitor, cells were co-treated with a 10 μ*M* concentration of the PPARα-selective antagonist GW6471 (Sigma-Aldrich Chemie GmbH). At the end of incubation, media was discarded, and cell layer was washed once with PBS. Afterward, plates including the attached cells were immediately stored at −80°C. All incubations were run in triplicate and each experiment was repeated 3 times.

For quantitative PCR (**qPCR**), total RNA was isolated, concentration and purity of isolated RNA were determined, and cDNA was synthesized as described recently in detail (Keller et al., 2012). Further details on RNA isolation and cDNA synthesis are provided in Supplemental Materials and Methods (available online at http://dx.doi.org/10.3168/jds.2013-7161). Quantitative PCR and normalization by geNorm normalization factor were also carried out as described recently in detail (Keller et al., 2012), with the exception that bovine gene-specific primer pairs were used according to Schlegel et al. (2012). Primer characteristics and qPCR performance are reported in Supplemental Table S1 (available online at http://dx.doi.org/10.3168/jds.2013-7161). The normalization factor was calculated as the geometric mean of expression data of the 3 most stable out of 5 tested potential reference genes. Means and standard deviations were calculated from normalized expression data for samples of the same treatment group. The mean of the vehicle control group was set to 1 and means and standard deviations of the WY-14,643 were scaled proportionally.

For immunoblot analysis, cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer [50 m*M* Tris (pH 7.5), 150 m*M* NaCl, 1 m*M* EDTA, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate] containing protease inhibitors (Sigma-Aldrich Chemie GmbH). Further details on immunoblot analysis are provided in Supplemental Materials and Methods (available online at http://dx.doi.org/10.3168/jds.2013-7161).

For carnitine uptake experiments, MDBK cells were washed 2 times with 1.5 mL of Hanks' balanced salts solution (HBSS; Biochrom AG, Berlin, Germany) with 5 m*M* HEPES (pH 7.4; Sigma-Aldrich Chemie GmbH) after reaching confluence, and then incubated at 37°C with a buffer containing a 10 n*M* concentration of methyl-L-[^{3}H]-carnitine (2.96 GBq/mmol; American Radiolabeled Chemicals Inc., St. Louis, MO) for 30 min. The buffer contained either 0, 25, or 125 m*M* NaCl and 4.8 m*M* KCl, 5.6 m*M* D-glucose, 1.2 m*M* $CaCl_2$, 1.2 m*M* KH_2PO_4, 1.2 m*M* $MgSO_4$, and 5 m*M* HEPES to study the Na^+ dependence of carnitine uptake according to Glube et al. (2007). Following incubation, the medium was aspirated and cells were washed 2 times with ice-cold buffered HBSS and thereafter dissolved with 0.5 mL of 1 *M* NaOH for 1 h with shaking at room temperature. Radioactivity in cell lysates was determined by scintillation counting (PerkinElmer Liquid Scintillation Analyzer Tri-Carb 2900TR; PerkinElmer LAS GmbH, Rodgau, Germany) and was related to protein content of cell lysates as determined by the bicinchoninic acid

protein assay with BSA as standard. Carnitine uptake is expressed as the amount of L-[^{3}H]-carnitine taken up per milligram of cell protein within 30 min. Statistical evaluation of treatment effects was carried out by one-way ANOVA and Duncan's multiple range test.

To first investigate whether PPARα in MDBK cells is activated, we studied the effect of 24-h treatment of 150 μ*M* WY-14,643 on the mRNA level of the known bovine PPARα target gene *CPT1A* in MDBK cells: We found that WY-14,643 treatment caused a pronounce increase in the mRNA level of *CPT1A* in MDBK cells compared with vehicle control treatment (WY-14,643: 17.3 ± 0.3; DMSO: 1.00 ± 0; $P < 0.05$). To next study whether activation of PPARα by WY-14,643 causes induction of OCTN2 in MDBK cells, we investigated the effect of WY-14,643 on relative mRNA and protein levels of *OCTN2* in MDBK cells. As shown in Figure 1A and B, both relative mRNA and protein levels of *OCTN2* were markedly greater in MDBK cells treated with WY-14,643 than in cells treated with vehicle alone for 24 h ($P < 0.05$). To further explore whether the upregulation of OCTN2 by WY-14,643 in MDBK cells is dependent on PPARα, we studied the effect of WY-14,643 on the expression of *OCTN2* in MDBK cells that were co-treated with the PPARα antagonist GW6471 (10 μ*M*) for 24 h. Co-treatment of MDBK cells with WY-14,643 and GW6471 caused a reduction in the relative mRNA level of *OCTN2* compared with treatment with vehicle alone (Figure 1C). The relative protein level of OCTN2 did not differ between MDBK cells

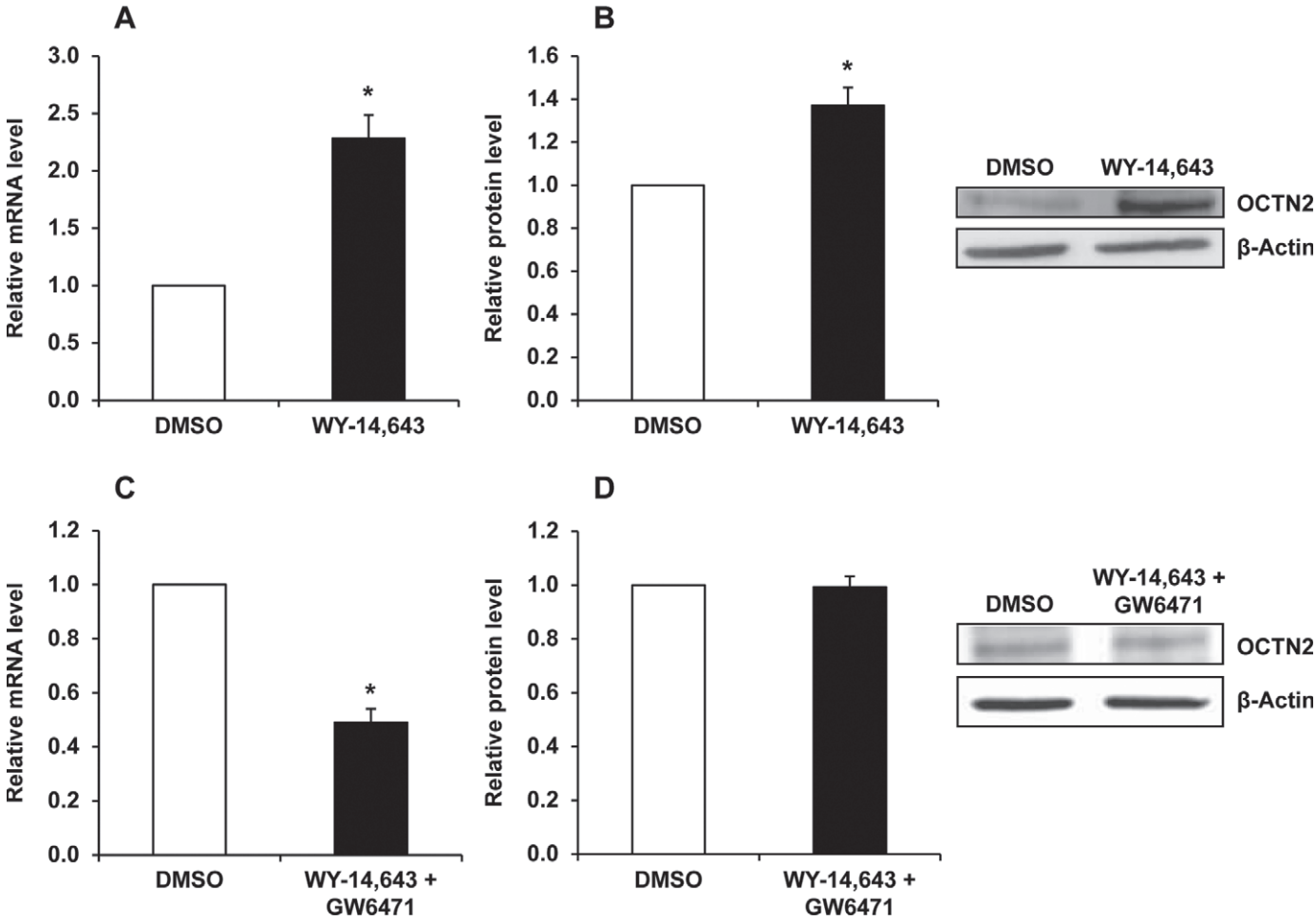

Figure 1. Effect of treatments with a 150 μ*M* concentration of WY-14,643 [a high-affinity ligand of peroxisome proliferator-activated receptor α (PPARα); Sigma-Aldrich Chemie GmbH, Steinheim, Germany] for 24 h in the absence (A and B) and presence (C and D) of PPARα-selective antagonist GW6471 (10 μ*M*; Sigma-Aldrich Chemie GmbH) on relative mRNA (A and C) and protein levels (B and D) of organic cation transporter 2 (OCTN2) in Madin-Darby bovine kidney (MDBK) cells. In panels A and C, bars represent means ± SD of 3 independent experiments and are expressed as fold of dimethyl sulfoxide (DMSO)-treated control cells. In panels B and D, bars represent data from densitometric analysis and are means ± SD of 3 independent experiments. Immunoblots specific to OCTN2 and β-actin as internal control are shown for 1 independent experiment; immunoblots for the other experiments revealed similar results. Data represent means ± SD of 3 independent experiments and are expressed as fold of DMSO-treated control cells. *Different from DMSO-treated control ($P < 0.05$).

co-treated with WY-14,643 and GW6471 and control cells (Figure 1 D). To investigate whether the upregulation of OCTN2 by WY-14,643 has an influence on Na^+-dependent carnitine uptake, which is characteristic for OCTN2-mediated carnitine uptake, we studied the uptake of methyl-L-[^{3}H]-carnitine into MDBK monolayers at different Na^+ concentrations in the incubation buffer. At 0 mM NaCl, L-carnitine uptake into MDBK cells was approximately 0.01 pmol/mg of protein per 30 min, representing non-OCTN2 dependent carnitine transport into MDBK cells. L-Carnitine uptake did not further increase in the presence of 25 m*M* NaCl, indicating that the Na^+ concentration was insufficient for facilitating Na^+-dependent OCTN2-mediated carnitine transport. At both NaCl concentrations (0 and 25 m*M*), WY-14,643 failed to increase L-carnitine uptake into MDBK cells, suggesting that WY-14,643 does not stimulate non-OCTN2-dependent carnitine transport. At 125 m*M* NaCl, the uptake of L-carnitine into MDBK cells increased in cells treated with vehicle alone to about 0.02 pmol/mg of protein per 30 min and additionally increased in cells treated with 150 μ*M* WY-14,643 to about 0.045 pmol/mg of protein per 30 min ($P < 0.05$; Figure 2A). These results indicate the presence of a Na^+-dependent transport system for L-carnitine, which applies to OCTN2, in MDBK cells and that the WY-14,643-induced L-carnitine uptake is likely mediated by OCTN2. To finally confirm the PPARα dependence of the WY-14,643-stimulated increase in L-carnitine uptake, we studied the effect of either WY-14,643 alone or WY-14,643 together with the PPARα antagonist GW6471 (10 μ*M*) on L-carnitine uptake. As shown in Figure 2B, the effect of WY-14,643 on L-carnitine uptake was completely blocked by GW6471, confirming that the effect of WY-14,643 on L-carnitine uptake is mediated by PPARα.

The main finding of the present study is that the PPARα ligand WY-14,643 increases mRNA and protein levels of OCTN2 in the bovine kidney cell line MDBK, whereas co-treatment of MDBK cells with WY-14,643 and the PPARα antagonist GW6471 blocks the WY-14,643-induced increase in mRNA and protein levels of OCTN2 in MDBK cells. A further important finding is that treatment of MDBK cells with the PPARα agonist stimulates specifically Na^+-dependent carnitine uptake in MDBK cells, which is likely a consequence of the increased carnitine transport capacity of cells due to the elevated expression of OCTN2. In addition, our data show that the WY-14,643-stimulated increase in L-carnitine uptake is completely blocked by treatment of cells MDBK with a PPARα antagonist. These findings indicate that OCTN2 expression and carnitine transport in cattle, as in rodents, are regulated by PPARα. The observed PPARα dependence of

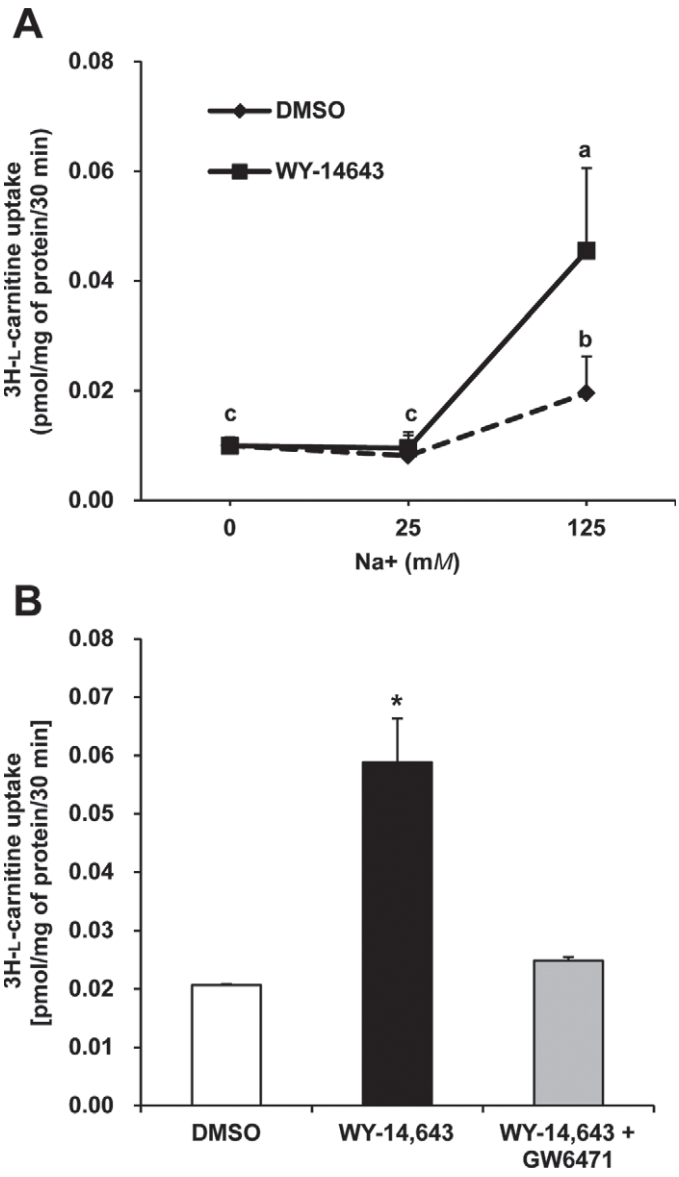

Figure 2. Effect of treatment with a 150 μ*M* concentration of WY-14,643 [a high-affinity ligand of peroxisome proliferator-activated receptor α (PPARα); Sigma-Aldrich Chemie GmbH, Steinheim, Germany] on uptake of L-[^{3}H]-carnitine (10 n*M*; specific radioactivity 2.96 GBq/mmol; American Radiolabeled Chemicals Inc., St. Louis, MO) by Madin-Darby bovine kidney (MDBK) cells. (A) Uptake of L-[^{3}H]-carnitine by MDBK cells treated for 24 h with either WY-14,643 or dimethyl sulfoxide (DMSO; control) at different Na^+ concentrations (0, 25, and 125 m*M* NaCl) was studied over 30 min. (B) Uptake of L-[^{3}H]-carnitine by MDBK cells treated for 24 h with WY-14,643, WY-14,643 together with PPARα-selective antagonist GW6471 (10 m*M*; Sigma-Aldrich Chemie GmbH), or DMSO (control) at 125 m*M* NaCl was studied over 30 min. Data represent means ± SD of 3 independent experiments, each performed in triplicate. *Different from DMSO-treated control ($P < 0.05$). Data with different letters (a–c) differ ($P < 0.05$).

OCTN2 expression provides a plausible explanation for the recent finding that OCTN2 in the liver is strongly upregulated during early lactation in high-producing

dairy cows (Schlegel et al., 2012). During early lactation, PPARα activation occurs physiologically due to the excessive flow of FA from adipose tissue to the liver where they bind to and activate PPARα (Loor et al., 2005; Loor, 2010). Thus, our observation in MDBK cells suggests that the bovine gene encoding OCTN2 is a target of PPARα. At least for the mouse gene encoding OCTN2, a functional binding site for PPARα, called peroxisome proliferator response element (**PPRE**), was identified in the first intron (Wen et al., 2010). This PPRE is responsible for direct transcriptional activation of the mouse *OCTN2* gene by PPARα. Although direct proof for the existence of a functional PPRE in the bovine *OCTN2* gene is missing, we have shown recently by sequence alignment that the functional PPRE identified in the mouse *OCTN2* gene is completely identical (100%) to a putative PPRE in the bovine *OCTN2* gene. This indicates that regulation of OCTN2 by PPARα between mouse and cattle is highly conserved and supports the assumption that the bovine *OTCN2* gene is a PPARα target gene.

In conclusion, the present study shows that expression of the carnitine transporter OCTN2 and OCTN2-mediated carnitine uptake are regulated by PPARα in bovine kidney cells. This suggests that the bovine gene encoding OCTN2, similar to the mouse *OCTN2* gene, is a target of PPARα. Future studies have to demonstrate the existence of a functional PPRE in the bovine *OCTN2* gene.

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BRIEF COMMUNICATION Open Access

Carnitine transporter OCTN2 and carnitine uptake in bovine kidney cells is regulated by peroxisome proliferator-activated receptor β/δ

Xiaodan Zhou, Robert Ringseis, Gaiping Wen and Klaus Eder*

Abstract

Background: Peroxisome proliferator-activated receptor α (PPARα), a central regulator of fatty acid catabolism, has recently been shown to be a transcriptional regulator of the gene encoding the carnitine transporter novel organic cation transporter 2 (*OCTN2*) in cattle. Whether PPARβ/δ, another PPAR subtype, which has partially overlapping functions as PPARα and is known to share a large set of common target genes with PPARα, also regulates *OCTN2* and carnitine transport in cattle is currently unknown. To close this gap of knowledge, we studied the effect of the PPARβ/δ activator GW0742 on mRNA and protein levels of *OCTN2* and carnitine uptake in the presence and absence of the PPARβ/δ antagonist GSK3787 in the bovine Madin-Darby bovine kidney (MDBK) cell line.

Findings: Treatment of MDBK cells with GW0742 caused a strong increase in the mRNA level of the known bovine PPARβ/δ target gene *CPT1A* in MDBK cells indicating activation of PPARβ/δ. The mRNA and protein level of *OCTN2* was clearly elevated in MDBK cells treated with GW0742, but the stimulatory effect of GW0742 on mRNA and protein level of *OCTN2* was completely blocked by GSK3787. In addition, GW0742 increased Na^+-dependent carnitine uptake, which is mediated by OCTN2, into MDBK cells, whereas treatment of cells with the PPARβ/δ antagonist completely abolished the stimulatory effect of GW0742 on carnitine uptake.

Conclusions: The present study shows for the first time that gene expression of the carnitine transporter OCTN2 and carnitine transport are regulated by PPARβ/δ in bovine cells. These novel findings extend the knowledge about the molecular regulation of the *OCTN2* gene and carnitine transport in cattle and indicate that regulation of *OCTN2* gene expression and carnitine transport is not restricted to the PPARα subtype.

Keywords: Bovine kidney cell, Novel organic cation transporter 2, Peroxisome proliferator-activated receptor β/δ

Findings

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors which play important roles in many metabolic and regulatory pathways through regulating the expression of a large set of target genes [1]. The ligands of PPARs include fatty acids and fatty acid derivatives and are therefore designated as transcriptional sensors of fatty acids [2]. In addition, synthetic compounds like the fibrate class of lipid lowering drugs or the insulin-sensitizing thiazolidinediones are well documented ligands of PPARs. From the PPARs, three different subtypes exist which have isotype-specific but also partially overlapping functions. For instance, both, the PPARα and the PPARβ/δ subtype are central regulators of fatty acid catabolism since both subtypes control the expression of genes encoding proteins involved in cellular fatty acid uptake, intracellular fatty acid transport, mitochondrial fatty acid uptake, and mitochondrial and peroxisomal fatty acid oxidation [1,3,4]. Recent studies convincingly demonstrated that PPARα is also a key regulator of genes involved in carnitine transport like novel organic cation transporter 2 (*OCTN2*) and carnitine synthesis like γ-butyrobetain dioxygenase (*BBD*) in many species including dairy cattle [5-8]. Activation of PPARα in dairy cattle occurs physiologically during early lactation due to extensive mobilization and release of fatty acids from adipose tissues which are taken up into the liver and non-hepatic tissues and bind to and activate PPARα [9,10]. The above

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mentioned PPARα dependence of OCTN2 and BBD expression provides a plausible explanation for the recent finding that OCTN2 and BBD in the liver are strongly upregulated during early lactation in high-producing dairy cows [11]. However, whether PPARβ/δ also regulates genes involved in carnitine homeostasis in cattle is currently unknown. PPARα and PPARβ/δ share a large set of common target genes involved in fatty acid catabolism. In addition, carnitine transport and synthesis are intrinsically linked to fatty acid catabolism, because fatty acid transport into the mitochondrial matrix is carnitine-dependent [12]. Thus, we hypothesized that PPARβ/δ also regulates genes involved in carnitine homeostasis in cattle. To verify our hypothesis we studied the effect of the PPARβ/δ activator GW0742 on mRNA and protein levels of *OCTN2* and carnitine transport in a bovine kidney cell line. Using this cell line we have very recently shown that *OCTN2* gene expression and carnitine transport are stimulated by a PPARα agonist [13]. However, kidney cells are also of relevance to study the effect of PPARβ/δ agonists in this regard because PPARβ/δ is known to be highly expressed in the kidney and OCTN2-mediated carnitine transport represents the transport mechanism for tubular reabsorption of carnitine in the kidney and is therefore fundamental for maintaining normal carnitine levels in serum [14]. We did not consider the effect of GW0742 on genes involved in carnitine synthesis in this cell line, because the kidney, unlike the liver, is not capable of synthesizing carnitine due to the lack of BBD.

Madin-Darby bovine kidney (MDBK) cells obtained from Cell Lines Service (Eppelheim, Germany) were cultivated in HyClone Minimum Essential Media/Earle's Balanced Salt Solution (MEM/EBSS) medium supplemented with 10% FBS and 0.05 mg/mL gentamicin (all from Invitrogen, Karlsruhe, Germany) at 37°C in a humidified atmosphere of 95% air and 5% CO_2 [13]. After reaching 80% confluence, MDBK cells were treated with 1 μM of the PPARβ/δ selective agonist GW0742 (Sigma-Aldrich, Steinheim, Germany) [dissolved in dimethylsulfoxide (DMSO); both from Sigma-Aldrich, Steinheim, Germany] in MEM/EBSS medium without FBS but 5 mg/L bovine insulin (Sigma-Aldrich, Steinheim, Germany) for 24 h. The incubation concentration of GW0742 was chosen based on published literature [15], in which treatment with 1 μM resulted in strong activation of PPARβ/δ. The incubation time was selected based on results from initial time course experiments (4 h, 24 h) demonstrating that the effect of GW0742 was stronger at 24 h (Figure 1A). Cells treated with vehicle alone (DMSO) were used as control. Incubation media of control cells contained the same vehicle concentration of 0.1% (v/v). For experiments using a PPARβ/δ inhibitor, cells were co-treated with 10 μM of the PPARβ/δ selective antagonist GSK3787 (Sigma-Aldrich) for 24 h. The incubation time and incubation concentration of GSK3787 was chosen also based on results from initial titration and time course experiments (concentration: 1 and 10 μM; time: 4 h, 24 h) demonstrating that inhibition of the agonist effect by GSK3787 was strongest at 10 μM and 24 h (Figure 1B). Following incubation, media was aspirated, the cell layer was washed once with phosphate-buffered saline, and plates including the attached cells were immediately stored at −80°C. All incubations were run in triplicate and each experiment was repeated three times. The mRNA levels of genes of interest (reference and target genes) in MDBK cells were determined by means of qPCR. Prior to qPCR, RNA from cells was isolated by adding TrizolTM reagent (Invitrogen, Karlsruhe, Germany) directly into the wells, and pipetting the lysed cells up and down 2–3 times. cDNA was synthesized in less than a week after RNA extraction from 1.2 μg of total RNA using 100 pmol dT18 primer (Eurofins MWG Operon, Ebersberg, Germany), 1.25 μL 10 mmol/L dNTP mix (GeneCraft, Lüdinghausen, Germany), 5 μL buffer (Fermentas, St. Leon-Rot, Deutschland), and 60 units M-MuLV Reverse Transcriptase (MBI Fermentas, St. Leon-Rot, Germany) at 42°C for 60 min, and a final inactivating step at 70°C for 10 min in a Thermal Cycler. qPCR was performed using 2 μL cDNA combined with 18 μL of a mixture composed of 10 μL KAPA SYBR FAST qPCR Universal Mastermix (Peqlab, Erlangen, Germany), 0.4 μL each of 10 μM forward and reverse primers and 7.2 μL DNase/RNase free water in 0.1 mL tubes (Ltf Labortechnik, Wasserburg, Germany). Ct-values of target genes and reference genes were obtained using Rotorgene Software 5.0 (Corbett Research). For determination of relative expression levels relative quantities were calculated using GeNorm normalization factor according to Vandesompele *et al.* [16]. The normalization factor was calculated as the geometric mean of expression data of the three most stable (*ACTB*, *ATP5B*, *SDHA*) out of five tested potential reference genes (*ACTB*, *ATP5B*, *PPIA*, *RPS9*, *SDHA*). Primer characteristics and qPCR performance data for the reference genes and target genes have been published recently [9]. Means and SD were calculated from normalized expression data for samples of the same treatment group. The mean of the vehicle (DMSO) control group was set to 1 and mean and SD of the GW0742 and GW0742 + GSK3787 groups were scaled proportionally. For immunoblot analysis, MDBK cells were lysed with RIPA lysis buffer (50 mM Tris, pH 7.5; 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) containing protease inhibitors (Sigma-Aldrich). Following determination of protein concentration of the cell lysates, 25 μg protein from the cell lysates were separated on a 10% SDS-PAGE, and proteins were transferred to a nitrocellulose membrane. Subsequently, membranes were blocked

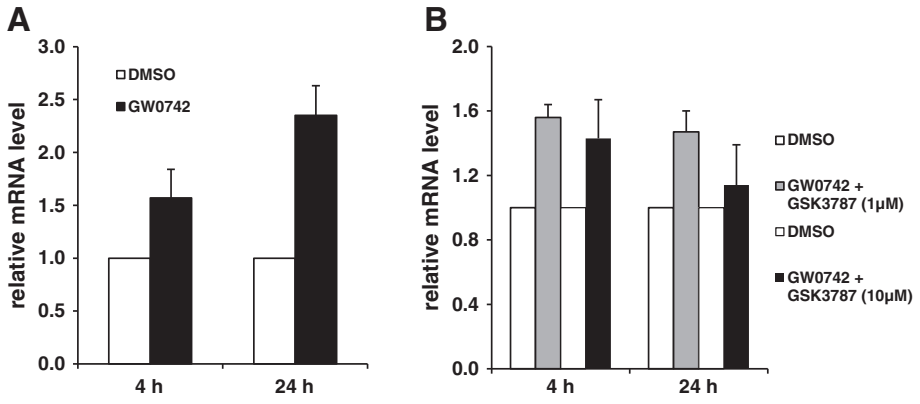

Figure 1 Effect of incubation time and effect of concentration of PPARβ/δ antagonist on relative mRNA level of OCTN2 in MDBK cells. **A**, Effect of treatment with 1 μM of PPARβ/δ agonist GW0742 for 4 and 24 h on mRNA level of *OCTN2*. **B**, Effect of treatment with 1 and 10 μM of PPARβ/δ selective antagonist GSK3787 for 4 and 24 h on mRNA level of *OCTN2*. Bars represent means ± SD of three independent experiments and are expressed as fold of DMSO-treated control cells.

overnight at 4°C in blocking solution (5% non-fat dried milk powder), and then incubated with antibodies against OCTN2 (polyclonal anti-OCTN2 antibody; dilution 1:500; Abcam, Cambridge, UK) and β-actin (monoclonal anti-β-actin; dilution 1:500, Abcam, Cambridge, UK) overnight at 4°C and for 2 h at RT, respectively. Following a washing step, the membranes were incubated with a horseradish peroxidase conjugated secondary monoclonal anti-mouse-IgG antibody (1:5000, Jackson Immuno Research, Suffolk, UK) for 1 h at room temperature. Finally, the blots were developed by using the Amersham^{TM} ECL Plus Western Blotting Detection System (GE Healthcare, Munich, Germany) and detected by a chemiluminescence imager (Syngene, Cambridge, UK). The signal intensities of specific bands were quantified using GeneTools software (Syngene, Cambridge, UK). Carnitine uptake experiments in MDBK cells using methyl-L-[^{3}H]-carnitine (80 mCi/mmol; American Radiolabeled Chemicals, St. Louis, USA) were performed as described recently in detail [13]. To study the Na^+ dependence of carnitine uptake, the incubation buffer contained either 0, 25 or 125 mM NaCl and 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 5 mM HEPES. Radioactivity in cell lysates determined by scintillation counting (Perkin Elmer Liquid Scintillation Analyzer Tri-Carb 2900TR, Rodgau, Germany) was related to protein content of cell lysates as determined by the bicinchoninic acid protein assay with BSA as standard. Carnitine uptake is expressed as the amount of L-[^{3}H]-carnitine taken up per mg cell protein within 30 min. Statistical evaluation of treatment effects was carried out by one-way ANOVA and Duncan's multiple range test.

In the first step, we investigated whether treatment of MDBK cells with the PPARβ/δ agonist GW0742 (1 μM) causes activation of PPARβ/δ. Activation of PPARβ/δ was evidenced by strongly increased mRNA levels of the known bovine PPARβ/δ target gene *CPT1A* in MDBK cells treated with GW0742 ($P < 0.05$; Figure 2A). The mRNA level of *PPARβ/δ* was slightly elevated by treatment with GW0742 ($P < 0.05$; Figure 2A). Next, we studied the effect of GW0742 on the mRNA level of *OCTN2* in MDBK cells. As shown in Figure 2B, the mRNA level of *OCTN2* was clearly elevated in MDBK cells treated with GW0742 ($P < 0.05$) indicating that bovine *OCTN2* gene transcription is regulated by PPARβ/δ. In addition, induction of the *OCTN2* gene by GW0742 in MDBK cells was also observed at the protein level ($P < 0.05$; Figure 2C). To further confirm the PPARβ/δ dependence of this effect, we studied the effect of GW0742 in the presence of GSK3787 (10 μM) on *OCTN2* gene expression. GSK3787 is a newly identified PPARβ/δ antagonist that can irreversibly attenuate the activity of PPARβ/δ by forming a covalent bond with a cysteine residue in the ligand binding domain of PPARβ/δ [17]. As illustrated in Figure 2D and E, the stimulatory effect of GW0742 on mRNA and protein levels of *OCTN2* in MDBK cells was completely blocked by the PPARβ/δ antagonist indicating that *OCTN2* gene expression is regulated by PPARβ/δ in bovine kidney cells. In a further step, we studied whether up-regulation of OCTN2 by GW0742 leads to an increased carnitine uptake. For this, we determined the uptake of methyl-L-[^{3}H]-carnitine into MDBK cells incubated with or without GW0742 at different NaCl concentrations in the incubation medium. As shown in Figure 2F, GW0742 increased carnitine uptake into MDBK cells at a NaCl concentration of 125 mM in the incubation media ($P < 0.05$) but not at 0 and 25 mM NaCl. This indicated that the PPARβ/δ agonist stimulates specifically the OCTN2-mediated carnitine uptake which is known to be sodium-dependent [18]. Finally, we provided clear evidence for the PPARβ/δ dependence of the GW0742-induced increase of carnitine uptake in showing that treatment of MDBK cells

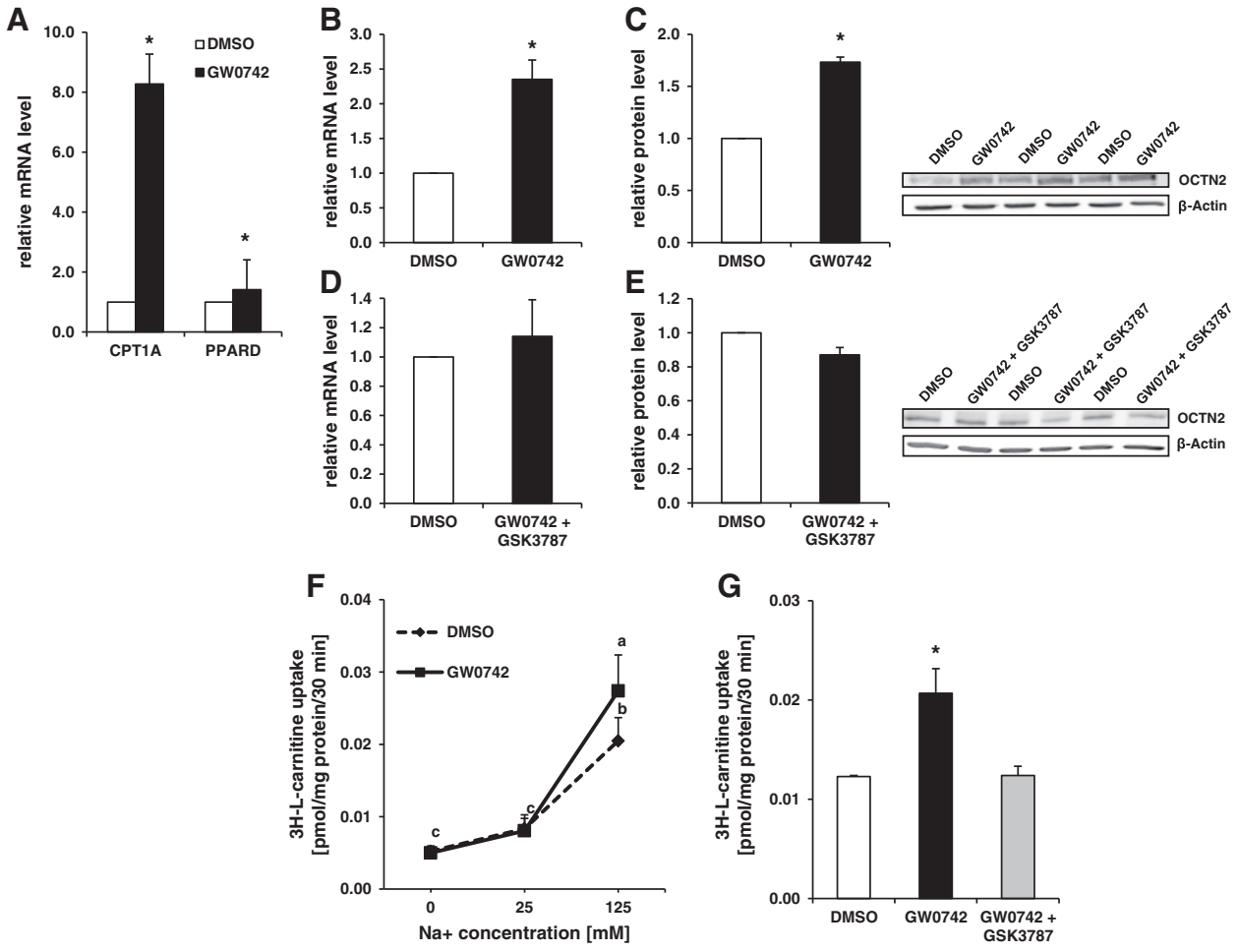

Figure 2 The carnitine transporter OCTN2 and carnitine uptake in MDBK cells is regulated by PPARβ/δ. A, Effect of treatment with 1 μM of GW0742 for 24 h on mRNA levels of *CPT1A* and *PPARD*. **B-E**, Effect of treatment with 1 μM of GW0742 for 24 h in the absence **(B, C)** and presence **(D, E)** of PPARβ/δ selective antagonist GSK3787 (10 μM) on relative mRNA **(B, D)** and protein levels **(C and E)** of *OCTN2*. **A**, **B** and **D**, Bars represent means ± SD of three independent experiments and are expressed as fold of DMSO-treated control cells. **C** and **E**, Bars represent data from densitometric analysis and are means ± SD of three independent experiments. Immunoblots specific to OCTN2 and β-Actin as internal control are shown for one independent experiment; immunoblots for the other experiments revealed similar results. Data represent means ± SD of three independent experiments and are expressed as fold of DMSO-treated control cells. *Different from DMSO-treated control, $P < 0.05$. **F** and **G**, Effect of treatment with 1 μM of GW0742 on uptake of L-[^{3}H]-carnitine (10 nM, specific radioactivity 80 Ci/mmol). F, Uptake of L-[^{3}H]-carnitine by MDBK cells treated for 24 h with either GW0742 or DMSO (control) at different Na^+ concentrations (0, 25 and 125 mM NaCl) was studied over 30 min. **G**, Uptake of L-[^{3}H]-carnitine by MDBK cells treated for 24 h with either GW0742, GW0742 together with GSK3787 (10 mM) or DMSO (control) at 125 mM NaCl was studied over 30 min. Data represent means ± SD of three independent experiments each performed in triplicate. ^{a,b,c}Data with different superscript letters differ, $P < 0.05$. *Different from DMSO-treated control, $P < 0.05$.

with the PPARβ/δ antagonist completely abolished the stimulatory effect of GW0742 on carnitine uptake (Figure 2G). In summary, these novel findings extend the knowledge about the molecular regulation of the OCTN2 gene and carnitine transport which have been convincingly demonstrated to be regulated by PPARα in cattle but also in several other species [6]. The fact that OCTN2 gene expression and carnitine transport are obviously regulated by both, PPARα and PPARβ/δ, is not surprising given that these two PPAR subtypes have partially overlapping functions. Namely, both PPAR subtypes play important roles in the regulation of mitochondrial fatty acid oxidation, which is dependent on the presence of carnitine, and therefore share a large set of common target genes involved in fatty acid oxidation [15]. Thus, our recent observation in high-producing dairy cows that OCTN2 is strongly up-regulated in the liver during early lactation [9] might be mediated by the activation of both PPAR subtypes because the fatty acids released from adipose tissue during this state are ligands and activators of both PPAR subtypes.

Conclusions

The present study shows for the first time that gene expression of the carnitine transporter OCTN2 and carnitine transport are regulated not only by PPARα but also by PPARβ/δ in bovine cells.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

XZ conducted the cell culture experiments, performed PCR analyses, immunoblot analyses, uptake experiments and statistical analyses and wrote the manuscript. GW supervised PCR analyses and helped to draft the manuscript. RR participated in the design and coordination of the study and helped to draft the manuscript. KE conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Study 3 (unpublished data)

The nuclear factor kappa B inducer TNFα increases expression of novel organic cation transporter 2 and carnitine uptake in bovine kidney cells

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ABSTRACT

Recent studies in bovine kidney cells demonstrated that peroxisome proliferator-activated receptor α (PPARα), a central regulator of energy homeostasis, is an important transcriptional regulator of the gene encoding the carnitine transporter novel organic cation transporter 2 (OCTN2). Less is known about the regulation of OCTN2 and carnitine transport in cattle by pro-inflammatory cytokines TNFα, which is a nuclear factor kappa B (NF-κB) inducer in non-ruminates. In order to explore the role of NF-κB for OCTN2 expression and carnitine transport in cattle, we studied the effect TNFα on the expression of OCTN2 in the presence and absence of NF-κB inhibitor and on OCTN2-mediated carnitine transport in the bovine Madin-Darby bovine kidney (MDBK) cell line. The results show that 5 ng/ml of TNFα increases mRNA and protein levels of OCTN2, whereas co-treatment of MDBK cells with TNFα and the NF-κB inhibitor Bay 11-7085 blocks the TNFα-induced increase of mRNA and protein levels of OCTN2 in bovine cells. In addition, treatment of MDBK cells with TNFα stimulates carnitine uptake in MDBK cells which is likely the consequence of the increased carnitine transport capacity of cells due to the elevated expression of OCTN2. In conclusion, our results indicate that OCTN2 expression and carnitine transport in cattle are regulated by NF-κB.

Keywords: bovine kidney cell, novel organic cation transporter 2, nuclear factor kappa B

INTRODUCTION

Dairy cows suffer multitudes of disorders during the peripartum, particularly high rates of mastitis, metritis, ketosis and fatty liver. It is well-documented that inflammation is involved in these infections and metabolic diseases. Although the immune function is suppressed by the negative energy balance, which resulting from the dramatically increase of energy requirements and the depression of the feed intake, inflammatory cytokines play a key role in the pathology of metabolic disorders in transition cows. Cytokines are cell signaling molecules, which can change many physiological systems for the wide expression of receptors in nearly all cell types. Tumor necrosis factor-α (TNFα) is one of the cytokines that has been demonstrated to activate nuclear factor kappa B (NF-κB) pathway via TNF receptor (TNFR) -TNF receptor-associated factor (TRAF) - inhibitor of kappa B kinase (IKK) pathway. Besides the crucial roles in the innate and adaptive immunity, cell proliferation and apoptotic processes, TNFα has the ability to directly interfere with the lipid metabolism. In adipose tissues, TNFα stimulates lipolysis via TNFR1 and involved the activation of mitogen-activated protein kinase family. In liver, TNFα increases the hepatic level of citrate consequently activating acetyl-CoA carboxylase, which is the rate limiting enzyme of free fatty acids synthesis.

OCTN2 is a member of the solute carrier 22A family, which is expressed in various organs, including the kidney. It has been confirmed to transport carnitine in a Na^+-dependent manner, guaranteeing carnitine absorption and distribution within the body (Tamai et al., 2000; Tamai et al., 2001). Carnitine is an essential cofactor for mitochondrial fatty acid oxidation by transferring long-chain fatty acids as acylcarnitine esters across the inner mitochondrial membrane (McGarry et al., 1997). The deficiency of

OCTN2 has been associated with many diseases, including systemic primary carnitine deficiency, Crohn's disease and diabetes. It has been reported that PPARα is the main regulator of OCTN2 (Eder and Ringseis, 2010). Our previous researches have shown that OCTN2 and carnitine uptake are regulated by PPARs in bovine kidney cells. Fujiya et al., (2011) has reported OCTN2 expression and carnitine uptake in human colonic epithelial cells are increased by proinflammatory cytokines TNFα. However, it is unclear whether the inflammation is able to alter OCTN2 and carnitine uptake in bovine kidney cells. We hypothesized TNFα could up-regulate OCTN2 expression and increase carnitine uptake in bovine kidney cells.

MATERIALS AND METHODS

Cell Culture

Madin-Darby bovine kidney (MDBK) cells obtained from Cell Lines Service (Eppelheim, Germany) were cultivated as described recently in detail. For experiments, cells were seeded out into 6-well plates at a density of 2.0×10^5 cells (for qPCR and immunoblot analysis experiments) or 24-well plates at a density of 7×10^4 cells (for L-carnitine uptake studies) or 96-well plates at a density of 1.2×10^4 cells (for Transient transfection and dual luciferase reporter gene assays) in HyClone MEM/EBSS complete medium. After reaching 80% confluence, MDBK cells were treated with 1, 5, 10, 20 ng/mL of TNFα (Sigma-Aldrich, Steinheim, Germany) [dissolved in dimethylsulfoxide (DMSO); both from Sigma-Aldrich, Steinheim, Germany] in MEM/EBSS medium without FBS but 5 mg/L bovine insulin (Sigma-Aldrich, Steinheim, Germany) for 24 h. Cells treated with vehicle alone (DMSO) were used as control. Incubation media of control cells contained the same vehicle concentration of 0.1 % (v/v). For experiments using a NF-κB inhibitor,

cells were co-treated with 1 µM of the BAY 11-7085(Merck, Bruchsal, Germany) in combination with 5 ng/mL of TNFα for 24 h. Following incubation, media was aspirated, the cell layer was washed once with phosphate-buffered saline (PBS), and plates including the attached cells were immediately stored at -80°C. All incubations were run in triplicate and each experiment was repeated three times.

RNA isolation, cDNA synthesis and qPCR analysis

Total RNA was isolated from MDBK cells after treatment using 500 µL Trizolreagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. RNA from cells was extracted within 3 days after the experiment. Isolated RNA was stored at -80°C until use. Concentrations and purity of isolated RNA were determined using an Infinite 200M microplate reader and a NanoQuant Plate (both from Tecan, Mannedorf, Switzerland). The A260/A280 ratios were 1.90±0.05 (mean±SD). The integrity of RNA was assessed by 1% agarose gel electrophoresis. RNA was considered to be suitable for use only if intact bands corresponding to 18S and 28S ribosomal RNA subunits were visible. First-strand cDNA was synthesized from 1.2 µg of total RNA using 100 pmolof dT18 primer (Eurofins MWG Operon, Ebersberg, Germany), 1.25 µl of 10 mM dNTP mix (GeneCraft, Lüdinghausen, Germany), 5 µl of buffer (Fermentas, St. Leon-Rot, Germany), and 60 unitsM-MuLV Reverse Transcriptase (MBI Fermentas, St. Leon-Rot, Germany) at 42°C for 60 min and a subsequent inactivating step at 70°C for 10 min in Biometra Thermal Cycler (WhatmanBiometra®, Göttingen, Germany). Finally, cDNA was preserved in aliquots at -20 °C. The mRNA levels of genes of interest (reference and target genes) in MDBK cells were determined by means of qPCR using gene-specific primer pairs as described in **Table 1**. For normalization of gene expression

levels the GeNorm normalization factor according to Vandesompele et al (2002). ACTB, ATP5B and RPS9 were calculated as the geometric mean of expression data of the three most stable out of five tested potential reference genes. Means and SD were calculated from normalized expression data for samples of the same treatment group. The mean of the vehicle (DMSO) control group was set to 1 and mean and SD of the TNFα groups were scaled proportionally.

Transient transfection and dual luciferase reporter gene assay

For transient transfections, MDBK cells were plated in 96 well plates at a density of 1.2×10^4. After plating for 15 h, according to the manufacture's protocol, cells were transiently transfected with 50 ng of NF-κB-Luc (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, Germany) for 8 h using FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany). NF-κB-Luc is a plasmid containing an NF-κB response element in front of the firefly luciferase reporter gene. Cells were also co-transfected with 5 ng of pGL4.74 Renilla luciferase (Promega, Mannheim, Germany), which was considered as an internal control reporter vector to normalize the differences in transfection efficiency. For measurement of NF-κB activation, the differentiated MDBK cells were treated with or without 5 ng/mL of TNFα for 24 h. Then, cells were washed with PBS and lysed with the passive lysis buffer (Promega). Normalized luciferase activities were determined with the dual luciferase reporter assay system (Promega) using a Mithras LB940 luminometer (Berthold Technologies, Bad Wildbad, Germany). Normalized luciferase activities are expressed as fold of control cells, and luciferase activity of control cells was set to 1.

Immunoblot analysis

MDBK cells were harvested and lysed with RIPA lysis buffer (50 MmTris pH 7.5, 150 Mm NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) containing protease inhibitor cocktail (Sigma-Aldrich, Steinheim, Germany). The concentration of the total protein was determined using the bicinchoninic acid protein assay kit according to the manufacturer's instructions (Interchim, Montluçon, France) with BSA as standard. 20 µg protein from the cell lysates were separated on a 10% SDS-PAGE. Afterwards proteins were transferred to a nitrocellulose membrane (Pall Corporation, Pensacola, FL, USA). Adding of equal amounts of protein in every line was verified by Ponceau S (Carl Roth, Karlsruhe, Germany) staining. The membranes were blocked overnight at 4°C in blocking solution (5% nonfat dried milk powder), and then incubated with the antibodies against OCTN2 (polyclonal anti-OCTN2 antibody; dilution 1:500; Abcam, Cambridge, UK) and β-actin (monoclonal anti-β-actin; dilution 1:500, Abcam, Cambridge, UK) overnight at 4°C and for 2 h at room temperature, respectively. The membranes were washed with TBS-T (50 mmol/L Tris, 150 mmol/L NaCl, pH 7.5, 0.2% Tween-20) and incubated with the horseradish peroxidase conjugated secondary monoclonal anti-mouse-IgG antibody (1:5000, Jackson Immuno Research, Suffolk, UK) for 1 h at room temperature. The blots were developed by using the Amersham^{TM} ECL Plus Western Blotting Detection System (GE Healthcare, Munich, Germany) and detected by a chemiluminescence imager (Syngene, Cambridge, UK). The signal intensities of specific bands were quantified using GeneTools software (Syngene, Cambridge, UK). The fold increase in band intensity for the treatment group was calculated and normalized against control.

Carnitine uptake

Carnitine uptake experiments in MDBK cells using methyl-L-[^{3}H]-carnitine (2.96 GBq/mmol; American Radiolabeled Chemicals, St. Louis, USA) were performed as described recently in detail (Zhou et al., 2014). Cells were washed 2 times with 1.5 mL of Hanks´ balanced salts solution buffer (HBSS, Biochrom AG, Germany) with 5 mM HEPES (Sigma, Steinheim, Germany) and co-incubated with 10 nM [^{3}H]-L-carnitine in a 37°C shaking water bath for 30 min. Afterwards, the medium was aspirated and the MDBK cells were immediately washed 2 times with 1 mL of ice-cold buffered HBSS before 0.5 mL of 1 M NaOH was added. The cells were incubated for 1 h at room temperature with shaking (75 rpm/min). The total volumes of samples were then collected, and 4 mL of Rotiszint Ecoplus scintillation fluid (Carl Roth GmbH, Germany) was added for scintillation counting (Perkin Elmer Liquid Scintillation Analyzer Tri-Carb 2900TR, U.S.A). Radioactivity in cell lysates was related to protein content of cell lysates as determined by the bicinchoninic acid protein assay with BSA as standard. Carnitine uptake is expressed as the amount of L-[^{3}H]-carnitine taken up per mg cell protein within 30 min.

Statistical Analysis

All data were analyzed by one-way analysis of variance (ANOVA) using the Minitab Statistical Software (Rel. 13.0 State College, PA, USA). Effects were considered significant when $P<0.05$. Data in the figures are presented as mean ± S.D.

RESULTS

Effects of the cytokine TNFα on NF-κB activation in bovine kidney cells

To determine if NF-κB in MDBK cells is activated, we studied the effect of the TNFα

on the known bovine NF-κB target genes IL6 and IL1B expression in MDBK cells. We found that TNFα increased IL6 and IL1B mRNA expression in a concentration-dependent manner at 24 h (Figure 1A). Compared to the control, the mRNA levels of the IL6 and IL1B were elevated about 10 fold by the treatment of 5 ng/ml of TNFα ($P < 0.05$; **Fig. 1A**). We further investigated the influence of the cytokine TNFα on NF-κB DNA-binding activity. The transactivation of NF-κB was significantly increased by the treatment of TNFα compared to the control in MDBK cells ($P < 0.05$; **Fig. 1B**). Our results show that 5 ng/ml of TNFα increased mRNA levels of NF-κB target genes and induced NF-κB transactivation.

Effects of the cytokine TNFα on transcription and protein levels of OCTN2 in MDBK cells

To study whether activation of NF-κB by TNFα causes induction of OCTN2, we investigated the effect of TNFα on relative mRNA and protein levels of OCTN2 in MDBK cells. As shown in **Fig. 2 A** and **C**, both, relative mRNA and protein levels of OCTN2 were markedly greater in MDBK cells treated with 5 ng/ml TNFα than in cells treated with vehicle alone for 24 h ($P < 0.05$). To further explore whether the up-regulation of OCTN2 by TNFα in MDBK cells is dependent on NF-κB, we studied the effect of TNFα on mRNA and protein levels of OCTN2 in the presence of the NF-κB inhibitor BAY 11-7085. The stimulatory effect of TNFα on transcription and protein levels of OCTN2 was completely abolished by BAY 11-7085 in MDBK cells (**Fig. 2 B and D**). The stimulatory effect of TNFα on mRNA and protein levels of OCTN2 in MDBK cells was completely blocked by the NF-κB inhibitor indicating that OCTN2 gene expression is regulated by NF-κB in bovine kidney cells.

Effects of the cytokine TNFα on [^{3}H] -L- carnitine uptake in MDBK cells

To investigate whether the up-regulation of OCTN2 by TNFα leads to an increased carnitine uptake, which is characteristic for OCTN2-mediated, we studied the uptake of methyl-L-[^{3}H]-carnitine into MDBK monolayers in the HBSS incubation buffer. Compared to the control, the carnitine uptake was increased in cells treated with 5 ng/ml of TNFα to about 0.060 pmol/mg protein/30 min ($P < 0.05$, **Fig. 3**). To further confirm the NF-κB dependence of the TNFα-stimulated increase of L-carnitine uptake, we studied the effect of TNFα in the presence of the BAY 11-7085(1 μ*M*) on L-carnitine uptake. As shown in **Fig. 3**, the effect of TNFα on L-carnitine uptake was completely blocked by BAY 11-7085 confirming that the effect of TNFα on L-carnitine uptake is mediated by NF-κB. The results from uptake experiments indicate that the up-regulation of OCTN2 by TNFα lead to an increase in carnitine uptake. Taken together, it is clear that bovine gene encoding OCTN2 is a target of NF-κB regulating carnitine reabsorption in kidney cells.

In conclusion, the present study shows for the first time that gene expression of OCTN2 and carnitine transport are both regulated by NF-κB inducer TNFα in bovine kidney cells. These findings indicate that inflammation may contribute to enhance the mitochondrial fatty acid oxidation.

Table 1. Primer characteristics used for qPCR assay

Gene[1]	Forward primer (from 5´ to 3’) Reverse primer (from 5’ to 3’)	PCR product size (bp)	NCBI GenBank accession no	Efficiency	Mean CT
Reference genes					
ACTB	ACTTGCGCAGAAAACGAGAT CACCTTCACCGTTCCAGTTT	120	AY141970	1.99	9.31
ATP5B	GGACTCAGCCCTTCAGCGCC GCCTGGTCTCCCTGCCTTGC	229	NM_175796.2	1.82	11.94
PPIA	GGCAAATGCTGGCCCCAACACA AGTACCACGTGCTTGCCATCCA	87	NM_178320.2	1.86	11.02
RPS9	GTGAGGTCTGGAGGGTCAAA GGGCATTACCTTCGAACAGA	108	BC148016	1.89	14.18
SDHA	GCAGAACCTGATGCTTTGTG CGTAGGAGAGCGTGTGCTT	185	NM_174178	1.71	15.82
Target genes					
IL1B	GCTGCATCCAACACCTGGA GGATGCTCCTCAGGTCATC	177	NM_174093.1	2.13	25.84
IL6	ACTCCCGCTTCACAAGCGCCTTC AAGTAGTCTGCCTGGGGTGGTGTCA	134	NM_173923.2	1.98	20.69
OCTN2	CACAGTGGTCAGGAACATGG AATGGTGTCTGGGAGTGGAG	181	BC105377	1.93	18.90

[1]*ACTB*=β-actin*; ATP5B*=ATP synthase, H+ transporting, mitochondrial F1 complex, β polypeptide; *IL1B*=interleukin 1 beta; *IL6*=interleukin 6; *PPIA*=peptidylprolylisomerase A; *RPS9*=ribosomal protein S9; *SDHA*=succinate dehydrogenase complex, subunit A; *OCTN2*=solute carrier family 22 (organic cation/carnitine transporter), member 5.

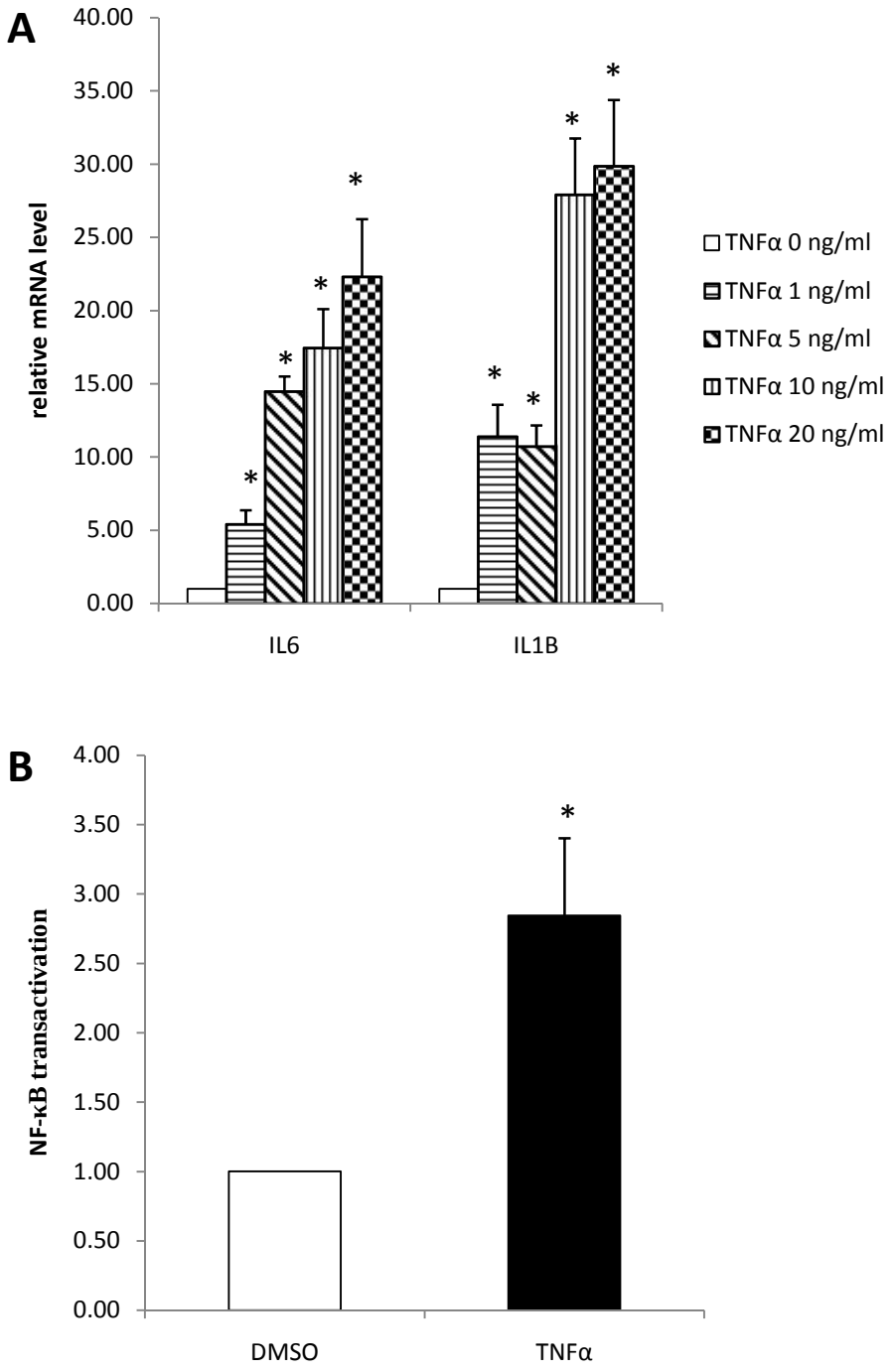

Figure 1: The activation of nulear factor κB (NF-κB) is induced by TNFα in MDBK cells. Effect of treatments with 0, 1, 5,10, 20 ng/ml of TNFα for 24 h on mRNA levels of IL6 and IL1B (A). Effect of treatment with 5ng/ml of TNFα for 24 h on NF-κB activation in MDBK cells (B). Bars represent means ± SD of three independent experiments and are expressed as fold of DMSO-treated control cells. *Different from DMSO-treated control, $P < 0.05$

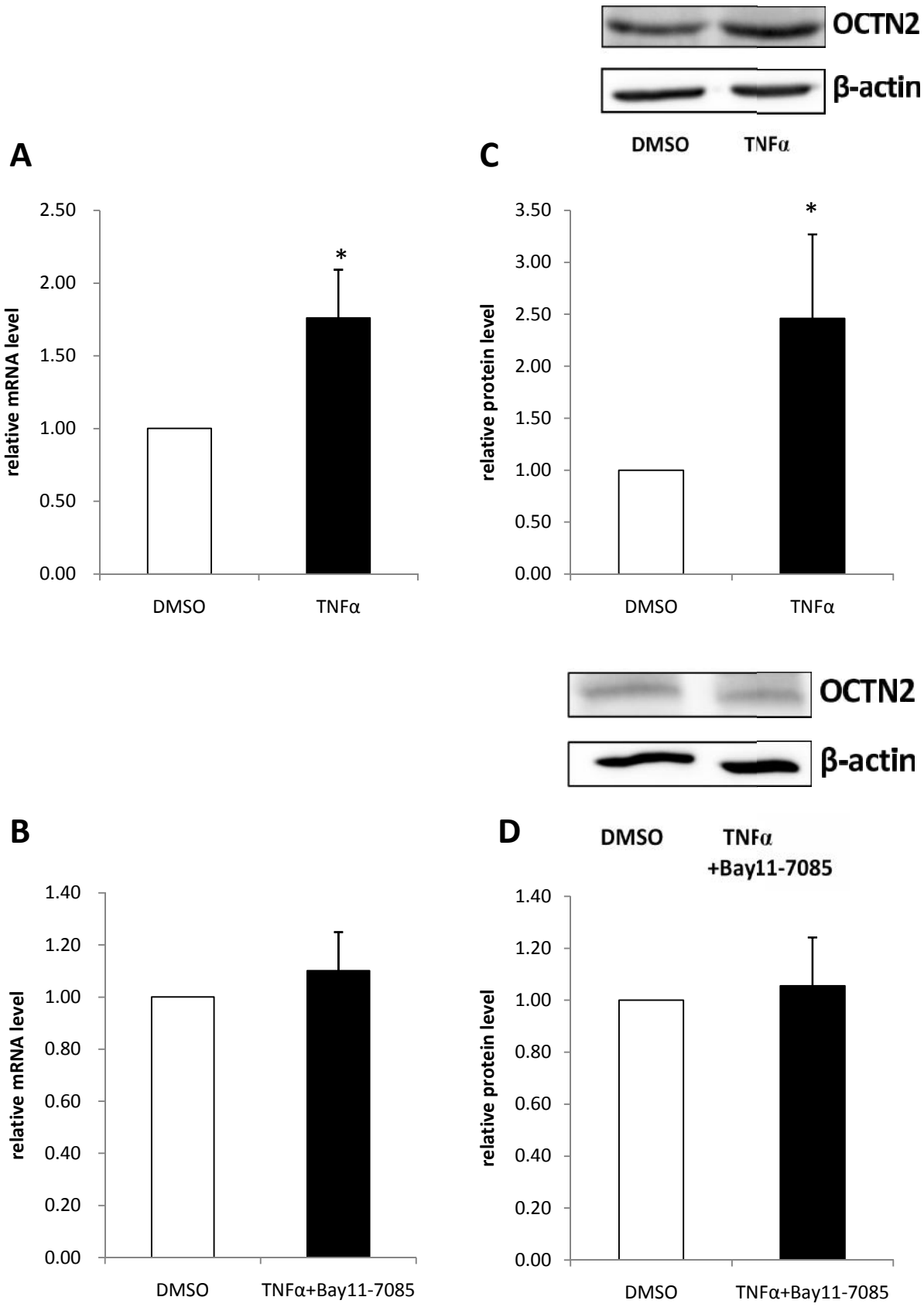
OCTN2
β-actin
DMSO
TNFα
A
C
relative mRNA level
relative protein level
2.50
2.00
1.50
1.00
0.50
0.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
*
*
DMSO
TNFα
OCTN2
β-actin
B
D
DMSO
TNFα
+Bay11-7085
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
DMSO
TNFα+Bay11-7085

Figure 2: The carnitine transporter OCTN2 in MDBK cells is regulated by NF-κB. Effect of treatment with 5ng/ml of TNFα for 24 h in the absence (A, C) and presence (B, D) of NF-κB inhibitor Bay 11-7085 (1 μ*M*) on relative mRNA (A, B) and protein levels (C and D) of OCTN2. A and B, Bars represent means ± SD of three independent experiments and are expressed as fold of DMSO-treated control cells. C and D, Bars represent data from densitometric analysis and are means ± SD of three independent experiments. Immunoblots specific to OCTN2 and β-Actin as internal control are shown for one independent experiment; immunoblots for the other experiments revealed similar results. Data represent means ± SD of three independent experiments and are expressed as fold of DMSO-treated control cells. *Different from DMSO-treated control, $P < 0.05$.

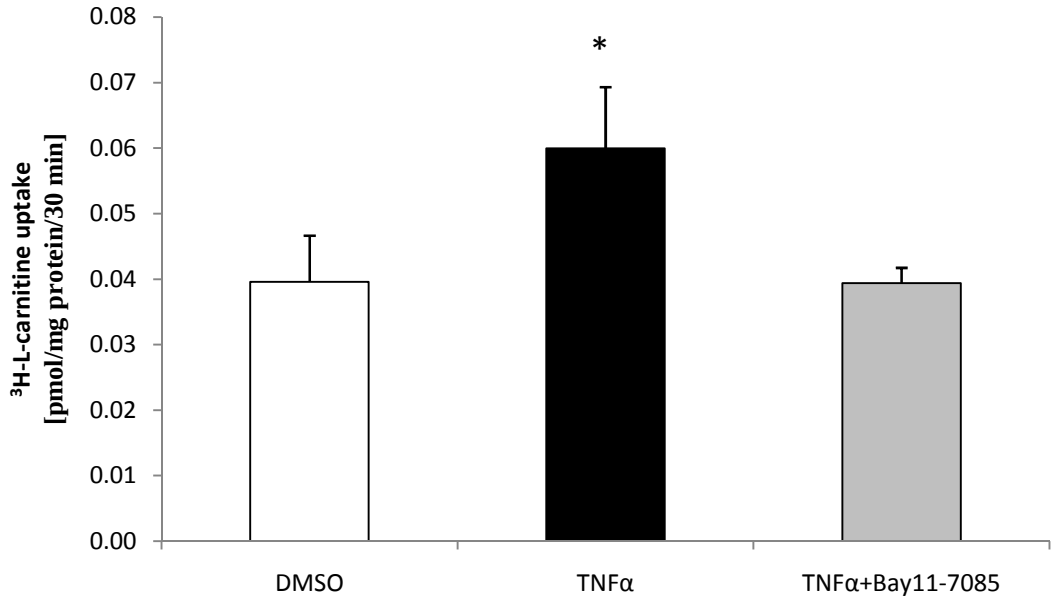

Figure 3: The carnitine uptake in MDBK cells is regulated by NF-κB. Effect of treatment with 5ng/ml of TNFα with or without Bay 11-7085 (1μ*M*) on uptake of L-[^{3}H]-carnitine (10 n*M*, specific radioactivity 2.96 GBq/mmol). Data represent means ± SD of three independent experiments each performed in triplicate. *Different from DMSO-treated control, $P < 0.05$.

4 DISCUSSION

The global aim of the present dissertation was to test the hypothesis that activation of nuclear transcription factors PPARα, PPARβ/δ and NF-κB, respectively, in bovine kidney cells enhances carnitine uptake by increasing transcription and protein levels of carnitine transporter OCTN2.

It is well known that CPT-1 is a PPARα downstream gene in both ruminant and non-ruminant animals (Desvergne et al., 2006; Bionaz et al., 2008). Our data in **study 1** show that expression of CPT-1 was strongly up-regulated in MDBK cells by treatment with PPARα agonist (150 µM of WY-14,643) indicating PPARα activation. This result is consistent with a number of studies (Bionaz et al., 2012; Thering et al., 2009; Bionaz et al., 2008), which have also demonstrated that WY-14,643 is an efficient PPARα activator in MDBK cells. **Study 1** shows that WY-14,643 leads to an up-regulation of mRNA and protein levels of OCTN2 in bovine kidney cells. This result is in agreement with previous studies that the transcription of OCTN2 is mediated by PPARα in non-ruminant cells and tissues including kidney, liver, heart, and intestine (Eder and Ringseis, 2010). In rat hepatoma (Fao) cells, treatment with 50 µM of WY-14,643 increased the mRNA concentration of OCTN2 (Luci et al., 2006). In the kidney of rats, the mRNA abundance of OCTN2 in the energy restriction group was 3.7-fold higher than in the control group (Luci et al., 2008). In wild-type mice, expression of OCTN2 in the kidney was increased by WY-14,643, whereas it remained unchanged in PPARα$^{-/-}$ mice (Koch et al., 2008). Although the regulation of carnitine homeostasis in ruminants has been less investigated, one resent study from our group reported that during early lactation mRNA abundances of PPARA and OCTN2 were increased 2.0-fold and 13-fold, respectively, in liver biopsy samples of dairy cows (Schlegel et al., 2012). To further explore whether the up-regulation of OCTN2 by WY-14,643 in MDBK cells is dependent on PPARα, we studied the effect of WY-14,643 on the expression of OCTN2 in MDBK cells that were co-treated with the PPARα antagonist GW6471. We first demonstrate that the antagonist GW6471 completely blocks the mRNA and protein expression of OCTN2, induced by PPARα agonist in bovine kidney cells. GW6471 has been widely used as a PPARα antagonist, which blocks the carboxy-terminal activation helix of PPARα by adopting the active position (Xu et al., 2002). Our finding that OCTN2 is strongly up-regulated by PPARα agonist and inhibited by PPARα antagonist in MDBK cells indicates that PPARα regulates the gene encoding OCTN2 in ruminant renal cells. The following study shows that the treatment of MDBK cells with

WY-14,643 increased [^{3}H]-L-carnitine uptake and the treatment with PPARα antagonist completely blocks the effect induced by WY-14,643. This is very important evidence that PPARα activation enhances carnitine reabsorption in bovine kidney cells. Consistent with our results, it has been reported that another PPARα agonist, fenofibrate, can increase the uptake of [^{3}H]-L-carnitine in freshly isolated hepatocytes from rats (Maeda et al., 2008). Furthermore, it has been widely confirmed that carnitine reabsorption in the kidney is mainly carried out via OCTN2 (Tamaiet al., 2001; Glube et al., 2007). Thus our results from **study 1** suggest that the increased carnitine uptake in response to WY-14,643 is due to the up-regulation of OCTN2. In **study 1**, we provide indirect evidence that OCTN2 is a PPARα target gene in bovine cells. Earlier studies in our group have elucidated that a functional PPRE is located in the first intron of mouse OCTN2 gene (Wen et al., 2012), indicated binding of PPARα/retinoid-X-receptor heterodimer directly to PPRE and activating OCTN2 expression. Although we did not further analyze the functional PPRE in the promoter/intron of OCTN2 in cattle, we found that cattle have an identical sequence with mouse functional PPRE in intron 1 of OCTN2 (Ringseis et al., 2012). We speculate that bovine OCTN2 perhaps has a similar regulation mechanism as the mouse OCTN2. However, this speculation has to be clarified in future studies. In addition, the observed PPARα dependence of OCTN2 expression provides a plausible explanation for the recent finding that OCTN2 in the liver is strongly up-regulated during early lactation in high-producing dairy cows (Schlegel et al. 2012).

An expanding body of literature has established the importance of PPARα in the regulation of carnitine homeostasis (Eder and Ringseis, 2010; Ringseis et al., 2012). However, a consensus on the role of other PPAR isotypes (PPARβ/δ and PPARγ) in regulating genes involved in carnitine uptake has not emerged. In **study 2**, we report that the transcription level of OCTN2 is markedly increased by the selective PPARβ/δ agonist GW0742. This finding indicates that regulation of OCTN2 expression by PPAR in kidney cells in ruminants is not restricted to the PPARα isotype. Given the fact that the mRNA of PPARβ/δ in cattle has lager abundance compared to PPARα in MDBK cells, it is not surprising that OCTN2 is also regulated by PPARβ/δ, because PPARβ/δ has partially overlapping functions as PPARα (Bionaz et al., 2013; Ringseis et al., 2012). For example, it is well established that muscle-type CPT and CACT are responsive to both PPARα and PPARβ/δ (Gilde et al., 2003; Gutgesell et al., 2009). On the contrary, the lack of response of OCTN2 to TGZ (data not shown) is an argument against the role for PPARγ in the regulation of carnitine homeostasis. D'Argenio et al. (2010) have demonstrated that PPARγ modulates colonic OCTN2 gene expression in humans and

mice. This could be partially explained by the fact that PPAR agonists exert distinct species-specific and tissue-specific actions (Ringseis et al., 2012), but it probably reflects the lower importance of PPARγ in bovine kidney cells, which primarily triggers the expression of genes responsible for de novo fatty acid synthesis and TG synthesis in mammary epithelial cells (Kadegowda, et al., 2009) and control of adipogenesis in adipose tissue (Rosen and Spiegelman, 2000). To further investigate whether the up-regulation of OCTN2 by GW0742 in MDBK cells is dependent on PPARβ/δ, we studied the effect of GW0742 on the expression of OCTN2 in MDBK cells that were co-treated with the PPARβ/δ antagonist GSK3787. GSK3787 is a newly identified PPARβ/δ antagonist that can irreversibly attenuate the activity of PPARβ/δ by forming a covalent bond with a cysteine residue in the LBD of PPARβ/δ (Shearer et al., 2010; Palkar et al., 2010). In the presence of GSK3787, the increased mRNA level of OCTN2 induced by GW0742 is eliminated. Taken together, our results suggest that transcription of carnitine transporter OCTN2 in bovine kidney cells is regulated by PPARβ/δ. In **study 2**, we determined OCTN2 relative protein concentration and [^{3}H]-L-carnitine uptake activity in MDBK cells with the treatment of PPARβ/δ agonist GW0742 in the presence or absence of PPARβ/δ antagonist GSK3787. Similar to the results in **study 1**, it shows the increase of OCTN2 protein level and [^{3}H]-L-carnitine uptake activity induced by PPARβ/δ agonist were totally abolished by its antagonist. These results provide indirect evidence indicating OCTN2 is a potential PPARβ/δ target gene in ruminants. To our knowledge, this is the first report that PPARβ/δ can regulate OCTN2 in mammalian cells.

The structural similarity in DNA binding domain between PPARs in cattle may explain that the activation of PPARα and PPARβ/δ could lead to similar outcomes in genes involved in fatty acid catabolism and carnitine transport. PPARα and PPARβ/δ have 59% similarity in amino acid residues. More than 80% of conservation is in the DNA binding domain, but less than 21% of conservation is in the A/B domain. The PPARα has 71% of conservation in the LBD with PPARβ/δ (Bionaz et al., 2013). However, the ligand-binding pocket of PPARβ/δ is narrower than those of PPARα and PPARγ. It inhibits the binding of PPARγ agonist TZD to PPARβ/δ and strongly decreases the affinity of PPARβ/δ towards PPARα agonist, because the bulky acid and alkyl groups on these compounds cannot be accommodated by the narrow pocket (Zoete et al., 2007; Bugge and Mandrup, 2010). Whereas the exact molecular mechanism underlying the activation of non-isoform selective target genes such as CPT1A and OCTN2 by PPARs is unknown, two factors may contribute to it: (i) The A/B domain of PPARs, which is responsible for the isotype specific transactivation, is dispensable to this

group of target genes for its lacking of the recruitment and tethering of histone acetylase complexes (Bugge and Mandrup, 2010). Histone acetylase complexes are a class of enzymes catalyzing the transfer of an acetyl group from acetyl-CoA to the lysine amino groups on the N-terminal tails of histones. These enzyme complexes are involved in transcriptional activation by neutralizing histone charge and forming acetyl-lysines on histone tails. Neutralized histones can weaken histone-DNA and internucleosome contacts and reduce chromatin compaction for the initiation of transcription. The acetyl-lysines on histone tails may provide recognition sites for factors involved in either the activation or repression of gene expression (Carrozza et al., 2003). (ii) PPARα and PPARβ share some coactivators. For instance, murine double minute 2, an ubiquitin ligase, is identified as an interacting protein in A/B domain of PPARs. Interestingly, this coactivator regulates PPARα and PPARβ/δ, but not PPARγ transcriptional activity (Gopinathan et al., 2009). It is in agreement with the fact that OCTN2 in bovine kidney cells is regulated by both PPARα and PPARβ/δ but has no effect in response to PPARγ activation.

It is well documented that inflammation is associated with hypertriglyceridemia (Sammalkorpi et al., 1988; Grunfeld et al., 1992; van Diepen et al., 2013). It has been confirmed that pro-inflammatory cytokines or LPS induce the elevation of serum TG. It is mainly caused by an increase of very low-density lipoprotein (VLDL), which is the transporter of TG within the water-based solution of the bloodstream. The increased VLDL results from either increased VLDL production or decreased VLDL clearance. For instance, pro-inflammatory cytokine TNFα can stimulate hepatic lipogenesis via increasing intracellular concentration of citrate, an allosteric activator of acetyl-CoA carboxylase in rodents, which is the rate-limiting enzyme of fatty acid synthesis and related to increased VLDL production (Feingold and Grunfeld, 1987). It can also inhibit lipoprotein lipase activity in cultured adipocytes (Patton et al., 1986) and decrease apoE mRNA in liver, which are required for the clearance of VLDL (Hardardottir et al., 1997; Lanza-Jacoby et al., 1992). Furthermore, pro-inflammatory cytokines can increase adipose tissue lipolysis, which provides free fatty acids for enhanced hepatic TG synthesis (Khovidhunkit et al., 2004). Massive infection such as sepsis decreases hepatic expression of both CPT-1 and CPT-2, which are necessary for translocation of long-chain fatty acids via carnitine across the mitochondrial membrane for β-oxidation (Barke et al., 1996). However, the concentration of carnitine in plasma is increased in heart failure patients, who are characterized by modestly elevating levels of pro-inflammatory cytokines such as TNFα (Vescovo et al., 2005). Na^+-

dependent carnitine uptake in human colonic epithelial cells is increased by treatment with IFN-γ and TNFα in a time-dependent manner (Fujiya et al., 2011). In vivo, TNFα antibody XT22 completely abolishes TNFα-induced carnitine uptake in both jejunum and ileum in mice (Fujiya et al., 2011). These findings indicate that the occurrence of inflammatory processes have a potential role in promoting fatty acid oxidation, which may relieve the pathogenic pressure caused by TG-rich lipoproteins (Malloy and Kane, 2001).

In **study 3**, a model of the activation of NF-κB in bovine kidney cells was established. We administered different concentration (from 1 to 20 ng/ml) of TNFα in the cell medium, and investigated the gene expression of NF-κB known target genes. In addition, we detected the influence of TNFα on NF-κB transactivation in MDBK cells, which were transiently transfected with a NF-κB-responsive reporter plasmid. TNFα is a well-known pro-inflammatory cytokine, which is secreted during the inflammation process in transition period in dairy cows. Several studies have shown that incubation of cells with TNFα can stimulate NF-κB activation (Gérardin et al., 2004; Schleser et al., 2006). Known target genes of NF-κB, including IL-6 and IL-1B encode their respective interleukins, which are first found in leukocytes and later have been confirmed to be produced by a wide variety of cells. Our results show that 5 ng/ml of TNFα increased mRNA levels of IL-6 and IL-1B, and induced NF-κB transactivation. These results are in agreement with previous studies in other cell types (Ringseis et al., 2006; Gessner et al., 2011), indicating that bovine kidney cells are a suitable model for the activation of NF-κB. We observed the effect of TNFα on relative mRNA and protein levels of OCTN2 in MDBK cells, in order to next investigate, whether activation of NF-κB by TNFα causes induction of OCTN2. **Study 3** shows that relative mRNA and protein levels of OCTN2 in MDBK cells treated with TNFα were markedly greater than in the control. The result of OCTN2 protein expression is consistent with one earlier observation that TNFα increased apical abundance of OCTN2 in human colonic epithelial cells (Fujiya et al., 2011). However, the data from OCTN2 gene expression is not in agreement with previous studies showing that mRNA level of OCTN2 is independent of TNFα (Maeda et al., 2007; Fujiya et al., 2011). This might be due to the fact that OCTN2 was measured in the human fibroblast-like synoviocyte cells and human colonic epithelial cells and not in bovine kidney cells. To further explore whether the up-regulation of OCTN2 by TNFα in MDBK cells is dependent on NF-κB, we studied the effect of TNFα on the expression of OCTN2 in MDBK cells that were co-treated with the NF-κB inhibitor BAY 11-7085. BAY 11-7085 is widely used NF-κB inhibitor that can selectively and irreversibly inhibit the phosphorylation of IκB, which

consequentially blocks the activation of NF-κB (Pierce et al., 1997; Reli et al., 2004). The stimulatory effect of TNFα on mRNA and protein levels of OCTN2 in MDBK cells was completely blocked by the NF-κB inhibitor indicating that OCTN2 gene expression is regulated by NF-κB in bovine kidney cells. The results from uptake experiments indicate that the up-regulation of OCTN2 by TNFα leads to an increase in carnitine uptake. It is noteworthy that we used a HBSS buffer instead of the 125 mM Na^+ incubation medium, which was used in **study 1** and **2,** due to the failure of detecting any difference between the treatment and control groups in 125 mM of Na^+ incubation buffer (data not shown). HBSS buffer has a higher concentration of Na^+, which is known as a driving force for OCTN2 mediated carnitine uptake (Tamai et al., 1998; Tamai et al., 2001; Glube et al., 2007). Taken together, it shows that bovine gene encoding OCTN2 is a target of NF-κB regulating carnitine reabsorption in kidney cells.

A variety of studies in non-ruminants have shown a negative cross-talk between PPARs and NF-κB. Activated PPARs can inhibit the activity of NF-κB in vitro (Bosscher et al., 2006; Staels et al., 1998; Eun et al., 2006). PPARα uses a direct protein-protein interaction mediating the repression of NF-κB signaling. PPARα interacts with RelA mainly in LBD of PPARα, because the truncated variant attenuates NF-κB repression. PPARα also interacts weakly with RelA in the 12-317 region, where it contains a Rel homology domain mediating DNA binding, dimerization and interaction with IκBα (Delerive et al., 1999). In addition, a later study from the same research group demonstrated in human aortic smooth muscle cells, that PPARα activators increase IκB transcription and protein levels, which keep NF-κB in an inactive form in cytoplasm (Delerive et al., 2002). In detail, PPARα activation enhances the occupancy of the NF-κB response element and recruits vitamin D_3 receptor-interacting proteins complexes onto the Sp1 site flanking the NF-κB site in the IκBα promoter. Besides PPARα, PPARγ also has an inhibitory effect on NF-κB. A novel mechanism is that PPARγ governs the distribution of RelA. PPARγ mediate the nuclear export of RelA, which may cause a lower concentration of active NF-κB in the nucleus (Kelly et al., 2004). In mouse macrophages another mechanism was indentified that PPARγ reduces the transcription of inflammatory target genes via a small ubiqutin-like modification dependent pathway (Pascual et al., 2005). Ligand dependent small ubiqutin-like modification occurs in LBD of PPARγ, which makes PPARγ a target to nuclear receptor corepressor (NCoR) on the promoter region of inflammatory genes. Normally NCoR is removed by ubiquitylation proteosome and in turn activates gene transcription. However, the PPARγ-NCoR complex prevents the recruitment of

the ubiquitylation proteasome machinery. Therefore, NCoR stays in the promoter and represses the target gene.

The negative cross-talk between PPARs and NF-κB in ruminants is not as obvious as in non-ruminants. Administration of PPARγ agonist TZD to cows during transition period did not decrease, but surprisingly increase the concentration of plasma TNFα (Schoenberg et al., 2011). In vitro, MDBK that was incubated with PPARα agonist WY-14643 has no effect on NF-κB target genes, including IL-6, HP (haptogblobin) and SAA3 (serum amyloid A3) (Bionaz et al., 2012). The exact relationship between PPARs and NF-κB in ruminants is unknown. Whether carnitine homeostasis can be affected by the combination effect of PPAR and NF-κB is not determined. Given the fact that during transition period both PPARs and NF-κB are activated in dairy cows, this area warrants further investigation.

In conclusion, the present dissertation shows that expression of the carnitine transporter OCTN2 and OCTN2-mediated carnitine uptake are regulated by PPARα, PPARβ/δ and NF-κB, respectively, in bovine kidney cells. This suggests that the bovine gene encoding OCTN2, like the mouse OCTN2 gene, is a target of PPARα (**study 1**). The novel findings in **study 2** extend the knowledge about the molecular regulation of the OCTN2 gene and carnitine transport which have been convincingly demonstrated to be regulated by PPARα in cattle and several other species. The fact that OCTN2 gene expression and carnitine transport are obviously regulated by both, PPARα and PPARβ/δ, is not surprising given that these two PPAR subtypes have partially overlapping functions. Namely, both PPAR subtypes play important roles in the regulation of mitochondrial fatty acid oxidation, which is dependent on the presence of carnitine, and therefore share a large set of common target genes involved in fatty acid oxidation. The findings in **study 3** show that the gene expression of OCTN2 and carnitine transport are both regulated by NF-κB in bovine cells. These findings indicate that inflammation may also contribute to enhance mitochondrial fatty acid oxidation. Therefore, the recent observation from our lab in high-producing dairy cows that OCTN2 is strongly up-regulated in the liver during early lactation (Schlegel et al., 2012) may be mediated by the activation of both PPARs (PPARα and PPARβ/δ) and NF-κB, because the fatty acids released from adipose tissue during this state are ligands and activators of both PPAR subtypes and NF-κB.

5 SUMMARY

L-Carnitine is a water soluble metabolite, serving as an essential cofactor for fatty acid β-oxidation by transferring long-chain fatty acids as acylcarnitine esters across the inner mitochondrial membrane. In the body, carnitine is derived from endogenous synthesis and from the intestinal absorption from the dietary sources. Tissues which cannot provide carnitine via endogenous synthesis, like skeletal muscle or myocardium, are dependent on carnitine uptake from the circulation. Carnitine transport is mediated by OCTN2, which is sodium-dependent and has a high-affinity to carnitine. OCTN2-mediated carnitine transport is also responsible for the tubular reabsorption of carnitine in the kidney and is therefore fundamental to maintaining normal carnitine levels in serum. Recent studies in rodents convincingly demonstrated that PPARα, which is a well-known central regulator of lipid metabolism and energy homeostasis, is an important transcriptional regulator of genes encoding OCTN2. Gene transcription by PPARα is initiated when a ligand, like fatty acids which are liberated from adipose tissue during energy deprivation and taken up into tissues during this state, or exogenous ligands such as fibrates (WY-14,643) bind to the LBD of this transcription factor. In contrast to rodents, little is known with regard to the regulation of OCTN2 by PPARα and its isoforms PPARβ/δ and their roles for carnitine transport in cattle. PPARβ/δ and PPARα have partially overlapping functions. For instance, they are central regulators of fatty acid catabolism since both subtypes control the expression of genes encoding proteins involved in cellular fatty acid uptake, intracellular fatty acid transport, mitochondrial fatty acid uptake, and mitochondrial and peroxisomal fatty acid oxidation. Although PPARs and NF-κB have a negative cross talk, in cattle both PPARs and NF-κB are activated during transition period. It has been confirmed that OCTN2 expression and carnitine uptake in human colonic epithelial cells are increased by NF-κB inducer TNFα. However, it is unclear whether NF-κB is able to alter OCTN2 and carnitine uptake in bovine kidney cells.

Study 1 aimed to investigate the hypothesis that PPARα, as in rodents, regulates OCTN2 involving in carnitine uptake in cattle. MDBK cells were incubated 24 h with 150 µM of PPARα agonist WY-14,643 in the absence and presence of 10 µM of PPARα antagonist GW6471. WY-14,643 increased mRNA and protein levels of OCTN2, whereas co-treatment of MDBK cells with WY-14,643 and GW6471 blocked the WY-14,643-induced increase of mRNA and protein levels of OCTN2. The treatment of MDBK cells with WY-14,643 stimulated specifically Na^+-dependent carnitine uptake in MDBK cells, which is likely the

consequence of the increased carnitine transport capacity of cells due to the elevated expression of OCTN2. In addition, WY-14,643-stimulated increase of carnitine uptake was completely blocked by treatment of cells with GW6471.

Study 2 aimed to investigate the hypothesis that PPARβ/δ, like PPARα, also regulates bovine OCTN2 involving in carnitine uptake. MDBK cells were incubated for 24 h with 1 μM of PPARβ/δ agonist GW0742 in the absence and presence of 10 μM of PPARβ/δ antagonist GSK3787. GW0742 increased mRNA and protein levels of OCTN2, whereas co-treatment of MDBK cells with GW0742 and GSK3787 blocked the GW0742-induced increase of mRNA and protein levels of OCTN2. The treatment of MDBK cells with GW0742 stimulated specifically Na^+-dependent carnitine uptake in MDBK cells. In addition, GW0742-stimulated increase of carnitine uptake was completely blocked by treatment of cells with GSK3787.

Study 3 aimed to investigate the hypothesis that NF-κB has a similar function as PPARs in regulating bovine OCTN2 and carnitine uptake. Dose-dependent test was performed to find the optimized concentration of TNFα. 5ng/ml of TNFα increased the transcription level of IL-6 and IL-1B, which are the well-known NF-κB target genes. 5 ng/ml of TNFα stimulated NF-κB transactivation in MDBK cells. MDBK cells were incubated 24 h with 5 ng/ml NF-κB activator TNFα in the absence and presence of 1 μM of NF-κB inhibitor BAY 11-7085. TNFα increased mRNA and protein levels of OCTN2, whereas co-treatment of MDBK cells with TNFα and BAY 11-7085 blocked the TNFα-induced increase of mRNA and protein levels of OCTN2. The treatment of MDBK cells with TNFα stimulated carnitine uptake in MDBK cells. In addition, TNFα-stimulated increase of carnitine uptake was completely blocked by treatment of cells with BAY 11-7085.

In conclusion, the overall finding of this dissertation is that PPARα, PPARβ/δ and NF-κB regulate OCTN2 and OCTN2-mediated carnitine uptake in bovine kidney cells. Therefore, it may provide an explanation for the recent observation from our lab that the mRNA level of OCTN2 and carnitine concentration are strongly up-regulated in the liver of high-producing dairy cows during early lactation. The increased carnitine transport might relieve the pathological pressure such as ketosis and fatty liver caused by negative energy balance and inflammation in dairy cows.

6 ZUSAMMENFASSUNG

L-Carnitin ist ein wasserlöslicher Metabolit, der als essenzieller Kofaktor bei der β-Oxidation von Fettsäuren dient, indem er langkettige Fettsäuren als Acylcarnitinester durch die innere mitochondriale Membran transferiert. Im Körper stammt Carnitin aus der endogenen Synthese und der intestinalen Resorption aus Nahrungsquellen. Gewebe, welche Carnitin nicht aus der endogenen Synthese gewinnen können, wie Skelett- und Herzmuskel, sind von der Aufnahme und Stoffverteilung im Körper abhängig. Der Carnitin-Transport wird durch den *novel organic cation transporter* (OCTN2) vermittelt, welcher natriumabhängig ist und eine hohe Affinität zu Carnitin aufweist. Der OCTN2-vermittelte Carnitintransport ist ebenso für die tubuläre Reabsorption von Carnitin in den Nieren verantwortlich und daher überaus bedeutsam, normale Carnitin-Spiegel im Serum aufrechtzuerhalten. Kürzlich durchgeführte Studien an Nagetieren haben überzeugend demonstriert, dass *peroxisome proliferator-activated receptor α* (PPARα), ein wesentlicher Regulator im Fettstoffwechsel und der Energiehomöostase, ein wichtiger transkriptionaler Regulator des OCTN2-Gens ist. Die Transkription wird durch PPARα initiiert, wenn Liganden, wie Fettsäuren, die während Energiemangel aus dem Fettgewebe freigesetzt und von anderen Geweben während dieses Zustands aufgenommen werden, oder exogene Liganden, wie Fibrate (WY-14,643), an die Ligandenbindungsdomäne dieses Transkriptionsfaktors binden. Im Gegensatz zu den Erkenntnissen bei Nagetieren, ist wenig bezüglich der Regulation von OCTN2 durch PPARα und seinen Isoformen PPARβ/δ und ihrer Rollen beim Carnitintransport von Rindern bekannt. PPARβ/δ und PPARα haben zum Teil überlappende Funktionen. Sie sind beispielsweise wesentliche Regulatoren im Fettsäurekatabolismus, da beide Subtypen die Expression von Genen kontrollieren, die Proteine kodieren, welche an der zellulären Aufnahme von Fettsäuren, dem intrazellulären Transport von Fettsäuren, der mitochondrialen Aufnahme von Fettsäuren und der mitochondrialen und peroxisomalen Fettsäureoxidation beteiligt sind. Obwohl die PPAR-Subtypen und *nuclear factor kappa B* (NF-κB), ein Schlüsselregulator der Entzündung, einen „negativen

Crosstalk“ aufweisen, werden während der Übergangszeit bei Rindern sowohl die PPAR-Subtypen, als auch NF-κB aktiviert. Es wurde nachgewiesen, dass die OCTN2-Expression und die Carnitin-Aufnahme in humanen Epithelzellen des Dickdarms durch den NF-κB-Induzierer *tumor necrosis factor-α* (TNFα) gesteigert werden. Jedoch ist unklar, ob NF-κB in der Lage ist, die OCTN2- und Carnitinaufnahme in Nierenzellen von Rindern zu verändern.

Ziel der ersten Untersuchung war es, die Hypothese zu überprüfen, dass PPARα, wie bei Nagetieren, OCTN2 reguliert, welcher bei der Carnitinaufnahme bei Rindern beteiligt ist. *Madin-Darby bovine kidney* (MDBK)-Zellen wurden für 24 Stunden mit 150 µM des PPARα-Agonisten WY-14,643 in Abwesenheit und Anwesenheit von 10 µM des PPARα-Antagonisten GW6471 inkubiert. WY-14,643 erhöhte die mRNA- und Proteinspiegel von OCTN2, wohingegen die Behandlung der MDBK-Zellen mit WY-14,643 und GW6471 die WY-14,643-induzierte Erhöhung von mRNA- und Proteinspiegeln des OCTN2 hemmte. Die Behandlung von MDBK-Zellen mit WY-14,643 stimulierte spezifisch die Na^+-abhängige Aufnahme der Zellen von Carnitin, was vermutlich die Folge der erhöhten Carnitintransportkapazität der Zellen auf Grund der gesteigerten Expression von OCTN2 ist. Darüber hinaus wurde die WY-14,643-stimulierte Erhöhung der Carnitinaufnahme durch die Behandlung der Zellen mit GW6471 vollständig gehemmt.

Ziel der zweiten Untersuchung war es, die Hypothese zu überprüfen, dass PPARβ/δ, wie PPARα, den bovinen OCTN2 reguliert. MDBK-Zellen wurden für 24 Stunden mit 1 µM des PPARβ/δ-Agonisten GW0742 in Abwesenheit und Anwesenheit von 10 µM des PPARβ/δ-Antagonisten GSK3787 inkubiert. GW0742 erhöhte die mRNA- und Proteinspiegel des OCTN2, wohingegen die Behandlung der MDBK-Zellen mit GW0742 und GSK3787 die GW0742-induzierte Erhöhung der mRNA- und Proteinspiegel von OCTN2 hemmte. Die Behandlung von MDBK-Zellen mit GW0742 stimulierte spezifisch die Na^+-abhängige Aufnahme der Zellen von Carnitin. Darüber hinaus wurde die GW0742-stimulierte Erhöhung der Carnitinaufnahme durch die Behandlung der Zellen mit GSK3787 vollständig gehemmt.

Ziel der dritten Untersuchung war es, die Hypothese zu überprüfen, dass NF-κB eine

ähnliche Funktion wie die PPARs bei der Regulation der bovinen OCTN2- und Carnitinaufnahme haben. Es wurden dosisabhängige Tests durchgeführt, um eine optimierte Konzentration von TNFα herauszufinden. 5 ng/ml TNFα erhöhten den Transkriptspiegel von *interleukin* (IL)-6 und IL-1B, welche Zielgene von NF-κB sind. 5 ng/ml TNFα stimulierten die Transaktivierung von NF-κB in MDBK-Zellen. MDBK-Zellen wurden für 24 Stunden mit 5 ng/ml NF-κB-Aktivator TNFα in Abwesenheit und Anwesenheit von 1 μM des NF-κB-Inhibitors BAY 11-7085 inkubiert. TNFα erhöhte die mRNA- und Proteinspiegel des OCTN2, wohingegen die Behandlung der MDBK-Zellen mit TNFα und BAY 11-7085 die TNFα-induzierte Erhöhung der mRNA- und Proteinspiegel von OCTN2 hemmte. Die Behandlung von MDBK-Zellen mit TNFα stimulierte die Carnitinaufnahme der Zellen. Darüber hinaus wurde die TNFα-stimulierte Erhöhung der Carnitinaufnahme durch die Behandlung der Zellen mit BAY 11-7085 vollständig gehemmt.

Zusammenfassend ist die Erkenntnis aus dieser Dissertation, dass PPARα, PPARβ/δ und NF-κB die OCTN2- und OCTN2-vermittelte Carnitinaufnahme in Nierenzellen von Rindern regulieren. Dies dürfte eine Erklärung für die kürzliche Beobachtung liefern, derzufolge der mRNA-Spiegel von OCTN2 und die Carnitin-Konzentration in der Leber von Hochleistungsmilchkühen während der frühen Laktation stark hochreguliert werden. Der erhöhte Carnitintransport könnte die Belastung durch Krankheiten, wie beispielsweise Ketose oder Fettleber vermindern, die bei Milchkühen durch eine negative Energiebilanz und Entzündungen hervorgerufen werden.

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Erklärung

„Ich erkläre: Ich habe die vorgelegte Dissertation „**Investigations on the effects of peroxisome proliferator-activated receptors and of nuclear factor kappa B on novel organic cation transporter 2 and carnitine uptake in bovine kidney cells“** selbstständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

Gießen, den .....

Xiaodan Zhou

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

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