Monocarboxylate transporters 1 and 4: expression and regulation by PPARα in ovine ruminal epithelial cells

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Running head: Regulation of MCT1 and MCT4 in ruminal epithelial cells

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ABSTRACT

In the intact rumen epithelium, isoforms 1 and 4 of the monocarboxylate transporter (MCT1 and MCT4) are thought to play key roles in mediating transcellular and intracellular permeation of short-chain fatty acids and their metabolites and in maintaining intracellular pH. We examined whether both MCT1 and MCT4 are expressed at mRNA and protein levels in ovine ruminal epithelial cells (REC) maintained in primary culture and whether they are regulated by peroxisome proliferator-activated receptor α (PPARα). Because both transporters have been characterized to function coupled to protons, the influence of PPARα on the recovery of intracellular pH after L-lactate exposure was evaluated by spectrofluorometry. MCT1 and MCT4 were detected using immunocytochemistry both at the cell margins and intracellularly in cultured REC. To test regulation by PPARα, cells were exposed to WY 14.643, a selective ligand of PPARα, for 48 hours. The subsequent qPCR analysis resulted in a dose-dependent upregulation of MCT1 and PPARα target genes, whereas response of MCT4 was not uniform. Protein expression of MCT1 and MCT4 quantified by Western blot was not altered by WY 14.643 treatment. L-lactate-dependent proton export was blocked almost completely by pHMB, a specific inhibitor of MCT1 and MCT4. However, L-lactate-dependent, pHMB-inhibited proton export in WY 14.643-treated cells was not significantly altered in comparison with cells not treated with WY 14.643. These data suggest that PPARα is particularly regulating MCT1 but not MCT4 expression. Extent of lactate-coupled proton export indicates that MCT1 is already working on a high level even under unstimulated conditions.

Keywords: rumen, sheep, monocarboxylate transporter, peroxisome proliferator-activated receptor α, intracellular pH
Ruminants meet the majority of their energy requirements through short-chain fatty acids (SCFA; mainly acetate, propionate and butyrate), which are released intraruminally in large amounts during the microbial breakdown of ingested carbohydrates (32). SCFA produced in the rumen are absorbed almost completely out of the rumen fluid (14). Some of the SCFA taken up luminally are degraded within the epithelial cells to β-hydroxybutyric acid, acetoacetic acid and lactic acid (27). Various studies suggest that monocarboxylate transporters 1 and 4 (MCT1 and MCT4) are directly or indirectly involved in the transmembranal transfer and intracellular handling of SCFA and their metabolites (9, 20, 24, 31). MCT isoform 1 was shown to be located on the basolateral cell membrane of the stratum basale of the rumen epithelium (15, 20, 24, 31) and to mediate the export of intracellular SCFA catabolic products such as β-hydroxybutyric acid, acetoacetic acid and lactic acid from the cytosol into the blood (31). MCT1 may also be able to accept acetate, i.e., non-metabolized SCFA (11, 29). Although the involvement of MCT1 in the transport of SCFA and/or their metabolites is strongly emphasized, the findings for MCT4 are controversial. Kirat et al. (21) strongly point to MCT4 being involved in the lumen-oriented uptake of SCFA, but Aschenbach et al. (2) could not detect any role for lumen-oriented MCT in SCFA uptake from the lumen to the blood.

The functions of both MCT isoforms are not restricted to the transfer of undegraded and/or degraded SCFA; the transporters also have an impact on the regulation of cytosolic pH (pH_i). Studies in various tissues including ruminal epithelial cells (REC) demonstrated that MCT1 functions either as a proton-coupled transporter (10, 16, 19, 29) or as a bicarbonate exchanger (11, 39). MCT4 has been described as a proton-coupled transporter thus far (17, 21, 36). Consequently, it must be assumed that any variations in expression and/or activity of MCT1 and MCT4 modulate pH_i homeostasis in REC.
Studies in the liver, kidney and intestinal epithelium of mice (26) and in the liver of pigs (4, 25-26) suggested that the expression of the \textit{MCT1} gene is regulated by peroxisome proliferator-activated receptor \(\alpha\) (PPAR\(\alpha\)). PPAR\(\alpha\) belongs to a ligand-activated nuclear receptor family and is thought to stimulate numerous genes involved in fatty acid metabolism (42). In the nucleus, PPAR\(\alpha\) heterodimerizes with retinoid X receptor (RXR), which binds to the PPAR response element located in the regulatory domain of the target genes (23). Transcriptional regulation of \textit{MCT4} expression has been poorly characterized thus far but is thought to be partially mediated by hypoxia-inducible factor-1\(\alpha\) (HIF1\(\alpha\); 6, 18, 46), which is, in turn, influenced by PPAR\(\alpha\) (50).

Based on these data, we aimed to investigate the involvement of PPAR\(\alpha\) in the regulation of \textit{MCT1} and \textit{MCT4} gene expression in REC. First we tested whether REC maintained in primary culture express both \textit{MCT} isoforms and the PPAR\(\alpha\) and RXR receptors. To assess the role of PPAR\(\alpha\), the cells were exposed to WY 14.643 (a specific synthetic ligand of PPAR\(\alpha\); Ref. 13) for 48 hours, and the expression levels of \textit{MCT1} and \textit{MCT4} were then measured using RT-qPCR and Western blot. PPAR\(\alpha\) activation was assessed by analysis of acyl-CoA oxidase (\textit{ACO}), carnitine palmitoyltransferase 1A (\textit{CPT1A}) and carnitine-acylcarnitine translocase (\textit{CACT}) gene expression, which are some of the main PPAR\(\alpha\) target genes (12, 28). To substantiate the results of PPAR\(\alpha\) agonist experiments, their effects were also tested in REC pretreated with GW 6471, a PPAR\(\alpha\) antagonist. Because both MCT isoforms have been characterized as proton-coupled lactate transporters (17), we also investigated lactate-dependent changes in pH\textsubscript{i} of REC treated with WY 14.643 to characterize functional consequences.

\textbf{MATERIALS AND METHODS}
Animals. Sheep (1-2 years) of both sexes had ad libitum access to hay and water for two weeks. The animals were stunned with a captive-bolt pistol and subsequently killed by exsanguination. The experimental procedures were approved by the regional council of Saxony (T86/10). At slaughter, the abdomen was opened, and pieces from the atrium ruminis were excised and stored on ice in Ca\(^{2+}\)- and Mg\(^{2+}\)-free Dulbecco’s phosphate-buffered saline (PBS) supplemented with 400 U/ml penicillin and 100 U/ml nystatin for 1 hour.

Cell culture. The cultivation of primary ruminal epithelial cells followed the procedure described by Müller et al. (30). Ruminal papillae were removed and subjected to fractional trypsinization to isolate cells of the stratum basale. Isolated cells were suspended in medium 199 (M199) containing 15% fetal calf serum (FCS), 20 mM HEPES, 50 µg/ml gentamicin and 2 mM L-glutamine and seeded at a density of \(10^5\) cells/cm\(^2\) in cell culture flasks (75 cm\(^2\); Greiner Bio One, Frickenhausen, Germany). The cells were incubated in a humidified 5% CO\(_2\) air atmosphere at 37°C. Forty-eight hours after seeding, the M199 was replaced with minimum essential Eagle’s nutrient medium (MEM) supplemented with 10% FCS, 20 mM HEPES, 50 µg/ml gentamicin and 2 mM L-glutamine. Six to nine days after seeding, cells were detached by trypsinization. The isolated cells were seeded at a density of \(2\cdot3\cdot10^4/cm^2\) either on 12-well plates (Greiner Bio One) for RT-qPCR, in cell culture flasks (75 cm\(^2\)) for Western Blot or on collagen-coated glass coverslips (10 mm diameter; Karl Hecht KG, Sondheim, Germany) for spectrofluorometry. Coverslips were placed in tissue culture dishes (35 mm diameter; Greiner Bio One). Three to four days after subcultivation, when confluence was >80%, WY 14.643 treatment was started. WY 14.643 was dissolved in Dimethylsulfoxid (DMSO). This stock solution was added to a low-serum medium containing MEM supplemented with 5% FCS, 20 mM HEPES and 2 mM L-glutamine to a final concentration of 25 or 100 µM. REC treated with the appropriate volume of DMSO were used as a control. Exposure to WY 14.643 and/or DMSO lasted for 48 hours. For experiments using a PPAR\(\alpha\)
inhibitor, cells were pretreated with 10 µM GW 6471, a selective PPARα antagonist (49) 4 h before treatment with 50 µM WY 14.643 for 48 hours. The concentration of WY 14.643 in combination with GW 6471 had to be lowered compared with the agonist experiments to prevent an effect on cell viability (tested by MTT test). Control cells were exposed to the appropriate volume of the solvent DMSO. All chemicals for cell culture were obtained from Sigma-Aldrich (Munich, Germany) and PAA (Pasching, Austria) if not stated otherwise.

Immunocytochemistry. Immunocytochemical staining was performed on control cells grown on coverslips. Following fixation with sodium metaperiodate-lysine-paraformaldehyde solution for 5 min, cells were washed 3 x 10 min in 0.1 M PBS and stored at 4°C in PBS containing 0.1% NaN₃. For immunofluorescence staining, cultured cells were preincubated for 60 min in PBS containing 4% goat serum, 4% horse serum and 0.5% Triton X-100. The same solution was used for the dilution of the primary and secondary antibodies. All chemicals were obtained from Sigma-Aldrich. Cultured REC were incubated for 12-16 hours at room temperature with the primary antibodies. Primary antibodies were chicken-anti-MCT1 (AB1286-I; Merck Millipore, Darmstadt, Germany) at a dilution of 1:100, rabbit-anti-MCT4 (sc-50329; Santa Cruz Biotechnology, Heidelberg, Germany) at a dilution of 1:100 and mouse-anti-Na⁺/K⁺-ATPase (alx-804-082; Enzo Life Sciences, Lörrach, Germany) at a dilution of 1:500. Combinations of up to two different primary antibodies raised in different host species were used per coverslip. After incubation with the primary antibodies, cultured cells were washed 3 x 10 min in PBS and incubated with the secondary antibodies goat-anti-chicken FITC at a dilution of 1:200, donkey-anti-rabbit Cy3 at a dilution of 1:500, donkey-anti-mouse Cy3 at a dilution of 1:500 and donkey-anti-rabbit Alexa488 at a dilution of 1:300 (all from Dianova, Hamburg, Germany) for 2.5 hours. Nonspecific binding of secondary antibodies was excluded by the application of the secondary antibodies to cultured cells without previous incubation with primary antibodies. For nuclear staining, 2 µg/ml 4’-6-
diamidino-2-phenylindole (DAPI, Invitrogen, Darmstadt, Germany) was used at a dilution of 1:500 for 1 minute. Finally, the cells were washed in PBS (3 x 10 min), mounted on slides, immersed in 80% PBS/glycerol solution and covered with a coverslip. Specimens were analyzed using an epifluorescence microscope (IX50, Olympus, Hamburg, Germany) with a black and white video camera (F-view, Olympus) attached to an image analysis system (cell F, Olympus). Analysis of co-localization of MCT1 and MCT4 was performed on 4 coverslips obtained from cultured REC of 4 different sheep. REC were stained against MCT1 and MCT4 and with DAPI. Ten photomicrographs were obtained from each coverslip. In the photomicrograph, cells clearly visible and at least immunoreactive for one of the MCT isoforms were analyzed for localization of MCT1 and MCT4 (in the cytoplasm and/or in the cell membrane) and for co-localization of both transport proteins.

Reverse transcription real-time PCR (RT-qPCR). Total RNA isolation from cells seeded on 12-well plates was performed after 48 hours of incubation. The RNeasy Micro Kit (Qiagen, Hilden, Germany) was used according to the manufacturer’s protocol. The quality and quantity of RNA were measured spectrophotometrically with a BioPhotometer (Eppendorf AG, Hamburg, Germany). Additionally, RNA integrity was checked by agarose gel electrophoresis. Reverse transcription (RT) was performed with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, St. Leon-Rot, Germany). Total RNA (500 ng) and 0.5 µl oligo (dT)₁₈ primer (0.5 µg/µl) were dissolved in H₂O to a total volume of 6 µl and incubated at 65°C for 5 minutes. Thereafter, 2 µl 5x Reaction Buffer, 0.5 µl RNase Inhibitor (20 U/µl), 1 µl 10 mM dNTP Mix and 0.5 µl Reverse Transcriptase (200 U/µl) were added for a total reaction volume of 10 µl. This mixture was incubated at 42°C for 60 minutes and then 5 minutes at 70°C to inactivate reverse transcriptase. The cDNA was stored at -20°C until further use.
For RT-PCR and RT-qPCR, primers (Table 1) were designed based on the sequences in the GenBank database using PrimerSelect (DNASTAR, Madison, WI, USA). All primers were synthesized by Eurofins MWG (Ebersberg, Germany). The PCR products of synthesized primer pairs were checked for specificity using agarose gel electrophoresis combined with sequencing. The obtained sequences were checked against the corresponding NCBI database entries (1). RT-qPCR was performed using SYBR® Green I and the Rotor Gene 6000 system (Corbett Research, Mortlake, NSW, Australia). The SensiMix SYBR No-ROX Kit (Bioline, Luckenwalde, Germany) was used according to the manufacturer’s protocol with a final reaction volume of 20 µl and a primer concentration of 112 nM, except for MCT4. Primer concentration for the MCT4 qPCR reaction was lowered to 84 nM to avoid primer-dimer formation. Each qPCR run contained a no-template control with DNase-free water instead of cDNA as well as a negative control using RNA instead of cDNA. Melting curve analysis was performed at the end of each qPCR reaction to confirm the amplification specificity. The reaction for each sample was performed in duplicate. Quantification cycle \( (C_q) \) and amplification efficiency of each amplification curve were determined using Rotor Gene Series Software 1.7 (Corbett Research). Expression ratios were calculated using the relative expression software tool (REST©; Ref. 33) with efficiency correction and normalization against the expression level of two averaged (geometric mean) reference genes, \( \text{GAPDH} \) and \( \text{Na}^+\text{K}^+-\text{ATPase} \). The control value was set to 1, and changes in the expression level of treated cells were presented as fold changes. Based on the \( C_q \) values, the expression of both reference genes was not different between control cells and treated cells (analyzed with BestKeeper; Ref. 34).

**RT-PCR.** Ruminal papillae were removed after slaughter, washed in PBS, immediately snap-frozen in liquid nitrogen and stored at -80°C. Specimens were processed with the RNeasy Midi Kit (Qiagen) following the manufacturer’s protocol. Reverse transcription of RNA was
performed as described in *RT-qPCR*. For PCR, the DyNAzyme II DNA Polymerase Kit (Thermo Scientific) was used to analyze cDNA from papillae and REC. The PCR mixture contained 10x Reaction Buffer (with 1.5 mM MgCl₂), 10 mM dNTPs, 1 U DNA Polymerase, 0.2 µM primer and 0.5 µl cDNA. The total reaction volume was 10 µl. The temperature protocol included denaturation for 1 min at 95°C followed by 30 cycles of 95°C (20 sec), annealing (30 sec, 58°C) and 72°C (35 sec), as well as a final elongation step at 72°C for 2 min. The PCR was performed with a Thermocycler PTC 200 (Biozym Scientific GmbH, Oldendorf, Germany). The products were loaded into an agarose gel (1% agarose) and separated by electrophoresis; PCR products were visualized by UV light and detected with a Bio Doc Analyze (Analytic Jena AG, Jena, Germany).

*Western blot Analysis.* For each treatment, two cell culture flasks (75 cm²) were incubated as described in *Cell culture*. Cells were washed with ice cold PBS, detached by cell scrapping and centrifuged at 1500 g for 5 min at 4 °C. Pellets were washed with ice cold PBS for two times and transferred to cryo container to store them at -80 °C until extraction of total protein. Protein extraction was carried out in a homogenizer with 400 µl of a lysis buffer consisting of 50 mM TRIS buffer, 4 mM EGTA, 10 mM EDTA, 0.1% Triton X-100, 100 mM β-glycerine-phosphate-disodium pentahydrate, 100 mM sodium pyrophosphate tetrabasic decahydrate, 15 mM sodium orthovanadate and 2.5 mM NaF (pH 7.4). After homogenization suspension was incubated for 1 h at 4°C under gentle agitation with subsequent centrifugation at 500g for 10 min at 4°C. The protein concentration of the supernatant was measured using the bicinchoninic acid (BCA) method with a Tecan Spectra Rainbow Microplate Reader (Tecan Deutschland GmbH, Crailsheim, Germany) and bovine serum albumin (BSA) as standard. Samples were adjusted to 3 µg in 26µl/well for analysis of MCT1 and to 20 µg in 44µl/well for MCT4 and denatured with Roti Load 1 (Carl Roth, Karlsruhe, Germany) for 10 min at room temperature followed by 5 min at 100 °C. The samples were subjected to 10% sodium
dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Afterwards, samples were transferred to nitrocellulose (Carl Roth) using the Mini-Protean© system (Bio-Rad laboratories, Hercules, CA, USA). The obtained blots were stained with Ponceau S solution to confirm equal sample loading and efficient transfer. Ponceau S destaining was carried out with TRIS-buffered saline (TBS) for 20 min at room temperature under gentle agitation. Blots were blocked in 3% BSA in TRIS-buffered saline containing 0.2% Tween-20 (TBST) before incubation with the primary antibodies (Table 2) at 4 °C over night. After washing five times with TBST, the blots were incubated with HRP-coupled secondary antibodies (Table 2) at room temperature for 1 h. Subsequently, the blots were rinsed again with TBST five times and once with TBS. Intensity of MCT1 and 4 protein bands was detected by enhanced chemiluminescence using a G:BOX Chemi XT4 (Syngene, Cambridge, UK) and analyzed with the GeneTools© software (Syngene). In a preliminary set of experiments, we found that the intensity of MCT1 protein band was linear from 1 to 6 µg and MCT4 protein band was linear from 10 to 30 µg of protein. The intensity of immunoreactive bands was normalized against the intensity of total proteins visualized by Ponceau S staining (40). Every Western Blot with treated and nontreated samples was applied and analyzed in triplicate on the same gel. All chemicals for Western Blot were obtained from Carl Roth and Sigma-Aldrich.

Spectrofluorometry. After 48 hours of treatment with 100 µM WY 14.643, coverslips covered with cells were mounted in an angled holder and washed one time in HEPES-buffered solution containing 20 mM L-lactate (= lactate buffer; see Buffer solutions). Afterwards, coverslips were inserted into a glass cuvette (10x10 mm; HELMA, Müllheim, Germany) and were incubated in L-lactate buffer supplemented with 10 µM 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein/acetomethylester (BCECF/AM; Calbiochem Merck, Darmstadt, Germany) for 45 minutes at 37°C to allow the pH-sensitive fluorescent dye to enter the cells (19, 31). To remove extracellular BCECF, cells were washed in lactate buffer without BCECF.
and subsequently transferred into another cuvette filled with lactate buffer free of BCECF. The cuvette was placed into a fluorescence spectrometer (LS 50B fluorescence spectrometer, PerkinElmer, Waltham, MA, USA) to record the emitted fluorescence. The recording was conducted at an emission of 530 nm in response to an excitation alternating between 495 nm (excitation maximum of BCECF when proton coupled) and 440 nm (excitation maximum of BCECF when uncoupled). The associated personal computer calculated the fluorescence ratio at 495 nm/440 nm excitation to evaluate the amount of free protons in the cell. The spectrometer was connected to a thermostatic holder to maintain the temperature of the buffer solutions at 37°C. After incubating for 220 seconds in lactate buffer, the cell-coated coverslips were transferred into another cuvette containing the preheated standard HEPES-buffered solution (= standard buffer; see Buffer solutions). The fluorescent signal was recorded for another 560 seconds. At the beginning of each measurement, either 600 µM p-hydroxymercuribenzoic acid (pHMB) dissolved in 0.1 N NaOH or the appropriate volume of NaOH was added to the buffer solution. For calculation of pHᵢ, the high potassium/nigericin method (45) was used after each measurement to calibrate pHᵢ. Cell-coated coverslips were then transferred into another cuvette containing calibration buffer with 121.5 mM KCl and 10 µM nigericin (see Buffer solutions). The pH of the buffer was decreased stepwise from pH 7.8 to pH 6.8 by adding 1 N HCl, and the fluorescent signal of the cells was measured. The resulting data were converted into a linear calibration curve, and the corresponding pHᵢ was calculated.

Buffer solutions. Measurements of pHᵢ were performed under bicarbonate-free conditions in the presence or absence of L-lactate. Lactate buffer contained (in mM): NaCl 60, KCl 5.4, CaCl₂ 0.6, MgCl₂ 1.2, NaH₂PO₄ 0.6, Na₂HPO₄ 2.4, N-methyl-D-glucamine 20, D-gluconate 20, Na-HEPES 20 and Na-L-lactate 20. In the standard buffer, L-lactate was replaced by 20 mM D-gluconate. The pH was adjusted to 7.2 by titration with 1 N HCl. The calibration
buffer contained (in mM): KCl 121.5, CaCl$_2$ 1, MgCl$_2$ 1, Na-HEPES 10, glucose 10 and 10 µM nigericin. All solutions were equilibrated at 37°C and 100% O$_2$. The osmolality was adjusted to 285 mosmol/kg with mannitol and was determined by freezing point depression with an osmometer (Knauer, Berlin, Germany). All chemicals for preparing buffer solutions were obtained from Sigma-Aldrich and VWR (Darmstadt, Germany).

Calculations and Statistics. To compare the results of spectrofluorometry, the increase in pH$_i$ after buffer change was quantified by calculating the slope of a linear regression line of pH$_i$ (slope). The period between 220 and 260 seconds covers the fast increase in pH$_i$ after change into L-lactate-free buffer; thus, the calculated slope represents the amount of L-lactate-dependent proton transport along the concentration gradient, which is further referred to as the slope of pH$_i$ recovery. Providing no change in intracellular buffer capacity, the slope of pH$_i$ recovery increases if more protons/L-lactate are transported out of the cell.

To estimate the co-localization of MCT1 and MCT4, the percentage of coincident staining was calculated for each sheep. Likewise percentage of membrane and/or cytosolic localization of MCT1 and MCT4 were calculated individually for each sheep.

The results are expressed as arithmetic means ± standard error of the mean (SEM). N indicates the number of animals used, and n is the number of specimens for each treatment.

The data obtained by RT-qPCR using two different concentration of WY 14.643 (agonist data) were compared by one-way analysis of variance (one-way ANOVA) followed by Dunnett’s Method to determine differences in the mean values among different treatments.

For analysis of RT-qPCR of the antagonist data, Western blot and spectrofluorometry, data were compared by paired Student’s $t$-test. The tests were performed using the software Sigma Plot 11.0 (Systat Software, Erkrath, Germany). Differences with $p<0.05$ were considered to be significant.
RESULTS

Detection and Localization of MCT1 and MCT4 in cultured REC by immunocytochemistry. REC showed immunoreactivity for MCT1 and/or MCT4 (Fig. 1). Both isoforms were localized in the cell membrane as well as in the cytoplasm. Membrane localization was confirmed by the co-localization of MCT with Na\(^+/K^+\)-ATPase (Fig. 2). Membrane localization for MCT1 and/or MCT4 was found in 53±6% of the cells examined. Of these cells (with membrane localization of MCT1 and/or MCT4) 44±5% expressed both MCT1 and MCT4 in their membrane. In 31±5% and 25±1% of the cells only MCT1 and MCT4 was found in the membrane, respectively.

Besides localization within the cell membrane, both MCT isoforms were expressed diffusely within the cytoplasm of all cells examined. In 65±9% of the cells examined, MCT1 seemed to be accumulated around the nucleus. In about half of these cells (35±10% of the cells examined) the perinuclear staining occurred also for MCT4.

Detection of PPAR\(\alpha\) and RXR by RT-PCR and sequence analysis. The presence of the respective mRNAs of PPAR\(\alpha\) and RXR in the rumen epithelium was determined by RT-PCR with cDNA templates derived from both intact ovine ruminal papillae and ovine REC maintained in culture. PCR products of both specimens showed a band at the expected molecular size of 400 bp for PPAR\(\alpha\) and 415 bp for RXR. The identity of the obtained products was confirmed by sequencing. The nucleotide sequence of the ovine ruminal PCR products shared 91% homology with bovine PPAR\(\alpha\) and 94% homology with bovine RXR (GenBank accession no. NM001034036 and XM002691687, respectively, NCBI).

Effect of WY 14.643 treatment on MCT1, MCT4, ACO, CPT1A and CACT gene expression. Compared with the untreated control, the abundance of MCT1 mRNA was slightly, but not
significantly, increased when the cells were treated with 25 µM WY 14.643 (Fig. 3). Likewise, mRNA abundance of the PPARα target genes ACO, CPT1A and CACT was slightly increased when cells were treated with 25 µM WY 14.643. Incubation with 100 µM WY 14.643 led to a significant increase in MCT1, ACO, CPT1A and CACT gene expression. Compared with the control, the expression levels of MCT1 increased by 31%. Expression levels of ACO, CPT1A and CACT increased by 28%, 356% and 234%, respectively. In contrast, the expression levels of MCT4 decreased significantly in a dose-dependent manner when cells were treated with 25 µM WY 14.643 (decrease: 14%) or 100 µM WY 14.643 (decrease: 50%).

**Effect of WY 14.643 in the presence and absence of PPARα antagonist GW 6471.** To explore the involvement of PPARα in the action of WY 14.643 on gene expression of MCT1, MCT4 and the PPARα target genes ACO, CPT1A and CACT, cells were pretreated without or with a selective PPARα antagonist 4 h before treatment with 50 µM WY 14.643. The WY 14.643 induced upregulation of the PPARα target genes CPT1A and CACT could be lowered by 38% (CPT) and 75% (CACT) when the cells were pretreated with the PPARα antagonist (Fig. 4). However, enhanced ACO gene expression after WY 14.643 treatment could not significantly be diminished by the PPARα antagonist. Increase of MCT1 gene expression after treatment with WY 14.643 was almost completely abolished by pretreatment with the PPARα antagonist. In contrast to the previous series (Fig. 3), MCT4 expression was stimulated in REC exposed to WY 14.643. This increase in MCT4 expression was not altered by GW 6471 pretreatment.

**Effect of WY 14.643 treatment on MCT1 and MCT4 protein expression.** To test if the changes in mRNA expression induced by WY 14.643 treatment, also lead to altered protein expression, protein abundance of MCT1 and MCT4 was analyzed by Western blot (Fig. 5). Staining with MCT1 antibody resulted in a single band at ~55 kDa, MCT4 antibody yielded a single band in Western blot at ~43 kDa like described in human skeletal muscle (43).
However, MCT1 and MCT4 protein expression was not altered by 48 h exposure to 100 µM WY 14.643.

$\text{pHi recovery after exposure to extracellular L-lactate.}$ Changes in MCT1 and MCT4 expression at the mRNA level induced by WY 14.643 may have led to functional consequences. Without differentiating between MCT1 and MCT4, Müller et al. (31) showed that lactate is removed from REC together with protons mainly by monocarboxylate transporters, at least in the absence of bicarbonate. Therefore, the L-lactate-coupled, bicarbonate-independent change in pH$_i$ provides a useful parameter for testing the activity of MCT located in the membrane. We first loaded the cells with L-lactate by exposing them to buffer solution containing 20 mM L-lactate for 45 minutes. After changing to L-lactate-free buffer, both control and WY 14.643 treated cells showed an increase in pH$_i$ in the first 60 seconds, which was followed by a slow decrease in pH$_i$ until the end of the measurement (Fig. 6).

To determine whether the proton-coupled L-lactate efflux was mediated by MCT1 and/or MCT4, 600 µM pHMB was added to the buffer. pHMB is a potent inhibitor of both MCT1 and MCT4 (48). Both in control and in WY 14.643-treated cells, pH$_i$ recovery after the change into lactate-free buffer was almost completely blocked by pHMB (Fig. 6 and Fig. 7).

To analyze differences between the two groups, the slope of pH$_i$ recovery was calculated as outlined in Statistics and Calculation. Calculation of slope began at 220 seconds after beginning of recording, when cells were exposed to the L-lactate-free solution, and ended at 260 seconds, to determine the immediate amount of extrusion of L-lactate independent from pH counter regulation mechanisms. pH$_i$ changes after L-lactate removal includes both MCT-dependent and MCT-independent mechanisms. To evaluate MCT-dependent changes of pH$_i$ recovery, the slope data of pH$_i$ recovery determined in pHMB exposed cells were subtracted from the corresponding slope data of pH$_i$ recovery in cells not treated with pHMB. The results
are presented in Fig. 7. The corrected slope (slope without pHMB - slope with pHMB) showed a numerical increase in WY 14.643-treated cells (0.0043 ± 0.00061 pH units/sec) in comparison with control cells (0.0026 ± 0.00069 pH units/sec) not treated with WY 14.643. The numerical increase, however, could not be proved to be significant (p=0.076; paired Student’s t-test).

**DISCUSSION**

In the present study, we demonstrated that ruminal epithelial cells maintained in culture express MCT1 and MCT4 both at the mRNA and protein levels. Thus, the MCT expression pattern of ovine REC maintained in culture resembles the pattern detected in the intact rumen epithelium (21, 31). In REC, MCT1 and MCT4 were expressed within the cell membrane as well as intracellularly.

The results of the RT-PCR analysis showed that PPARα and RXR are present in ruminal papillae as well as in REC at the mRNA level. The functionality of PPARα and RXR is underlined by the finding that the expression of PPARα target genes CPT1A and CACT increased significantly after WY 14.643 treatment. Considering the decreased response to WY 14.643 and the missing inhibition by the antagonist, ACO seems to play a minor role as a PPARα target gene in REC. Changes in the abundance of MCT1 mRNA after WY 14.643 treatment show that PPARα activation causes alterations in the mRNA expression pattern of this MCT isoform. PPARα-induced upregulation of MCT1 in the ruminal cells is at least qualitatively similar to that observed in the livers of rodents and pigs (25) and in the small intestine of mice (26). However, whether the observed changes are direct or indirect consequences of PPARα activation cannot be definitively determined. With respect to MCT1, König et al. (25) did not detect an induction of MCT1 promoter activity by WY 14.643, suggesting that MCT1 is not a direct target gene of PPARα. Thus, the upregulation of MCT1
may be induced by secondary factors downstream from PPARα activation. In our study the
effect of WY 14.643 on MCT1 expression was almost totally abolished by a PPARα
antagonist. This finding supports the assumption that ruminal MCT1 expression may be very
effectively upregulated by PPARα in a direct or indirect manner. Nevertheless, the natural
ligand that influences MCT1 expression via PPARα remains unknown. Thus far, a multitude
of natural ligands, such as fatty acids and their catabolites, have been characterized to bind to
PPARα (37, 47). Taking the variety of ligands into account, especially fatty acids, one might
speculate that SCFA themselves and/or their intracellular catabolites serve as natural ligands
for MCT1 regulatory pathways in the rumen epithelium. Studies in human intestinal epithelial
cells were able to show upregulation of MCT1 by butyrate (5). By contrast, Baldwin et al. (3)
did not detect a change in the expression of MCT1 24-168 hours after intraruminal butyrate
infusion when studying butyrate-induced changes in the transcriptome of the rumen
epithelium. Thus, SCFA-induced effects may also be modulated and counteracted by other
substrates, hormones and triggers located on the luminal and blood-oriented sides of the
epithelium. This assumption is confirmed by our previous studies that clearly showed both the
localization and protein expression intensity of MCT1 in the forestomach changes drastically
during the development of newly born calves, i.e., in the complete absence of SCFA (35).

By contrast to MCT1, the response of MCT4 to WY 14.643 was not uniform and could not be
modulated by exposure to a PPARα antagonist. Therefore, regulation of MCT4 by PPARα
seems to be unlikely. The independence of MCT1 and MCT4 regulation is in contrast to other
studies pointing to co-regulation of both isoforms. Co-regulation of both isoforms has been
demonstrated to occur in hypoxic and/or cancer cells (8, 38) and under the influence of acute
and chronic exercise in skeletal muscle cells (44). In these studies, both isoforms were
generally upregulated under the conditions applied.

The difference in the regulatory response between REC and the other cell types mentioned
may be because, in cancer and skeletal muscle cells, MCT1 and MCT4 mainly serve as
mediators of lactate transfer between glycolytic and oxidative cells (7), whereas in the intact ruminal epithelium, MCT1 and MCT4 are thought to be important for transepithelial and intraepithelial shuttling of SCFA and/or their metabolites (14, 21).

In the intact rumen epithelium of adult ruminants, MCT1 is localized on the blood-oriented side (15, 31) and is strongly involved in the extrusion of products of intracellular SCFA catabolism (31) and undegraded SCFA (11, 29). Thus, any upregulation of blood-oriented MCT1 might be interpreted as a protective mechanism of the cells themselves to avoid intracellular accumulation of undegraded SCFA and products of intracellular SCFA degradation. MCT4 was shown to be localized on the lumen-oriented side in the intact ruminal epithelium (21), but functional studies suggest only a minor influence of MCT on the lumen-oriented uptake of acetate and lactate (2). In the present study, MCT1 and MCT4 were detected in the membrane of REC as indicated by their co-localization with the Na⁺/K⁺-ATPase. Studying the co-localization of MCT1 and MCT4 within the membrane revealed that both isoforms were co-localized in a significant amount of cells. This finding is in contrast to observations from the intact ruminal epithelium showing MCT1 localization in the basolateral membrane and MCT4 localization mainly in the apical membrane (21, 31). However, in the cultured REC, a strict polarization of an apical and basolateral membrane compartment has not yet been shown. Additionally, one must keep in mind that the REC used in this study may not be all on the same differentiation level. In newborn calves, we recently showed that MCT1 localization within the epithelial membranes changed around the time of birth (35).

In the majority of immunoreactive cells, MCT1 and 4 were co-localized within the cytoplasm. At least for MCT1, this finding does not resemble the distribution found in the intact epithelium where MCT1 has been found to be located predominantly in the cell membranes (15, 20, 31). The intracellular distribution of MCT1 and MCT4 in REC could be due to the storage of these proteins in the endoplasmic reticulum as proposed by Kirk et al. (22). Storage within the endoplasmic reticulum would most likely explain the perinuclear accumulation of
MCT1 in 65% and MCT4 in 35% of the cells. MCT might not only be stored intracellularly but also function as mitochondrial lactate transporters as suggested by Kirat et al. (21) for MCT4. However, Halestrap (17) strongly opposed the assumption that any MCT plays a role in mitochondrial transfer of lactate or ketone bodies.

Despite the questionable role of intracellular located MCT, we examined the involvement of membrane located MCT in the regulation of pHᵢ in cultured REC. MCT1 works as a proton cotransporter (16, 29) and/or as a bicarbonate exchanger (11, 39). MCT4 is described to work as a proton cotransporter (16). Under certain conditions, H⁺ removal by MCT may even exceed the sum of H⁺ removal via other regulatory systems such as the Na⁺/H⁺ exchanger (44). Thus, if the upregulation of mRNA expression is followed by functional changes, this should help the epithelium increase the capacity for proton extrusion. However, although treatment with 100 µM WY 14.643 changed the mRNA expression of MCT1, the treatment did not affect the protein expression of either transporter and only slightly increased the ability for lactate dependent proton extrusion.

Nevertheless, as shown in Fig 6 and 7, our study points to a prominent role of MCT in H⁺ removal out of REC even under unstimulated conditions. Proton transfer was blocked almost completely by pHMB, suggesting that MCT are important pHᵢ regulatory proteins in the membrane, at least under the conditions used in this study. Thus, the upregulation of mRNA expression of MCT1 may not have led to functional alterations, because MCT1 was already expressed and/or working at a high level.

**PERSPECTIVES AND SIGNIFICANCE**

Our data demonstrate that the mRNA expression of MCT1 in ruminal epithelial cells is regulated by PPARα. However, upregulation on the mRNA level did not lead to an increase in protein expression or an increase in MCT-mediated proton transfer. Nevertheless, studies on proton extrusion under control conditions showed that more than 60% of pHᵢ recovery is
mediated by MCT. The effective proton extrusion underlines the fact that MCT are expressed and/or are working at a high level even under unstimulated conditions and thus do not respond to enhanced mRNA expression. Furthermore, we demonstrated that MCT1 and MCT4 are not only located in the cell membrane but, to a large extent, are also stored intracellularly in the REC. The intracellular localization of MCT1 and MCT4 points to these proteins playing a role not only in transcellular but also in intracellular transport processes. The results of this study also underline the assumption that MCT proteins play crucial and most likely multiple roles in the transfer of protons, SCFA and their catabolites and thus are kept at a high protein and/or activity level, even under unstimulated conditions.

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DISCLOSURE
No conflicts of interest are declared by the authors.

REFERENCES


2. Aschenbach JR, Bilk S, Tadesse G, Stumpff F and Gabel G. Bicarbonate-dependent and bicarbonate-independent mechanisms contribute to nondiffusive uptake of acetate in the


42. Schoonjans K, Staels B and Auwerx J. The peroxisome proliferator activated receptors (PPARS) and their effects on lipid metabolism and adipocyte differentiation. *Biochim Biophys Acta* 1302: 93-109, 1996.


47. Varga T, Czimmerer Z and Nagy L. PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. *Biochim Biophys Acta* 1812: 1007-1022, 2011.


49. Xu HE, Stanley TB, Montana VG, Lambert MH, Shearer BG, Cobb JE, McKee DD, Galardi CM, Plunket KD, Nolte RT, Parks DJ, Moore JT, Kliwer SA, Willson TM and


Figure captions

Fig. 1. Immunocytochemical staining against MCT1 (A, C) and MCT4 (B, D) in REC. Nuclei are stained with DAPI (blue). (A-B) clarify the variety of MCT1 and MCT4 distribution in the membrane. Cells co-expressing MCT1 and MCT4 at their margins (presumably within the cell membrane) are indicated by a solid arrow. Expression of either MCT1 or MCT4 in the cell membrane is indicated by an open arrowhead or an open arrow, respectively. Nearly all the cells express MCT1 and MCT4 intracellularly. (C-D) are showing a strong cytoplasmic perinuclear staining of MCT1 and MCT4 (indicated by a solid arrowhead). Scale bars represent 50 µm.

Fig. 2. Immunocytochemical staining against MCT1 (A), Na⁺/K⁺-ATPase (B, D), MCT4 (C) in REC. Nuclei are stained with DAPI (blue). (A-B) MCT1 (green) can be found in the cytoplasm and co-localized with Na⁺/K⁺-ATPase (red) in the membrane (indicated by an open arrowhead). (D-F) MCT4 (green) is co-localized with Na⁺/K⁺-ATPase (red) in the membrane (indicated by a solid arrowhead) and, furthermore, localized in the cytoplasm. Scale bars represent 50 µm.

Fig. 3. RT-qPCR showing the relative expression of MCT1, MCT4 (A), ACO, CPT1A and CACT (B) mRNA. Values of the group only treated with DMSO (solvent) are set to 1. The relative mRNA abundance of REC treated with two different concentrations of WY 14.643 (WY) for 48 hours is shown in the graphs. (A) is showing the expression of the genes MCT1 and MCT4. In (B) relative expression of PPARα target genes ACO, CPT1A and CACT is shown. GAPDH and Na⁺/K⁺-ATPase were used as reference genes. Vertical bars represent means ± SEM of N=4 animals. The asterisks indicate significant differences compared with the control value (* p<0.05; *** p<0.001; One-way ANOVA followed by Dunnett’s Method).
**Fig. 4.** RT-qPCR showing the relative expression of \( \text{MCT1, MCT4, ACO, CPT1A and CACT} \) mRNA treated with 50 µM WY 14.643 after 4h preincubation with the PPAR\(\alpha\) selective antagonist GW6471 dissolved in DMSO (WY 50 + GW 6471) or after 4h preincubation with DMSO (WY 50) only. Control cells were treated only with DMSO. Results of control cells were set to 1. GAPDH and Na\(^+\)/K\(^-\)-ATPase were used as reference genes. Vertical bars represent means ± SEM of N=5 animals. The asterisks indicate significant differences compared with the control value (* \(p<0.05\); ** \(p<0.001\); paired Student’s \(t\)-test).

**Fig. 5.** Western blot analysis of MCT1 and MCT4 in cells exposed to DMSO (control) or to 100 µM WY 14.643 (WY 100) for 48 h. Representative immunoblots specific for MCT1 and MCT4 are shown on the left side. Intensity of bands was normalized against intensity of total proteins visualized by Ponceau S staining. Results are shown in the graph on the right side. Vertical bars represent means ± SEM of n=18 specimens obtained from N=6 animals.

**Fig. 6.** \(p\text{Hi}\) recovery of L-lactate (20 mM)-loaded REC after transfer to L-lactate-free solution in the presence and absence of 600 µM pHMB. Before measuring \(p\text{Hi}\) recovery, REC were exposed to 100 µM WY 14.643 dissolved in DMSO (WY) or the appropriate amount of the solvent DMSO (control) for 48 hours. Values are presented as the means ± SEM of n=20 coverslips obtained from N=10 animals.

**Fig. 7.** Comparison of the slopes of \(p\text{Hi}\) for control cells and cells treated with 100 µM WY 14.643 (WY 100 µM). The slope of a linear regression was calculated for the data points between 220 and 260 sec. The slope of \(p\text{Hi}\) recovery determined in pHMB-inhibited cells was significantly lower in both control and WY 14.643-treated cells. Vertical bars represent means ± SEM of n=20 coverslips obtained from N=10 animals. Asterisks indicate significant differences (* \(p<0.05\); ** \(p<0.001\); paired Student’s \(t\)-test).

**Table 1.** Specific primers used for RT-PCR and RT-qPCR

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<th>Gene</th>
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<td>PPAR(\alpha)</td>
<td>5’ CATGTGAGGGCTGCAAGGGTTTCT 3’</td>
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<td>58°C</td>
<td>NM_001034036</td>
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<tr>
<td></td>
<td>5’ CTCGGCCCATACACAGCGTCTCCAT 3’</td>
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Table 2. Antibodies for Western blot

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<th>Purchased from</th>
<th>Secondary antibody</th>
<th>Dilution</th>
<th>Purchased from</th>
<th>Protein size (kDa)</th>
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<tr>
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<td>Dianova, #103-035-155</td>
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<td>Donkey-anti-goat HRP</td>
<td>1:5000</td>
<td>Santa Cruz Biotechnology sc-3851</td>
<td>43</td>
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1^Annealing temperature (T_a)