Identification of differentially expressed proteins in ruminal epithelium in response to a concentrate-supplemented diet

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Submitted 25 June 2010; accepted in final form 6 May 2011

Ruminants are of major worldwide interest and economic importance because of increasing meat and milk production. A substantial improvement in the productivity and performance of ruminants can only be achieved with adequate dietary supplementation. When high-yielding cattle, milk sheep, or goats merely consume forage, the intake of energy and protein is too low for the desired rates of animal performance. Hence, supplementation of forage-fed ruminants with sufficient amounts of energy and protein is necessary and significantly improves milk production or daily weight gain (41), but, particularly, a surplus of easily fermented carbohydrates or low-fiber intake will place the animals at risk. Such a change in the diet is a challenge for rumen microbes (31) and especially for the ruminal epithelium with respect to adaptation to the new fermentation pattern (47, 55). Insufficient adaptation to diet may result in disintegration of the epithelium (22), translocation of lipopolysaccharides (30), and disturbed transport mechanisms (22) and can further cause subacute ruminal acidosis (14, 49). This disease not only can lead to depressed feed intake and milk production, but can also be associated with laminitis, inflammation, and liver abscesses (14, 42, 49).

Hence, one objective of attaining an adequate diet composition for ruminants is to maintain the physiological functions of the forestomach, such as optimal fermentation and absorptive properties (15, 20). The phenotypic effects of diets, particularly of concentrate supplementation on rumen morphology and physiology, have been well investigated (38, 55) and reviewed (7). In particular, feeding a diet containing high-concentrate rations increases the number and/or size of papillae in the rumen and influences the transport properties of the ruminal epithelium (19, 26). Furthermore, a diet rich in concentrate increases short-chain fatty acid (SCFA) concentration and leads to a decrease of pH (47).

A better understanding of these processes at the molecular level is helpful in order, subsequently, to increase and/or modulate animal performance, but, concomitantly, to minimize negative implications for health, productivity, and animal welfare. Recently, microarray studies of ruminal tissue samples from cattle revealed that genes involved in mitogen-activated protein kinase signaling, regulation of actin cytoskeleton, and focal adhesion are dominantly affected in response to high-concentrate diet (58). Furthermore, gene expression changes for cholesterol homeostasis are associated with rumen epithelial adaptation to diet (57). However, no corresponding proteomic study focusing on rumen has been reported to date. Two-dimensional gel electrophoresis is a holistic protein characterization method that might help to decipher proteomic changes in the ovine rumen. It was, therefore, the aim of this study 1) to determine global protein expression changes in the ruminal epithelium in response to a concentrate diet; and 2) to identify specific targets underlining the well-characterized changes in rumen morphology, metabolism, and absorption.

MATERIAL AND METHODS

Animals and feeding. A group of German dairy sheep of different sex were used. Animals were 9–10 mo old at the time of the experiment, and their weight ranged between 33 and 40 kg at the beginning of the experiment. Experiments were conducted in accordance with German law for the care and use of experimental animals, as attested by the Animal Welfare and Ethics Representative of the Veterinary Faculty/FU Berlin. No procedures were conducted with live animals. The sheep were slaughtered according to German slaughter regulations (after stunning; permit no. T0064/99 from the Landesamt für Gesundheit, Berlin), and the material used for scientific purposes was taken from the gastroesophageal vestibule of the dead animals. A full description of the experimental procedures,
feeding regime, and diet composition has recently been described in detail (19). Briefly, before implementation of the experimental protocol, all animals were fed a pure hay diet ad libitum over a period of 6 wk to ensure adaptation to a low-energy roughage diet. The hay contained per kg dry matter (DM) 144 g crude protein, 28 g fat, 277 g crude fiber, 89 g ash, 29 g potassium, 2.2 g sodium, and 8.5 MJ metabolizable energy (ME). At the beginning of the experimental period, hay intake was 1,000 g per animal and day (88% DM) and was offered in two portions at 7:00 AM and 3:00 PM, equaling an intake of 7.5 MJ ME, which is slightly above the requirement for the maintenance of sheep (40 kg body wt), according to the Gesellschaft für Ernährungsphysiologie (20a). Following this period, the animals were randomly assigned to four groups, according to the type and duration of feeding, as follows: six animals of one group (control) were fed a pure hay diet, as described above, and the other groups received a mixed diet (hay/concentrate) for 2 days (6 animals) and 6 wk (5 animals), respectively. The sheep were kept individually in pens on straw to control the feed intake. The supplemented concentrate contained per kg DM: 176 g crude protein, 143 g crude fiber, 53 g fat, 104 g ash, and 6.5 MJ ME. Animals received 400 g hay and 400 g concentrate twice a day (7:00 AM and 3:00 PM), which amounted to 11.2 MJ ME per animal and day and permitted a growth rate of ~200 g/day (20a). No significant variation of feed intake was observed between the feeding groups (19). At the end of each feeding period, the sheep were killed by captive bolt stunning, followed by exsanguination from the carotid arteries. Within 5 min after slaughter, a segment of the ventral rumen wall was taken and immediately cleaned in phosphate-buffered saline buffer, and the ruminal epithelium was separated from the muscular and serosal layers by blunt dissection. The tissue was then cut into smaller pieces of ~2 × 2 cm, and the papillae were removed and washed three times in sterile phosphate-buffered saline. These samples were frozen immediately in liquid nitrogen and stored in aliquots of 250 mg until analysis.

Sample preparation. Protein extraction from the samples was performed by the addition of 1 mL lysis buffer (9 M urea, 2% wt/vol CHAPS, biolyte, pH 3–10, supplemented with 60 mM DTT, 5 μM PMPSF) and protease inhibitor mixture (Calbiochem). The proteins were extracted by using FastPrep FP120 homogenizer (MP Biomedicals) with appropriate lysing matrix tubes. The tissue extract was incubated for 1 h on ice and centrifuged at 10,000 g, 4°C, for 10 min. The supernatant was stored at ~80°C until analyzed. Protein lysates were further purified by a modified TCA-acetone precipitation method (2-D-Clean Up kit, GE Healthcare) and finally resuspended in differential in gel electrophoresis (DIGE) labeling buffer (8 M urea, 4% wt/vol CHAPS, 30 mM Tris, pH 8.5). Protein concentration was determined by using a 2-D Quant Kit (GE Healthcare).

Two-dimensional DIGE analysis. For two-dimensional (2D) DIGE, a pool consisting of equal amounts of each animal sample (rumen tissue) was prepared as an internal standard. Thus every protein from every sample was represented in this standard on all gels. Each sample (60 μg protein) was labeled with 480 pmol appropriate CyDye following the manufacturer’s protocol. The total volume of each labeled protein sample (3 × 60 μg) was adjusted to 300 μl with DeStreak Rehydration Solution (GE Healthcare). Samples were applied to isoelectric focusing (IEF) strips (3–10 pH range, nonlinear, 17 cm; Bio-Rad), which were overloaded with 2.5 mL DryStrip Cover Fluid (GE Healthcare) and equilibrated for 14 h at 50 V and isoelectrically focused at 1 h 200 V, 1 h 500 V, and finally at 10,000 V for 7 h by using a Protean IEF cell (Bio-Rad). Thereafter, the strips were equilibrated for 15 min with gentle shaking in 50 mM Tris-HCl (pH 8.8) containing 6 M urea, 4% SDS, 65 mM DTT, 30% glycerol, and 0.02% bromophenol blue; 135 mM iodoacetamide were added to the second equilibration solution instead of DTT, and the strips were further incubated for 15 min in this solution.

Subsequently, strips were loaded onto 12% acrylamide vertical gels. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for the second dimension was carried out in an ETTAN DALT six electrophoresis unit (GE Healthcare), first at 0.2 W/gel for 1 h and thereafter at 2 W/gel for 18 h.

Image acquisition and analysis. Protein spots were visualized by using Typhoon 9400 (GE Healthcare), choosing an appropriate wave-length for Cy2, Cy3, and Cy5 dyes (Cy2 = 520 nm; Cy3 = 580 nm; Cy5 = 670 nm) at a resolution of 100 μm and were cropped and imported into DeCyder V.6.5 software (GE Healthcare). During spot detection by a codetection algorithm in the software, the estimated number of spots was set at 2,500, and the exclude filter was set a slope >1.7 and area <200. Because of an intrinsic variability associated with animals, we chose a stringent criterion, for which only those proteins present in at least five of the six gels (or four of the five gels) were considered for further analysis. The DeCyder differential in the gel analysis module was used to process the images from a single gel and enables the pairwise comparison of each control (hay-fed sample) and treated sample (concentrate-fed) to the pooled internal standard. The abundance of each protein spot was determined as a ratio to its corresponding spot present in the internal standard on the same gel. The DeCyder biological variation analysis module was used to standardize the ratios across the gels, accounting for the differences in the internal standard.

Liquid chromatography electrospray ionization mass spectrometry. Changes of protein expression in response to dietary alterations detected by 2D-DIGE analysis were matched with silver-stained protein patterns (400 μg protein), and some selected spots that showed at least 1.5 or more times enhanced/decreased expression were selected for identification. Spots of interest were excised from the gel. The nano-liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS/MS) analysis was performed by Proteome Factory Berlin (Proteome Factory) using an Agilent 1100 nano-LC system (Agilent), PicoTip emitter (New Objective), and an Esquire 3000 plus ion trap MS (Bruker). Protein spots were in-gel digested by trypsin (Promega), and proteins were identified using MS/MS ion search of Mascot search engine (Matrix Science) and nr protein database (National Center for Biotechnology Information, Bethesda, MD). Ion charge in search parameters for ions from ESI-MS/MS data acquisition were set to “+1, +2, or +3”, according to the instrument’s and method’s common charge state distribution.

Quantitative reverse transcription-polymerase chain reaction. Total RNA was extracted from tissue samples by using an Invisorb Spin Cell RNA Mini Kit (Invitrek), according to the manufacturer’s instructions. A sample of 1 μg total RNA was treated with 3.75 μM random hexamers (GE Healthcare) and 200 units of Moloney murine leukemia virus reverse transcriptase (Fermentas) in a 60-μl reaction mixture to generate single-stranded cDNA (45). Possible genomic DNA contamination was removed before reverse transcription by performing a DNase treatment.

Real-time PCR in the presence of SYBR Green I was performed by using a Rotor-Gene 3000 (Corbett Research), as previously described (45). Used primers are indicated in Table 1.

SDS-PAGE and immunoblotting. Tissues were homogenized in DIGE buffer containing protease inhibitor cocktail (Merck Biosciences). Sample proteins (20 μg) and a prestained protein-weight marker (Bio-Rad) were resolved by SDS-PAGE in 12% polyacryl-amide gels and transferred onto nitrocellulose membranes in Tris-glycine buffer with 20% (vol/vol) methanol. The membrane was saturated with 5% (wt/vol) nonfat milk powder (Roth) prepared in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature and then incubated with primary antibody overnight at 4°C. The primary antibodies employed were mouse monoclonal actin-related protein 3 (ARP3) (Abcam) used at a 1:5,000 dilution,
and rabbit polyclonal peroxiredoxin 6 (PRDX6) (LIFESPAN Biosciences) used at a 1:500 dilution. The carbonic anhydrase I (CAI) antibody (antibodies online; the online version of this article contains supplemental data) was labeled by using the horseradish peroxidase Conjugation Kit (Serotec) and diluted to 1:500. After several washes with Tris-buffered saline containing 0.1% Tween 20, membranes were incubated in a 1:20,000 dilution of an anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (GE Healthcare). The signals were detected by chemiluminescence with ECL Advance (GE Healthcare), according to the manufacturer’s instructions. Finally, membranes were incubated with a 1:2,000 dilution of mouse monoclonal D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-antibody (Acris) to normalize the results. Densitometry analysis of the protein bands was performed using ImageQuant TL software (GE Healthcare).

Statistics. Using Decyder software, proteins were defined as differentially regulated if the observed fold-change calculated as the ratio of the average standardized abundances corresponding to the groups of samples (hay and concentrate) was > 1.5, with P values of <0.05 (Student’s t-test).

The quantity of each specific mRNAs was normalized with the program GeNorm and the selected control genes 18S rRNA, GAPDH, succinate dehydrogenase complex subunit A (SDHA), β2-micro-globulin (B2M), and β-actin (ACTB) according to Ref. 61. The normalized values were used for statistical assessment and for the generation of box and whisker plots. The diagrams present the median values with 50% of all data within the box. The normalized means obtained from the duplicates were analyzed by the Kruskal-Wallis H-test. When this test indicated significant differences, the Mann-Whitney U-test was used to compare the samples from the control (hay feeding) with samples obtained at distinct points in time during the feeding of the concentrate-supplemented diet. Diagrams and statistical tests of mRNA expression were performed by using SPSS 18.0 (SPSS). Statistical analysis of Western blots was performed using Student’s t-test for unpaired samples. A P value <0.05 was considered as significant.

RESULTS

Proteome analysis. Highly sensitive 2D-DIGE technology has been applied to evaluate the time-dependent effects of dietary change on protein expression in ovine ruminal epithelium. The overall number of proteins separated by 2D-DIGE was ~2,200 from each tissue sample, independent of experimental conditions and feeding regime. Figure 1A shows one representative 2D gel of a rumen protein extract of sheep fed for 2 days with a concentrate-supplemented diet. Analysis of
A selection of highly separated differentially expressed protein spots (15) was excised from the gels. By using LC-ESI-MS/MS and data-based researches, these spots were identified and assigned in a single representative 2D gel (Fig. 2). Proteins corresponding to the numbered spots in Fig. 2 are listed in Tables 2 and 3. It should be mentioned that PRDX6 and annexin 5 (ANXA5) were identified in three (1, 2, 3) and two (4, 5) spots, respectively (Fig. 2).

On the basis of information in the literature, mainly from the National Center for Biotechnology Information, USA (NCBI), the 15 identified proteins were classified according to their biological function as follows: proteins with a structural function, proteins involved in signal transduction, stress response, metabolic enzymes, and transporter proteins. Each protein was assigned to one major function (Table 3).

In particular, ARP3 is involved in the regulation of cellular reorganization and the cytoskeleton. ANXA1 and ANXA5 have mainly been implicated in signal transduction. Two proteins are stress-related proteins [thioredoxin-domain-containing protein 5 precursor (TXNDC5P) and PRDX6], and five proteins have direct metabolic functions, namely $\delta$-adenosyl-homocysteine hydrolase (AHCY), ATP synthase $\gamma$-subunit precursor (ATP5G), isocitrate dehydrogenase (IDH), thiopurine $S$-methyltransferase (TPMT) and CAI. Figure 3 shows details of 2D-DIGE images and the corresponding three-dimensional views of two representative differentially expressed proteins in response to concentrate diet, as analyzed by DeCyder software. The expression levels of some proteins were decreased, e.g., CAI (Fig. 3A), whereas other spots showed a higher expression level, e.g., AHCY (Fig. 3B). The changes in the abundance of these proteins in all corresponding gels of the experiment (hay-fed animals/concentrate-fed animals), as analyzed by the biological variation analysis module of DeCyder software, are indicated in Fig. 3, right.

Table 2 summarizes the distinct protein expression levels of the identified proteins of ovine ruminal epithelium and indicates those that are increased (+) or decreased (−) in comparison of the tissue samples from animals fed the concentrate-supplemented diet vs. the control hay diet at the indicated points in time. Some of the identified proteins, namely PRDX6, TXNDC5P, ATP5G, and IDH, significantly change their ex-

Table 2. Differentially expressed proteins identified in the rumen of concentrate-fed sheep compared with hay-fed controls after 2 days or 6 wk of diet

<table>
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<tr>
<th>Spot No.</th>
<th>pI</th>
<th>Mass, Da</th>
<th>Score</th>
<th>Accession No.</th>
<th>Average Ratio After 2 Days</th>
<th>Average Ratio After 6 wk</th>
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<td>+1.65*</td>
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<td>8</td>
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<td>+1.24†</td>
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expression only after 2 days of the concentrate diet, and three proteins, namely CAI, esterase D (ESD), and ARP3, exhibit significant differences exclusively after 6 wk. Pre-proserum albumin, ANXA1, and AHCY are significantly more expressed at 2 days and 6 wk, whereas thiopurine S-methyltransferase is decreased at both points in time.

**Analysis of rumen gene expression.** Quantitative RT-PCR analysis was performed on all rumen tissue samples used for 2D-DIGE experiments to confirm differences in specific protein expression, as indicated in Fig. 4. Whereas the level of mRNA for ANXA5 was found to be decreased after 2 days in concentrate-fed sheep compared with hay-fed animals (Fig. 4A), the metabolic enzyme ESD was upregulated after 6 wk (Fig. 4B). These results at the mRNA level were consistent with the respective results of protein expression (Table 2), thus reflecting transcriptional regulation. However, no significant expression alteration was found for the transcripts of the other 13 genes in response to dietary change (data not shown).

**Western blot confirmation.** To examine further whether proteomic changes in response to concentrate diet revealed by 2D-DIGE were accompanied by corresponding expression, one representative protein of each of three different functional groups (see Table 3), PRDX6, CAI, and ARP3, was examined by Western blotting. PRDX6, ARP3, and CAI were chosen based on their obvious expression level in 2D-DIGE and on their function and time course of expression upon diet and antibody availability. As shown in Fig. 5, good agreement exists in the expression levels of the proteins between 2D gels and corresponding Western blot analysis. The 25-kDa band of PRDX6 showed a significant downregulation in concentrate-fed diet sheep compared with controls (hay-fed sheep) after 2 days (Fig. 5A). ARP3 revealed a single band at 47 kDa and was significantly upregulated in concentrate-fed animals after 6 wk (Fig. 5B), whereas CAI was downregulated (Fig. 5C).

**DISCUSSION**

The response of the ruminal epithelium to an increase of energy (and protein) has been previously studied in sheep, cattle, and goats (19, 47, 55).

The data presented here provide, to our knowledge, the first proteomic study characterizing comprehensive protein expression patterns in the ruminal epithelium of sheep in response to dietary changes. With the help of the DIGE technology, we have been able to detect 60 differentially expressed proteins between hay-fed and concentrate-fed sheep after 2 days. In contrast, only 14 proteins are differentially expressed after 6 wk. This difference in the number of differentially expressed proteins supports the hypothesis that an adaptation of the epithelium to this diet change takes place.

We have identified 15 differentially expressed proteins known to be involved in different cellular functions that will be discussed in detail below, according to their proposed physiological functions.

**Structural proteins.** In particular, the three cytoskeleton-related proteins, ARP3, ANXA1, and ANXA5, have been identified as being differentially regulated, in response to dietary change. The ARP2/3 complex is a ubiquitous and essential component of the actin cytoskeleton, which determines the shape, motility, and internal organization of eukaryotic cells. It is an essential effector of signal transduction networks controlling cellular differentiation in eukaryotes (40). Exclusively, after 6 wk of concentrate diet, ARP3 has been found to be upregulated at translational level, as validated by Western blot. One can speculate that ARP3 is involved in the well-known studied diet-dependent morphological alterations of the ruminal epithelium (18, 26, 54, 55). Supporting this hypothesis, recently, gene ontology analysis after comparison of gene expression profiles in ruminal tissue from cows fed with low- or high-concentrate diets revealed that the regulation of the actin cytoskeleton is one of the most effected pathways (58). Obvious expression changes have been found in this study for members of the multifunctional annexin family (ANXA1, ANXA5), further supporting significant diet-dependent remodeling events in ruminal epithelium. Particularly for ANXA1, it has been shown that it plays an important role in actin remodeling (66). Various isoforms have been observed with respect to ANXA5, since spots (designated as spots 4 and 5 in Fig. 2) with different isoelectric points have been identi-
fied as the same protein. These isoforms possibly reflect distinct levels of phosphorylation (37), suggesting functional fine-tuning.

Annexins can form ion channels, which has also been confirmed for ANXA1 and ANXA5 (29, 44). We should mention in this context that ruminal Ca\(^{2+}\) transport is increased in concentrate-fed sheep (60), indicating possible interactions between annexin and Ca\(^{2+}\) transport. The interaction between these proteins and Ca\(^{2+}\) is also known from other studies reviewed by Gerke et al. (23). Furthermore, ANXA1 and ANXA5 can inhibit phospholipase A2 (32, 53). Since cPLA2 is a key enzyme responsible for signal transduction in inflammation, and the annexins have been

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Fig. 3. Expression analysis of two selected rumen proteins, which are differentially expressed \((P < 0.05)\) in response to dietary change, as revealed by DeCyder software. Spots of the selected proteins, the corresponding three-dimensional views, and the changes of the standard abundance (control = hay fed, treated = concentrate-supplemented fed) are shown. A: one example [carboanhydrase 1 (CAI)] of three downregulated proteins of a sheep rumen tissue sample after 6 wk of administration of a concentrate diet compared with a hay diet. B: one example [S-adenosylhomocysteine hydrolase (AHCY)] of 40 upregulated proteins after 2 days of administration of a concentrate diet.

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Fig. 4. Temporal changes of mRNA levels of selected differentially expressed proteins from sheep rumen tissue samples in response to dietary change. Quantitative RT-PCR was performed with corresponding tissue samples (Table 2); relative RNA levels were normalized with GeNorm to the level of the selected control genes [18S rRNA, GAPDH, succinate dehydrogenase complex subunit A (SDHA), β₂-microglobulin (B2M), and β-actin (ACTB)], according to Ref. 61. *Significant differences between samples from hay- and concentrate-fed sheep \((P < 0.05)\).
credited with oxidative stress and anti-inflammatory properties, it might be that they are involved in triggering the observed immune response (local and systemic) by increasing the proportion of concentrate in the diet of ruminants (30, 68).

Nevertheless, the variety of the proposed functions of the proteins of the annexin family makes it difficult to define the exact function of these proteins in diet response of ruminal epithelium.

Stress response. Two days after the change of diet, a cluster of stress response proteins has been found to be modulated, of which TXNDC5 and PRDX6 are important antioxidant enzymes protecting cells against oxidative injury by reducing endogenous levels of peroxides. Both proteins catalyze the degradation of H$_2$O$_2$, organic hydroperoxides, and peroxinitrites and are involved in signal transduction, cell proliferation, differentiation, and apoptosis (51, 65). It is well known that immune cells can produce large amounts of ROMs, usually referred as “reactive oxygen metabolites,” when stimulated. In light of this fact, it has recently been reported that feeding cattle with concentrate initiates inflammatory response (30, 68).

Furthermore, thioredoxin acts not only as an antioxidant and antiapoptotic molecule, but also as an anti-inflammatory molecule (67). Since concentrate supplementation has been proved to increase the degradation of antioxidants in the rumen (64), we hypothesize that the reverse regulation of TXDNDC5 and PRDX6 after 2 days might be involved in maintaining cellular homeostasis in the ruminal epithelium during adaptation to concentrate-supplemented diet.

Metabolic proteins. The major group of proteins identified in our study is involved in cellular metabolism. Among these, IDH and ATP5G have been identified as being upregulated after 2 days of dietary change. The elevated expression of these enzymes may be correlated with the increased energy requirement during enhanced proliferation and/or for a transient increase of SCFA production. Furthermore, the NADP-IDH in ruminal mucosa may be important for the essential NADPH supply to feed the above-mentioned upregulated thioredoxin system (43).

CAI is a member of a well-characterized isoenzyme family previously described in the rumen (1, 9, 62). It catalyzes the hydration of carbon dioxide and the dehydration of bicarbonate. The general role of CAI in the regulation of pH, cell growth, urea synthesis, gluconeogenesis, and lipogenesis is well established (11). In particular, for the rumen, CA is a crucial enzyme because the maintenance of pH is of great importance to resident microorganisms of the rumen (52) and for the host (46). Furthermore, the absorption of SCFA is linked to HCO$_3^-$ secretion (3). Surprisingly, in our study, CAI has been found to be significantly downregulated in the rumen after 6 wk of concentrate feeding. Notably, in addition to its well-characterized function in pH regulation and ion transport, CAI is also a c-Myb target involved in regulating cell proliferation and differentiation (12, 34, 50). Since high-concentrate diet leads to an increase in the size and number of ruminal papillae (18), we propose that the decreased expression of CAI might be linked to the c-Myb pathway. In the intestinal cells of monogastic mammals, decreased CAI expression is accompanied by malignant transformation (6, 8), and thus CAI might be involved in the regulation of the proliferation and differentiation of rumen epithelial cells.

AHCY is a cytoplasmic and ubiquitously expressed enzyme that catalyzes the reversible hydrolysis of S-adenosylhomocysteine (SAH) to adenosine and L-homocysteine (59). AHCY plays an important role in methionine metabolism by disposal...
of SAH. Additionally, intracellular SAH concentrations regulate processes such as trans-methylation (48), trans-sulfuration (5), and purine metabolism (17).

ESD has been found to be significantly upregulated over the time of the supplementary diet (6 wk) in the rumen of concentrate-fed animals. It is highly conserved and forms a part of the formaldehyde detoxification pathway (16). Verification of differentially regulated proteins. As known from other studies, changes in protein levels do not automatically correlate well with mRNA levels, indicating that post-transcriptional events participate in such discrepancies. This supports the general view that gene expression does not necessarily predict the expression level of proteins (27). However, changes in the mRNA levels of ANXA5 and ESD have been found to be consistent with the protein expression seen after dietary change. Furthermore, in those cases in which specific antibodies are commercially available for immunoblotting, changes in the expression level of proteins (ARP3, PRDX6, CAI) are fully consistent with the 2D gel electrophoresis results.

In general, a major bottleneck in proteomic studies in sheep is the limited availability of antibodies. Hence, only a limited number of identified proteins can be validated by Western blotting. Therefore, the combination of the three chosen techniques (2D-DIGE, immunoblotting, and quantitative reverse transcription-polymerase chain reaction) used in this study provides a more reliable way for the identification and verification of differentially regulated proteins in ovine ruminal epithelium in response to dietary changes.

In conclusion, this proteomic study indicates an adaptation of protein expression in ovine ruminal epithelium to a concentrate-supplemented diet. The precise determination of the total number of proteins of the sheep rumen tissue involved in the dynamic response to a high-energy diet is difficult because of some limitations of the 2D technology for the separation of proteins with extreme molecular weights and isoelectric points. Unfortunately, membrane proteins are difficult to separate and identify, because they are typically of low abundance and high hydrophobicity. Thus, despite their described well-known role in cellular adaptation to a high-energy diet, membrane proteins, such as the various transporters of the rumen epithelial cells, are underrepresented in the current proteomic study. However, our proteomic analysis has permitted the identification of 15 differentially expressed proteins involved in rumen adaptation to a concentrate-supplemented diet.

Remarkably, our work has identified some expression changes of proteins that have been previously reported in relation to cancer, especially those that are involved in the stress response, proliferation, and differentiation. These proteins may be associated with the well-known morphological alterations of the ruminal epithelium in response to a concentrate diet and should be in the focus of further research. The results obtained in our study should provide a reliable basis with regard to the changes in the expression of single proteins involved in the diet-specific response of ruminal epithelium to an increase of energy and protein intake.

ACKNOWLEDGMENTS

We thank Christoph Holder for excellent technical assistance.

GRANTS

This study was supported by the Margarete-Marcus Charity and partly by Bundesminister für Bildung und Forschung Fugato-plus and SFB 852.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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