The pro-inflammatory cytokine tumor necrosis factor α stimulates expression of the carnitine transporter OCTN2 (novel organic cation transporter 2) and carnitine uptake via nuclear factor- κ B in Madin-Darby bovine kidney cells

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ABSTRACT

Carnitine uptake into tissues is mediated mainly by the novel organic cation transporter 2 (OCTN2), whose expression is upregulated in the liver of early-lactating dairy cows. It has been shown recently that pro-inflammatory cytokines, including tumor necrosis factor α (TNF α), stimulate *OCTN2* expression and carnitine uptake in intestinal cells and inflamed intestinal mucosa. Given that many early-lactating dairy cows show typical signs of hepatic and systemic inflammation, such as elevated concentrations of circulating TNF α and activation of the key regulator of inflammation, nuclear factor κ B (NF- κ B), in tissues, it is possible that upregulation of *OCTN2* and increase of carnitine uptake by TNF α is mediated by NF- κ B, a mechanism that might contribute to the upregulation of *OCTN2* in the liver of early-lactating dairy cows. Thus, in the present study, we tested the hypothesis that TNF α stimulates *OCTN2* gene expression and carnitine uptake via NF- κ B in the bovine Madin-Darby bovine kidney (MDBK) cell line. Treatment with TNF α caused activation of NF- κ B, increased the mRNA and protein concentration of *OCTN2*, and stimulated the uptake of carnitine in MDBK cells. In contrast, combined treatment of MDBK cells with TNF α and the NF- κ B inhibitor BAY 11-7085 completely blocked the effect of TNF α on *OCTN2* mRNA and protein concentration and uptake of carnitine. These findings suggest that the bovine *OCTN2* gene and carnitine uptake are regulated by NF- κ B. Future studies are required to show the in vivo relevance of this regulatory mechanism in cattle.

Key words: carnitine uptake, novel organic cation transporter 2, nuclear factor- κ B, dairy cow

INTRODUCTION

Carnitine is a water-soluble quaternary amine that is essential for normal function of all tissues. Carnitine

is derived from endogenous biosynthesis, which occurs mainly in the liver, and from intestinal absorption of carnitine from the diet (Vaz and Wanders, 2002). Tissues that are incapable of synthesizing carnitine, such as skeletal muscle or myocardium, are dependent on active carnitine uptake from the circulation. Active carnitine uptake is mainly mediated by the novel organic cation transporter 2 (OCTN2), a transporter that is sodium-dependent, high affinity, and broadly distributed in tissues (Tamai et al., 1998). The essential role of OCTN2 for carnitine homeostasis is shown by the fact that lack of OCTN2 due to a mutation in the *OCTN2* gene leads to systemic carnitine deficiency (Lahjouji et al., 2001).

It was recently demonstrated that *OCTN2* expression and carnitine concentration are elevated in the liver of high-yielding dairy cows during early lactation (Schlegel et al., 2012). It is known that the *OCTN2* gene of many species, including cattle, is regulated by the transcriptional regulator peroxisome proliferator-activated receptor α (PPAR α ; Luo et al., 2014), that PPAR α activation stimulates *OCTN2* expression and carnitine uptake in bovine kidney cells (Zhou et al., 2014), and that PPAR α is activated in the liver of dairy cattle during early lactation (Lor et al., 2005; Schlegel et al., 2012; Akbar et al., 2013). Based on this, it has been proposed that PPAR α activation represents the molecular basis for the above-mentioned increased *OCTN2* expression and elevated carnitine concentration in the liver of lactating dairy cows (Schlegel et al., 2012).

However, apart from the established PPAR α -dependent regulation of the *OCTN2* gene, it has been shown recently that pro-inflammatory cytokines, such as tumor necrosis factor α (TNF α), increase *OCTN2* expression and sodium-dependent carnitine uptake in intestinal cells (Fujiya et al., 2011). Moreover, Fujiya et al. (2011) demonstrated that colonic *OCTN2* expression is increased during active inflammation in the inflamed intestinal mucosa, where pro-inflammatory mediators are found at elevated concentrations. Interestingly, many high-yielding dairy cows show typical signs of hepatic and systemic inflammation, such as elevated concentrations of acute phase proteins and cytokines, including TNF α , during early lactation (Bionaz et al.,

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2007; Vels et al., 2009). This is attributed to the exposure to diverse inflammatory challenges, such as TNF α and microbial components such as LPS, which arise from infectious diseases such as mastitis (Vels et al., 2009; Jørgensen et al., 2012), endometritis (Burke et al., 2010; Akbar et al., 2014), and rumen digestive disorders, such as subacute rumen acidosis (Plaizier et al., 2008; Zebeli and Metzler-Zebeli, 2012) and abomasal displacement (Guzelbektes et al., 2010), that frequently occur during parturition or at the onset of lactation.

In addition, it has been reported recently that nuclear factor- κ B (NF- κ B) is activated in different tissues, including the liver, of dairy cows during early lactation (Farney et al., 2013; Gessner et al., 2013). Nuclear factor- κ B is a key regulator of inflammation due to stimulating the transcription of genes involved in the inflammation process, including cytokines, chemokines, adhesion molecules, and acute phase proteins (Karin and Delhase, 2000). Many pro-inflammatory stimuli, such as TNF α and LPS, mediate their pro-inflammatory actions to a large extent by activating the NF- κ B pathway (Baud and Karin, 2001; Vallabhapurapu and Karin, 2009). In view of this, it is possible that upregulation of *OCTN2* gene expression and increase of carnitine uptake by TNF α is mediated by NF- κ B, a mechanism that might also contribute to upregulation of *OCNT2* in the liver of early-lactating dairy cows. Thus, in the present study, we tested the hypothesis that TNF α stimulates *OCTN2* gene expression and carnitine uptake via NF- κ B in a bovine cell line.

MATERIALS AND METHODS

Cell Culture and Treatments

The Madin-Darby bovine kidney (MDBK) cell line, a frequently used cell model to address mechanistic questions in bovine tissues (Bionaz et al., 2008), was obtained from Cell Lines Service (Eppelheim, Germany) and cultivated in HyClone Minimum Essential Medium/Earle's Balanced Salt Solution (MEM/EBSS) supplemented with 10% fetal bovine serum (FBS) and 0.05 mg/mL gentamicin (all from Invitrogen, Karlsruhe, Germany) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. For experiments, cells were seeded out into 6-well plates at a density of 2.0×10^5 cells (for PCR and Western blotting experiments), 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 reporter gene assays) in HyClone MEM/EBSS complete medium. After reaching 80% confluence, MDBK cells were treated with dif-

ferent concentrations of TNF α (0, 1, 5, 10 and 20 ng/mL; Sigma-Aldrich, Steinheim, Germany) dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) in MEM/EBSS medium without FBS but with 5 mg/L bovine insulin (Sigma-Aldrich) for 24 h. Cells treated with vehicle alone (DMSO) were used as control. Incubation medium of control cells contained the same vehicle concentration of 0.1% (vol/vol). For experiments using the NF- κ B inhibitor, cells were co-treated with 5 ng/mL TNF α and 1 μ M BAY 11-7085 (Merck, Bruchsal, Germany). All experiments were performed 3 times from a different cell passage number (= 3 independent experiments). An independent experiment was defined as an experiment performed with cells of a specific passage number and included seeding, treatment, and analysis.

Quantitative Real-Time PCR

Total RNA was isolated from the MDBK cell layer after a washing step with PBS using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The RNA from cells was extracted within 3 d 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 integrity of RNA was assessed by 1% agarose gel electrophoresis, and RNA was considered to be suitable for use only if intact bands corresponding to 18S and 28S rRNA subunits were visible. First-strand cDNA was synthesized from 1.2 μ g of total RNA using 100 pmol of dT18 primer (Eurofins MWG Operon, Ebersberg, Germany), 1.25 μ L of 10 mM dNTP mix (GeneCraft, Lüdinghausen, Germany), 5 μ L of buffer (Fermentas, St. Leon-Rot, Germany), and 60 units of M-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 (Whatman Biometra, Göttingen, Germany). Finally, cDNA was preserved in aliquots at -20°C. Quantitative real-time (q)PCR was performed using 2 μ L of cDNA combined with 18 μ L of a mixture composed of 10 μ L of KAPA SYBR FAST qPCR Universal Mastermix (Peqlab, Erlangen, Germany), 0.4 μ L each of forward and reverse primers (10 μ M), and 7.2 μ L of DNase- and RNase-free water in 0.1-mL tubes (Ltf Labortechnik, Wasserburg, Germany). Primer pairs were designed using Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA) to have melting temperatures of about 60°C, and, if possible, both primers of a primer pair were designed to be located in different exons. Characteristics of primer pairs and qPCR performance data are listed in Table

Table 1. Primer characteristics used for quantitative PCR

Gene ¹	Forward primer (5' to 3') Reverse primer (5' to 3')	PCR product size (bp)	GenBank accession no.	Efficiency	Mean cycle threshold
Reference genes					
<i>ACTB</i>	ACTTGCGCAGAAAACGAGAT CACCTTCACCGTTCCAGTTT	120	AY141970	1.99	9.31
<i>ATP5B</i>	GGACTCAGCCCTTCAGCGCC GCCTGGTCTCCCTGCCTTGC	229	NM_175796.2	1.82	11.94
<i>PPIA</i>	GGCAAATGCTGGCCCCAACACA AGTACCACGTGCTTGCCATCCA	87	NM_178320.2	1.86	11.02
<i>RPS9</i>	GTGAGGTCTGGAGGGTCAAA GGGCATTACCTTCGAACAGA	108	BC148016	1.89	14.18
<i>SDHA</i>	GCAGAACCTGATGCTTTGTG CGTAGGAGAGCGTGTGCTT	185	NM_174178	1.71	15.82
Target genes					
<i>IL6</i>	ACTCCCGCTTCACAAGCGCCTTC AAGTAGTCTGCCTGGGGTGGTGTCA	134	NM_173923.2	1.98	20.69
<i>OCTN2</i>	CACAGTGGTCAGGAACATGG AATGGTGTCTGGGAGTGGAG	181	BC105377	1.93	18.90
<i>BBOX1</i>	TCCAGCTGCCTACTCTGGAT AGCTGAACCTTACCCCAGGT	292	BC149884.1	—	—

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

1. Quantitative PCR runs were performed with a Rotorgene 2000 system (Corbett Research, Mortlake, Australia) using the following protocol: 3 min at 95°C, followed by 40 cycles of a 2-step PCR consisting of 5 s at 95°C (denaturation) and 20 s at 60°C (annealing and extension). Subsequently, melting curve analysis was performed from 50°C to 95°C to verify the presence of a single PCR product. Calculation of gene expression data and normalization by GeNorm normalization factor was carried out as described recently in detail (Keller et al., 2012). The normalization factor was calculated as the geometric mean of expression data of the 3 most stable out of 5 tested [β -actin (*ACTB*), ATP synthase, H⁺ transporting, mitochondrial F1 complex, β polypeptide (*ATP5B*), peptidylprolylisomerase A (*PPIA*), ribosomal protein S9 (*RPS9*), succinate dehydrogenase complex, subunit A (*SDHA*)] potential reference genes. Normalized gene expression level of treated cells (TNF α , TNF α + BAY 11-7085) is presented relative to that of control cells (DMSO), which was set to 1.0. In each independent experiment, all treatments (dose-response relationship: 0, 1, 5, 10, 20 ng of TNF α /mL; other experiments: DMSO, TNF α , TNF α + BAY 11-7085) were represented once (1 well per treatment) in each 6-well plate. Three identical 6-well plates were included in each independent experiment. Within one independent experiment, all wells from the three 6-well plates were analyzed and a single mean representing the experimental unit was calculated for each treatment from the three 6-well plates. Three independent experiments were run; thus, the total number of experimental units was 3.

Transient Transfection and Reporter Gene Assay

Madin-Darby bovine kidney cells with a confluency of 65% were transiently transfected with 50 ng of NF- κ B-luciferase reporter plasmid (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, Germany), a plasmid containing an NF- κ B response element in front of the firefly-luciferase reporter gene, and 5 ng of pGL4.74 *Renilla*-luciferase plasmid (Promega, Mannheim, Germany), a control vector used for normalization, using FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) for 8 h. Subsequently, the MDBK cells were treated without or with 5 ng/mL of TNF α for 24 h as described above. Following incubation, cells were washed with PBS and lysed with passive lysis buffer (Promega). Luciferase activities were measured using Beetle-Juice and Renilla-Juice Kits from PJK (Kleinblittersdorf, Germany) using a Mithras LB940 luminometer (Berthold Technologies, Bad Wildbad, Germany). Data were normalized for transfection efficiency by dividing firefly-luciferase activity by that of *Renilla*-luciferase activity. Normalized luciferase activity of TNF α -treated cells is shown relative to that of control cells (DMSO), which was set to 1.0. In each independent experiment, all treatments (DMSO, TNF α) were represented in 3 wells (3 technical replicates per treatment) in each 96-well plate. Within one independent experiment, all technical replicates were analyzed and a single mean representing the experimental unit was calculated for each treatment. Three independent experiments were run; thus, the total number of experimental units was 3.

Immunoblotting

The MDBK cells were washed once with PBS and then lysed with radioimmunoprecipitation assay (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). Determination of protein concentration of cell lysates, protein separation by SDS-PAGE, protein transfer to nitrocellulose membranes, and immunodetection of OCTN2 and β -actin as reference protein were carried out as described recently (Zhou et al., 2014). For calculation of protein expression data, the band intensity of OCTN2 was normalized to that of β -actin and normalized protein expression level of treated cells (TNF α , TNF α + BAY 11-7085) is presented relative to that of control cells (DMSO), which was set to 1.0. In each independent experiment, all treatments (DMSO, TNF α , TNF α + BAY 11-7085) were represented once (one well per treatment) in each 6-well plate. Three identical 6-well plates were included in each independent experiment. Within one independent experiment, all wells from the three 6-well plates were analyzed and a single mean representing the experimental unit was calculated for each treatment from the three 6-well plates. Three independent experiments were run; thus, the total number of experimental units was 3.

Carnitine Uptake

The MDBK cells were washed 2 times with 1.5 mL of Hanks' balanced salts solution (HBSS, Biochrom AG, Berlin, Germany) with 5 mM HEPES (pH 7.4; Sigma, Steinheim, Germany), and then incubated in a buffer containing 10 nM methyl-L-[^{3} H]-carnitine (80 mCi/mmol; American Radiolabeled Chemicals, St. Louis, MO), 125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, and 5 mM HEPES at 37°C for 30 min according to the method of Glube et al. (2007). Following incubation, the buffer 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 (Liquid Scintillation Analyzer Tri-Carb 2900TR, Perkin Elmer, Rodgau, Germany) and related to protein content of cell lysates as determined by the bicinchoninic acid protein assay with BSA as standard. Carnitine uptake was expressed as the amount of L-[^{3} H]-carnitine taken up per milligram of cell protein within 30 min. In each independent experiment, all treatments (DMSO, TNF α , TNF α + BAY 11-7085) were represented in 3 wells (3 technical replicates per treatment) in each 24-well plate. Within

one independent experiment, all technical replicates were analyzed and a single mean representing the experimental unit was calculated for each treatment. Three independent experiments were run; thus, the total number of experimental units was 3.

Statistics

All data represent means and standard deviations (SD), which were calculated from the means for the same treatments of 3 independent experiments. Statistical analysis was performed using the Minitab statistical software (release 13.0; Minitab Inc., State College, PA). Data of each independent experiment were analyzed for normality of distribution (Anderson-Darling test). Because all data showed a normal distribution, one-way ANOVA was applied to evaluate the effect of treatment. Effects were considered significant if $P < 0.05$. If one-way ANOVA revealed significant effects, a post hoc comparison (Fisher's multiple comparison) was applied.

RESULTS

To investigate the suitability of TNF α as an activator of NF- κ B in MDBK cells, the dose-response relationship between TNF α (0, 1, 5, 10, 20 ng/mL) and the mRNA concentration of the known NF- κ B target gene *IL6* in MDBK cells was studied first. As shown in Figure 1A, treatment of MDBK cells with TNF α dose-dependently increased the *IL6* mRNA concentration compared with vehicle control treatment. Considering that 5 ng/mL of TNF α caused a strong (~15-fold) increase in the *IL6* mRNA concentration and higher TNF α concentrations (10 and 20 ng/mL) caused only a modest additional increase in the *IL6* mRNA concentration (Figure 1A), 5 ng/mL TNF α was used for NF- κ B activation in subsequent experiments.

To demonstrate direct activation of NF- κ B by TNF α in MDBK cells, we next investigated the effect of TNF α on activation of a luciferase reporter plasmid containing the consensus NF- κ B response element in MDBK cells transiently transfected with this plasmid. This experiment revealed that the NF- κ B-driven luciferase reporter was activated about 3-fold by TNF α in MDBK cells, indicating that TNF α directly activates NF- κ B (Figure 1B).

To further explore whether the stimulatory effect of TNF α on *IL6* mRNA concentration in MDBK cells is mediated via NF- κ B, we studied the effect of blocking NF- κ B activation by the NF- κ B inhibitor BAY 11-7085 (1 μ M). BAY 11-7085 inhibits NF- κ B through irreversible inhibition of I κ B α phosphorylation, thereby preventing activation of NF- κ B by TNF α and other

Figure 1. (A) Effect of 24-h treatment with different concentrations of tumor necrosis factor α (TNF α ; 0, 1, 5, 10, 20 ng/mL) on relative *IL6* mRNA concentration in Madin-Darby bovine kidney (MDBK) cells. Cells treated with vehicle (dimethyl sulfoxide, DMSO) only (0 ng of TNF α /mL) served as control. (B) Effect of 24-h treatment with TNF α (5 ng/mL) compared with treatment with vehicle only (DMSO) on nuclear factor- κ B (NF- κ B) transactivation in MDBK cells. (C) Effect of 24-h treatment with either TNF α (5 ng/mL) alone or TNF α (5 ng/mL) and NF- κ B inhibitor BAY 11-7085 (BAY; 1 μ M) compared with treatment with vehicle only (DMSO) on relative *IL6* mRNA concentration in MDBK cells. In all panels, bars represent means $\pm$ SD of 3 independent experiments ($n = 3$) and are expressed as fold of DMSO-treated control. *Different from DMSO-treated control, $P < 0.05$.

cytokines, as well as LPS. As shown in Figure 1C, the stimulatory effect of TNF α on *IL6* mRNA concentration was completely abolished by BAY 11-7085, suggesting that TNF α stimulates *IL6* gene transcription via NF- κ B in MDBK cells.

Based on the finding that NF- κ B is activated by TNF α in MDBK cells, we next studied the effect of NF- κ B activation by TNF α on gene expression of *OCTN2* in MDBK cells. This experiment demonstrated that treatment of cells with TNF α increased the mRNA and protein concentration of *OCTN2* in MDBK cells by 1.7- and 2.5-fold, respectively (Figure 2A and 2B), indicating that *OCTN2* gene expression in bovine MDBK cells is regulated by the pro-inflammatory cytokine TNF α . To explore a mechanistic role of NF- κ B for this effect of TNF α , we investigated the effect of TNF α on *OCTN2* gene expression during blockage of NF- κ B by BAY 11-7085. This experiment revealed that BAY 11-7085 completely abolished the stimulatory effect of TNF α -induced NF- κ B activation on *OCTN2* mRNA and protein concentrations (Figure 2C and 2D), suggesting that *OCTN2* gene expression in MDBK cells is regulated by NF- κ B. In contrast to the mRNA expression of *OCTN2*, no mRNA expression of the γ -butyrobetaine dioxygenase (*BBOX1*) gene, which encodes the enzyme catalyzing the final step of carnitine biosynthesis, could be detected in MDBK cells by means of qPCR and conventional PCR (data not shown).

To finally investigate whether the upregulation of *OCTN2* by TNF α has an influence on carnitine uptake, which is largely mediated by OCTN2 under physiological conditions, we studied the uptake of methyl-L-[^{3} H]-carnitine into MDBK monolayer. As illustrated in Figure 3, the uptake of L-carnitine into MDBK cells increased about 35% in cells treated with TNF α compared with cells treated with vehicle alone ($P < 0.05$). To study the NF- κ B dependence of the TNF α -stimulated increase of L-carnitine uptake, we also studied the effect of TNF α on L-carnitine uptake by MDBK cells during blockage of NF- κ B by BAY 11-7085. As shown in Figure 3, the effect of TNF α on L-carnitine uptake was completely blocked by BAY 11-7085, suggesting that the effect of TNF α on L-carnitine uptake is mediated by NF- κ B.

DISCUSSION

The main finding of the present study is that the pro-inflammatory cytokine TNF α stimulates gene expression of *OCTN2* and carnitine uptake in the bovine MDBK cell line, and co-treatment of cells with an NF- κ B inhibitor blocks the TNF α -induced stimulation of *OCTN2* gene expression and carnitine uptake in this cell line. The OCTN2 protein is essential for carnitine homeostasis through its important role in

Figure 2. Effect of 24 h treatment with either tumor necrosis factor α (TNF α ; 5 ng/mL) alone (panels A and B) or TNF α (5 ng/mL) and nuclear factor- κ B inhibitor BAY 11-7085 (BAY; 1 μ M) (panels C and D) compared with treatment with vehicle only (dimethyl sulfoxide, DMSO) on relative mRNA (A and C) and protein concentrations (B and D) of *OCTN2* [solute carrier family 22 (organic cation/carnitine transporter), member 5] in Madin-Darby bovine kidney (MDBK) cells. In all panels, bars represent means $\pm$ SD of 3 independent experiments ($n = 3$) and are expressed as fold of DMSO-treated control. In panels B and D, immunoblots specific to OCTN2 and β -actin (as internal control) including the size marker (M) are shown for one independent experiment; immunoblots for the other experiments revealed similar results. *Different from DMSO-treated control, $P < 0.05$.

carnitine distribution between tissues and the tubular carnitine reabsorption process in the kidney, which is evidenced by the fact that mutations in the *OCTN2* gene lead to systemic carnitine deficiency, at least in humans (Lahjouji et al., 2001). Given this important role of OCTN2 for carnitine uptake, it is likely that the TNF α -stimulated carnitine uptake observed in this

study was due to upregulation of *OCTN2*. Considering that inhibition of NF- κ B, which is known to be activated by TNF α and other pro-inflammatory cytokines (Baud and Karin, 2001; Vallabhapurapu and Karin, 2009), completely abolished the effect of TNF α on *OCTN2* expression and carnitine uptake, it is likely that *OCTN2* is regulated by the NF- κ B pathway, at

Figure 3. Effect of 24-h treatment with either tumor necrosis factor α (TNF α ; 5 ng/mL) alone or TNF α (5 ng/mL) and nuclear factor- κ B inhibitor BAY 11-7085 (BAY; 1 μ M) compared with treatment with vehicle only (dimethyl sulfoxide, DMSO) on uptake of L-[³H]-carnitine (10 nM, specific radioactivity 80 Ci/mmol) by Madin-Darby bovine kidney (MDBK) cells; uptake of L-[³H]-carnitine was studied over 30 min. Bars represent means $\pm$ SD of 3 independent experiments ($n = 3$). *Different from DMSO-treated control, $P < 0.05$.

least indirectly. In line with a regulatory role of pro-inflammatory cytokines for *OCTN2* expression, it has been demonstrated recently that pro-inflammatory cytokines, such as TNF α and interferon- γ , increase *OCTN2* expression and sodium-dependent carnitine uptake, which is characteristic for OCTN2-mediated carnitine transport, in human and mouse intestinal cells (Fujiya et al., 2011). Those authors also demonstrated that colonic *OCTN2* expression is increased during active inflammation in the inflamed intestinal mucosa of mice, where pro-inflammatory mediators are generated and present at elevated concentrations. Fujiya et al. (2011) proposed that the upregulation of *OCTN2* in the inflamed mouse intestine plays a role in mediating host response to injury and stress. This assumption is based on the knowledge that OCTN2 also mediates the uptake of small bacterial peptides in human and mouse intestinal cells, resulting in the induction of cytoprotective heat-shock proteins and activation of key signaling pathways [Akt/PKB (protein kinase B), MAPK (mitogen-activated protein kinase)]. These events are supposed to confer protection of intestinal epithelial cells to the injurious effects of reactive oxygen species and contribute to restoration of intestinal homeostasis (Fujiya et al., 2007). Although speculative, upregulation of OCTN2 might be also a means to increase ATP synthesis from fatty acid oxidation and energy status in

the stressed cell due to the role of carnitine as a shuttling molecule for the import of long-chain fatty acids into the mitochondrial matrix. It is widely known that many high-yielding dairy cows show typical signs of systemic inflammation, such as elevated concentrations of acute phase proteins, during early lactation (Bionaz et al., 2007; Vels et al., 2009). This is attributed to the exposure to diverse inflammatory challenges, such as pro-inflammatory cytokines including TNF α , reactive oxygen species, and microbial components, which arise from infectious diseases, such as mastitis and endometritis, but also subacute rumen acidosis and abomasal displacement that occur frequently during parturition and onset of lactation. Moreover, it has been reported that signaling pathways, which are involved critically in stress and inflammatory response, such as the NF- κ B (Gessner et al., 2013), the Nrf2 (Moyes et al., 2010; Gessner et al., 2013), and the ER stress-induced unfolded protein response (UPR) pathway (Invernizzi et al., 2012; Gessner et al., 2014) are activated in different tissues of dairy cows during early lactation. Thus, pro-inflammatory cytokines might play a role in upregulating *OCTN2* and increasing carnitine uptake in tissues of dairy cows during early lactation, and the upregulation of *OCTN2* might be involved in the restoration of tissue homeostasis under conditions of inflammation-associated stress.

To the best of our knowledge, expression of *OCTN2* in rumen epithelium has not been described yet, but considering the ubiquitous expression of *OCTN2* in tissues of other species (Alnouti et al., 2006), this is not unlikely. It remains to be seen whether OCTN2 mediates protective functions in the rumen epithelium of cattle, as has been shown in human and mouse intestinal cells (Fujiya et al., 2007, 2011). This question is relevant considering the critical role of an intact barrier function of the rumen epithelium for preventing inflammation and other health-related problems in ruminants (Zebeli and Metzler-Zebeli, 2012). In goats, feeding a high energy diet and the subsequent rumen acidosis caused a downregulation of several protective proteins in the rumen epithelium (Hollmann et al., 2013). This might impair the cellular defense system of the rumen epithelium, thereby increasing the risk for local inflammation and allowing infectious agents to enter the systemic circulation.

We have recently shown that *OCTN2* expression and carnitine uptake in bovine MDBK cells are also regulated by PPAR α (Zhou et al., 2014), and at least the murine, porcine, and bovine *OCTN2* genes have been convincingly identified as direct PPAR α target genes (Wen et al., 2010; Luo et al., 2014). Moreover, it has been demonstrated that PPAR α is activated in the liver of dairy cows during early lactation (Loor

at al., 2005; Schlegel et al., 2012; Akbar et al., 2013), providing 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). Nonetheless, the present findings from cell culture experiments suggest that activation of NF- κ B might also contribute to upregulation of *OCTN2* in the liver of cows during lactation. Despite data showing negative crosstalk between PPAR α activation and NF- κ B signaling (Delerive et al., 1999), evidence shows that both pathways are activated in different tissues, including the liver, of dairy cows during early lactation (Loor et al., 2005; Farney et al., 2013; Gessner et al., 2013).

The present study has one limitation: Due to the lack of a bovine hepatocyte cell line, a bovine kidney cell line (MDBK) was used for the experiments. Nevertheless, we are confident that MDBK cells, which are widely used to investigate mechanistic questions in bovine tissues (Bionaz et al., 2008; Thering et al., 2009), are suitable for addressing this specific question, because *OCTN2* expression is regulated similarly, at least by PPAR α , in different cell types including hepatocytes and kidney cells (Luci et al., 2006; Zhou et al., 2014). Thus, we expect the same mechanism of regulation by TNF α in liver cells. It is well known that carnitine synthesis occurs in both liver and kidney in several species (Vaz and Wanders, 2002). To the best of our knowledge, it remains to be shown whether the bovine kidney is able to synthesize carnitine. Interestingly, our experiments with MDBK cells revealed that this cell line does not express *BBOX1* mRNA, which is essential for carnitine homeostasis because it catalyzes the final step of carnitine biosynthesis. This indicates that at least this bovine cell line lacks the ability to synthesize carnitine. It has been demonstrated that cellular carnitine uptake and carnitine synthesis can be stimulated in parallel (Luci et al., 2006) and no study has yet demonstrated that carnitine synthesis reduces carnitine uptake or vice versa. Thus, these findings indicate that MDBK cells are a suitable cell model to study regulation of *OCTN2* expression by TNF α and that the lack of carnitine synthesis does not undermine the present observations.

CONCLUSIONS

The present study shows that expression of the carnitine transporter *OCTN2* and *OCTN2*-mediated carnitine uptake are stimulated by the pro-inflammatory cytokine TNF α in bovine MDBK cells, an effect that is blocked by inhibition of NF- κ B. This suggests that the bovine *OCTN2* gene is regulated by NF- κ B. Future studies must clarify whether regulation of the *OCTN2*

gene by NF- κ B is mediated directly through binding to a NF- κ B response element in the regulatory region of the *OCTN2* gene. In addition, studies are required to show the in vivo relevance of this regulatory mechanism in cattle.

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