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 (PPAR α), 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 PPAR α and its role for carnitine transport in cattle, even though PPAR α activation physiologically occurs in the liver of high-producing cows during early lactation. To explore the role of PPAR α for OCTN2 expression and carnitine transport in cattle, we studied the effect of the PPAR α activator WY-14,643 on the expression of OCTN2 in the presence and absence of PPAR α 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 PPAR α 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 expression of OCTN2. In conclusion, our results indicate that OCTN2 expression and carnitine transport in cattle, as in rodents, are regulated by PPAR α .

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 co-factor 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 (PPAR α ; 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 PPAR α 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 PPAR α and its role for carnitine transport

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in cattle. In cattle, PPAR α 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 PPAR α (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 PPAR α also regulates carnitine homeostasis in cattle. However, more convincing evidence is necessary to clearly establish a role for PPAR α as a regulator of OCTN2 and carnitine transport in cattle, especially because it is well documented that the response to PPAR α 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 PPAR α (WY-14,643) on the expression of OCTN2 in the presence and absence of PPAR α 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 PPAR α -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% CO₂. 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 PPAR α 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 PPAR α inhibitor, cells were co-treated with a 10 μ M concentration of the PPAR α -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 im-

mediately 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 mM Tris (pH 7.5), 150 mM NaCl, 1 mM 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 mM HEPES (pH 7.4; Sigma-Aldrich Chemie GmbH) after reaching confluence, and then incubated at 37°C with a buffer containing a 10 nM concentration of methyl-L-[³H]-carnitine (2.96 GBq/mmol; American Radiolabeled Chemicals Inc., St. Louis, MO) for 30 min. The buffer contained either 0, 25, or 125 mM NaCl and 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 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-[^3H]-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 PPAR α in MDBK cells is activated, we studied the effect of 24-h treatment of 150 μM WY-14,643 on the mRNA level of the known bovine PPAR α target gene *CPT1A* in MDBK cells: We found that WY-14,643 treatment caused a pronounced 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 PPAR α 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 PPAR α , we studied the effect of WY-14,643 on the expression of *OCTN2* in MDBK cells that were co-treated with the PPAR α antagonist GW6471 (10 μM) 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 μM concentration of WY-14,643 [a high-affinity ligand of peroxisome proliferator-activated receptor α (PPAR α); Sigma-Aldrich Chemie GmbH, Steinheim, Germany] for 24 h in the absence (A and B) and presence (C and D) of PPAR α -selective antagonist GW6471 (10 μM ; 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 $\pm$ 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 $\pm$ 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 $\pm$ 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-[^3H]-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 mM NaCl, indicating that the Na^+ concentration was insufficient for facilitating Na^+ -dependent OCTN2-mediated carnitine transport. At both NaCl concentrations (0 and 25 mM), 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 mM 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 μM 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 PPAR α 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 PPAR α antagonist GW6471 (10 μM) 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 PPAR α .

The main finding of the present study is that the PPAR α 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 PPAR α 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 PPAR α 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 PPAR α antagonist. These findings indicate that OCTN2 expression and carnitine transport in cattle, as in rodents, are regulated by PPAR α . The observed PPAR α dependence of

Figure 2. Effect of treatment with a 150 μM concentration of WY-14,643 [a high-affinity ligand of peroxisome proliferator-activated receptor α (PPAR α); Sigma-Aldrich Chemie GmbH, Steinheim, Germany] on uptake of L-[^3H]-carnitine (10 nM; specific radioactivity 2.96 GBq/mmol; American Radiolabeled Chemicals Inc., St. Louis, MO) by Madin-Darby bovine kidney (MDBK) cells. (A) Uptake of L-[^3H]-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 mM NaCl) was studied over 30 min. (B) Uptake of L-[^3H]-carnitine by MDBK cells treated for 24 h with WY-14,643, WY-14,643 together with PPAR α -selective antagonist GW6471 (10 mM; Sigma-Aldrich Chemie GmbH), or DMSO (control) at 125 mM NaCl was studied over 30 min. Data represent means $\pm$ 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, PPAR α activation occurs physiologically due to the excessive flow of FA from adipose tissue to the liver where they bind to and activate PPAR α (Loor et al., 2005; Loor, 2010). Thus, our observation in MDBK cells suggests that the bovine gene encoding OCTN2 is a target of PPAR α . At least for the mouse gene encoding OCTN2, a functional binding site for PPAR α , 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 PPAR α . 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 PPAR α between mouse and cattle is highly conserved and supports the assumption that the bovine *OCTN2* gene is a PPAR α target gene.

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

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