Modulation of renal calcium and phosphate transporting proteins by dietary nitrogen and/or calcium in young goats¹

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ABSTRACT: In young goats, a reduction in dietary nitrogen (N) had an impact on mineral homeostasis although ruminants are able to recycle N effectively due to rumino-hepatic circulation. A solitary calcium (Ca) reduction stimulated calcitriol synthesis and Ca concentrations remained unchanged, whereas a dietary N reduction led to a decrease in calcitriol, which could not be prevented by a simultaneous reduction of N and Ca. In a previous study, it was shown that a reduced dietary N intake caused a decrease in intestinal Ca absorption due to a reduction of intestinal Ca transporting proteins. As no data on the potential role of the kidneys are available, it was the aim of the present study to evaluate whether an N- and/or Ca-reduced diet had an impact on renal Ca and phosphate (P_i) transporting protein expression in young goats. The animals were divided into 4 feeding groups, each receiving an adequate N and Ca supply, a reduced N supply, a reduced Ca supply, or a combined N and Ca reduction for 6 to 9 wk. The protein expression of the renal Ca channel transient receptor potential cation channel subfamily V member 5 (TRPV5) was diminished in N-reduced fed goats

($P = 0.03$), whereas in Ca restricted animals, the expression remained unaltered. The mRNA and protein expression of the Ca-binding protein calbindin-D28K (CaBP_{D28K}) and the sodium–Ca exchanger 1 (NCX1) were significantly decreased due to the N-reduced feeding (mRNA, $P = 0.003$; $P < 0.0001$; protein, $P = 0.002$; $P = 0.02$), whereas dietary Ca reduction increased the CaBP_{D28K} and NCX1 mRNA expression ($P = 0.05$; $P = 0.01$). The mRNA and protein expression of the parathyroid hormone receptor (PTHr) decreased due to the N-reduced feeding ($P = 0.02$; $P = 0.03$). These results confirm that a reduced dietary N intake led to decreased TRPV5, CaBP_{D28K}, PTHr, and NCX1 expression levels, contributing to low levels of calcitriol and plasma Ca. In contrast to this, sodium–phosphate cotransporter type IIa expression and plasma P_i concentration were increased during dietary N reduction, thus indicating that P_i homeostasis is modulated in a calcitriol-independent manner. In conclusion, the modulation of Ca transporting proteins expression in the kidney is not able to prevent changes in mineral homeostasis in young goats receiving an N-reduced diet.

Key words: calcitriol, calcium transporting protein, goat, nitrogen, phosphate transporter

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INTRODUCTION

In ruminant feeding, a reduction in dietary nitrogen (N) is desirable for economic and environmental reasons. Intake of N is strongly correlated with renal N excretion in ruminants (Silanikove, 1984). Due to rumino-hepatic circulation, ruminants are able to recycle N more effectively than monogastric species to ensure an adequate N supply for the microbes as long as energy supply is adequate.

However, reducing dietary N had an impact on mineral homeostasis in young goats (Muscher and Huber, 2010; Muscher et al., 2011; Elfers et al., 2015). It was demonstrated that a dietary N reduction led to a decrease in blood Ca and calcitriol concentrations (Muscher and Huber, 2010; Elfers et al., 2015). Moreover, a dietary N reduction diminished the intestinal absorption of Ca due to a decrease in intestinal Ca transporting proteins, probably mediated by reduced calcitriol levels (Elfers et al., 2015).

From monogastric animals, it is known that the kidneys are involved in modulating Ca and phosphate (P_i) homeostasis by changes in renal handling of Ca and P_i . In addition, the renal regulation of calcitriol synthesis plays an important role because calcitriol regulates the expression of proteins involved in intestinal and renal Ca and P_i transport.

It is hypothesized that a dietary N and/or Ca reduction influences the expression of renal Ca and P_i transporting proteins and therefore contributes to the changes in mineral homeostasis in young goats. Therefore, the aim of the present study was to investigate the expression of renal Ca and P_i transporting proteins as affected by changes in dietary N and/or Ca intake in young goats. Furthermore, the expression of the Ca-sensing receptor (CaR) and the parathyroid hormone receptor (PTHr) were examined because both are involved in the modulation of renal Ca and P_i transporting proteins.

MATERIALS AND METHODS

The experimental conditions and the animal treatment were approved, and the study conducted was supervised by the Animal Welfare Commissioner of the University of Veterinary Medicine Hannover, Germany (permit number: 33.12-42502-04-13/1045) in accordance with the German Animal Welfare Law.

Animals and Feeding Regime

The samples used in this study derive from a previous experiment (Elfers et al., 2015). When

working with an experimental design using a large animal model, it is necessary to keep the number of animals as low as possible for animal welfare-based reasons (3R principles).

Twenty-six male colored German goats aged 8 wk were randomly divided into 4 feeding groups with 6 to 7 animals (n) in each group. Dietary ingredients and exact conditions of feeding regimes were published recently in Elfers et al. (2015). Briefly, all groups received an isoenergetic feed containing approximately 12.5 MJ metabolizable energy/kg DM. Depending on the feeding group, the animals received either an adequate N and Ca supply [control group (N^+/Ca^+), 21% CP, 1% Ca], a reduced N supply [(N^-/Ca^+) , 8% CP, 1% Ca], a reduced Ca supply [(N^+/Ca^-) , 22% CP, 0.4% Ca], or a combined N and Ca reduction [(N^-/Ca^-) , 8% CP, 0.4% Ca] for 6 to 8 wk. To create the different N content, the feed for the control (N^+/Ca^+) and (N^-/Ca^-) group was enriched with urea as an N source, whereas the N-reduced diets were fortified with Sipernat 22S to adjust the weight. The animals were fed 3 times a day. Chopped wheat straw was offered at 25% of the concentrate weight. Water was freely available, and the goats were weighed weekly.

Blood Samples

Blood samples were taken shortly before slaughtering, always at the same time in the morning to avoid circadian effects. Samples were taken by puncturing the *Vena jugularis* with lithium heparinate-coated syringes and serum syringes (Sarstedt, Nümbrecht, Germany). Plasma and serum samples were separated by centrifugation [$2,000 \times g$ at room temperature (RT), 15 min] and stored at -20°C until further analysis.

Tissue and Urine Sampling

At the end of the feeding period, 1 animal a day was sacrificed by exsanguination after captive bolt stunning in a group-alternating manner to avoid time effects. Urine samples were collected after exsanguination by aspiration from the bladder except for 1 animal in the (N^-/Ca^-) group which had no urine in the bladder. Urine samples were used immediately for measuring osmolarity, Ca, and P_i and then stored at -20°C for creatinine analyses. The kidneys were removed within 5-min postmortem, and tissue samples were taken from the cortex. These tissue samples were then rinsed with ice-cold saline (0.9% NaCl, wt/vol), immediately frozen in liquid N_2 , and stored at -80°C until further preparation.

Biochemical Determination

Plasma urea concentrations were measured using a commercial kit (r-Biopharm, Darmstadt, Germany; interassay CV 3.6%; intra-assay CV 5.8%) to define the N status of the animals.

Concentrations of Ca and inorganic P_i were measured colorimetrically in plasma and urine samples by standard spectrometric techniques [Sarkar and Chauhan, 1967; Kruse-Jarres, 1979; interassay CV 8.2% (P_i and Ca); intra-assay CV 4.9% (Ca), 1.7% (P_i)]. Plasma and urine creatinine concentrations were analyzed commercially (ABX Pentra Creatinine 120 CP, Horiba, Montpellier, France) in the Clinic for Cattle of the University of Veterinary Medicine, Hannover, Germany. Plasma albumin concentrations were detected by using a bromocresol green albumin assay kit (Sigma, Taufkirchen, Germany). The total serum concentrations of thyroid hormones, triiodothyronine (T₃), were analyzed in the Endocrinology Laboratory, Clinic for Cattle, University of Veterinary Medicine, Hannover, by competitive chemiluminescence immunoassay (interassay CV 7.0% to 13.2%).

Urine and plasma osmolarity were measured by freezing point depression (micro-osmometer; Roebling, Germany).

Calculation of Fractional Excretion

The fractional excretion (FE) of total Ca was determined at the end of the experimental period using the following formula:

$$\text{FE Ca} = \frac{\text{Creatinine}_{\text{plasma}} [\text{mM}] \cdot \text{Ca}_{\text{urine}} [\text{mM}]}{\text{Creatinine}_{\text{urine}} [\text{mM}] \cdot \text{Ca}_{\text{plasma}} [\text{mM}]} \cdot 100 [\%]$$

The FE of P_i was calculated according to the same equation, but with the respective P_i concentrations.

Measurement of Na⁺/K⁺-ATPase Activity

Measurement of the activity of the basolateral located sodium–potassium adenosine triphosphatase (Na⁺/K⁺-ATPase) was conducted using 0.5-g renal cortical tissue that was homogenized in 5-mL ice-cold homogenization buffer containing 20 mM Tris base, 250 mM sucrose, 5 mM sulfuric acid, 5 mM ethylene glycol tetraacetic acid, and 0.8 M phenylmethanesulfonyl fluoride. After centrifugation (600 × g at 4 °C for 10 min), Na⁺/K⁺-ATPase activity was measured in the supernatant, in accordance with the method described by Mircheff and Wright (1976).

RNA Isolation and Reverse Transcription

The total RNA was isolated from renal cortical tissue using the RNeasy Mini-Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. The RNA concentrations were measured by UV absorbance (BioPhotometer plus, Eppendorf AG, Hamburg, Germany). The quality and integrity of the RNA were assessed using an RNA 6000 nanoassay for an Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany).

Using random hexamers, oligo-dt primers, and TaqMan-Reverse Transcription Reagents (Applied Biosystems, Darmstadt, Germany), 200 ng of isolated RNA was reverse transcribed for quantitative PCR (qPCR) in accordance with the manufacturer's protocol.

Semiquantification of TRPV5, CaBP_{D28K}, NCX1, NaPi IIa, Na⁺/K⁺-ATPase, CaR, and PTHR mRNA Expression

To evaluate mRNA expression of the transient receptor potential cation channel subfamily V member 5 (TRPV5), the Ca-binding protein calbindin-D28K (CaBP_{D28K}), the sodium–Ca exchanger 1 (NCX1), the sodium–phosphate cotransporter type IIa (NaPi IIa, SLC34A1), and the Ca-sensing receptor (CaR), caprine gene-specific TaqMan primers and probes were synthesized by TIB MOLBIOL (Berlin, Germany; Table 1). Reaction mixture (20 µL each) contained TaqMan Universal PCR Master Mix (Applied Biosystems), 16 ng cDNA, 300 nM specific primers, and 100 nM specific probes. Polymerase chain reaction products were amplified (50 °C, 2 min; 95 °C, 10 min; 40 cycles of 95 °C, 15 s and 60 °C, 1 min) and analyzed on a real-time PCR cycler (CFX96TM, Bio-Rad).

For quantifying Na⁺/K⁺-ATPase and PTHR mRNA expression, SYBR Green PCR assays were performed with specific primers from Life Technologies (Darmstadt, Germany; Table 2). Reaction mixtures (20 µL) contained KAPA SYBR FAST Universal Master Mix (PEQLAB Biotechnologie GmbH, Erlangen, Germany), 200 nM specific primers, and 16 ng reverse transcribed cDNA. The PCR products were amplified (95 °C, 3 min; 40 cycles of 95 °C, 10 s and 60 °C, 30 s) and detected on the same real-time PCR cycler (CFX96TM, Bio-Rad). The thermal profile for determining the melting curve began with an incubation of 10 min at 55 °C with a gradual increase in temperature (0.5 °C/10 s). Absolute copy numbers were determined using calibration

Table 1. Primers and probes used for TaqMan assays

Gene ¹	Sense and antisense primers and probes (5'→3')	Source
CaBP _{D28K}	GTGGATCAGTATGGGCAGAGAGAT GAAGTTCTCTTCTGTGGGTAACACAT FAM-AGCCAACTCTACAATTCCTATTTTCCAT-BBQ	Herm et al. (2015)
CaR	ATCGGGTCCTCCTCGTGTTT GGACTTGAAGGCGAAGAAGAA FAM-TGTCATCTGTGCCATTGGCTCAACAC-TMR	Elfers et al. (2014)
NaPi IIa	CCACCGTACACGACTGCTTTAA CATTTCTCCAGGGTGGCATT FAM-TGGCTGTCAGTTCTGGTGCTGCT-BBQ	Herm et al. (2015)
NCX1	AGAAGCTGCCCTCCTGTTTT GCCAATCATGAGGATGGAGA FAM-ACGTGATGCACTTTCTGACTGTGTTCT-BBQ	Wilkens et al. (2011)
TRPV5	GGACAGGGAAGGGGTCG AGGAGGAAGGACAAGGGCAT FAM-CGGTGAAGTAAAGCGGGCTTCT-BBQ	Herm et al. (2015)
18S	AAAAATAACAATACAGGACTCTTTTCG GCTATTGGAGCTGGAATTACCG FAM-TGGAATGAGTCCACTTTAAATCCTTCCGC-BBQ	Wilkens et al. (2018)

¹CaBP_{D28K} = Ca-binding protein calbindin-D28K; CaR = Ca-sensing receptor; NaPi IIa, SLC34A1 = sodium-phosphate cotransporter type IIa; NCX1 = sodium-Ca exchanger 1; TRPV5 = transient receptor potential cation channel subfamily V member 5; 18S = 18S ribosomal RNA.

Table 2. Primers used for SYBR Green assays

Gene ¹	Sense and antisense primers (5'→3')	Source
Na ⁺ /K ⁺ -ATPase	TGGAACTCGGAGAAGAAGGA GCCACTCGGTCCTGATATGT	Elfers et al. (2015)
PTHr	ATCAACATCGTGCGGGTTCT AGCCTGTACCTCACCATTGC	Herm et al. (2015)

¹Na⁺/K⁺-ATPase = sodium-potassium adenosine triphosphatase; PTHR = parathyroid hormone receptor.

curves generated with cloned PCR fragment standards (Wilkens et al., 2009). Specificity of the amplicons was verified by sequencing (GATC, Konstanz, Germany) and using NCBI Blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Expression of genes of interest was normalized to 18S as a constantly expressed housekeeping gene. Reactions were performed in duplicate and included a no-template water control.

Semiquantification of TRPV5, CaBP_{D28K}, NCX1, NaPi IIa, Na⁺/K⁺-ATPase, CaR, and PTHR Protein Expression

For the abundance of TRPV5 and NaPi IIa, brush border membranes (BBM) were prepared using the method described by Wilkens et al. (2009). Cytosolic proteins were isolated for quantifying CaBP_{D28K}, and for NCX1, CaR and PTHR crude membranes (CM) were isolated as described by Wilkens et al. (2011). Determination of Na⁺/

K⁺-ATPase protein expression was carried out in whole cell lysates from kidney cortex used as described previously.

Protein concentrations of all cell fractions were measured with a commercial Coomassie blue protein assay (Bio-Rad).

For TRPV5, 20 µg of BBM was separated by 8.5% SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare, Freiburg, Germany) using a tank-blotting system (Bio-Rad). Membranes were blocked in PBS containing 0.1% Tween 20 (PBST) and 5% fat-free milk powder for 2 h at RT. The membranes were incubated overnight with anti-TRPV5 antibody (Alomone, Jerusalem, Israel) diluted 1:1,000 in PBST. Immunoreactivity of the primary antibody was detected using horseradish peroxidase (HRP)-coupled secondary antibody against rabbit (diluted 1:20,000).

The specificity of the TRPV5 antibody was successfully demonstrated by preincubating the primary antibody with the corresponding antigenic peptide (data not shown).

For NCX1, 25 µg of CM was separated by 8.5% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked in PBST and 5% fat-free milk powder for 2 h at RT. The NCX1 protein was detected in CM from renal cortical tissue by incubating with the diluted antibody (Swant, Marly, Switzerland) 1:1,000 in PBST overnight at 4 °C. Immunoreactivity of the primary antibodies was detected using HRP-coupled secondary

antibody against mouse (diluted 1:10,000). The bound antibodies were visualized using enhanced chemiluminescence (SuperSignal, Thermo Fisher Scientific, Bremen, Germany) in accordance with the manufacturer's protocol and a ChemiDoc system (Bio-Rad). For anti-NCX1 antibody, cross-reactivity with bovine tissue was reported by the manufacturer.

Immunoblot assays detecting the expression of CaBP_{D28K}, NaPi Ila, Na⁺/K⁺-ATPase, CaR, and PTHR proteins in caprine tissue were performed as described elsewhere (Muscher et al., 2008; Elfers et al., 2014, 2015; Herm et al., 2015).

Densitometric measurements of proteins were performed using Image Lab 5.2.1 software (Bio-Rad). For semiquantification of TRPV5, NCX1, NaPi Ila, and CaR, values of the investigated protein were normalized to the amount of β -actin (anti- β -actin, AC-15; Sigma-Aldrich). For CaBP_{D28K}, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal standard with constant expression level (anti-GAPDH; Merck Millipore), and for Na⁺/K⁺-ATPase and PTHR, signal intensities per millimeter were measured for determining relative amounts of protein expression.

Statistical Analysis

All data were given as means with their pooled standard errors (SEM) if not stated otherwise. Data were analyzed using GraphPad Prism version 6.05 (GraphPad Software; www.graphpad.com) by 2-way ANOVA with Tukey's multiple comparisons test. The $P < 0.05$ was set to be significantly different, and $P < 0.1$ was used to define trends.

To test for a linear relationship between mRNA and protein expression levels, a simple correlation analysis with Pearson's correlation coefficient was calculated.

RESULTS

Intake, Body Weight, and Daily Weight Gain

All results concerning feed intake, BW, and daily weight gain were previously published in Elfers et al. (2015). Briefly, all animals were clinically healthy throughout the study. Daily energy and P_i intake of all the feeding groups and Ca supply in the (N⁺/Ca⁺) and (N⁻/Ca⁺) groups met the recommendations of the Society of Nutrition Physiology (GfE) for young ruminants (Society of Nutrition Physiology, 2003). Daily weight gain was not affected by the different diets and ranged from 0.15 to 0.18 (SEM 0.04) kg/d. The animals receiving an N-reduced feed finally had a significant lower BW of about 11% in comparison to the goats receiving the (N⁺/Ca⁺) and (N⁺/Ca⁻) diet.

Biochemical Determination

Blood and urine parameters are shown in Tables 3 and 4. Some of the blood parameters were published in Elfers et al. (2015). Plasma creatinine concentrations increased significantly in both groups receiving a reduced dietary N supply. Plasma albumin concentrations increased significantly in both groups being fed a reduced Ca intake. No significant differences were detected regarding plasma osmolarity between the 4 feeding groups. Total plasma Ca concentrations were significantly reduced in the goats fed a reduced N diet and were not changed due to dietary Ca reduction. Plasma P_i concentrations were increased when dietary N supply was reduced and remained unaffected by dietary Ca reduction. Total serum concentrations of T3 were not affected by any of the dietary interventions.

Table 3. Blood parameters of young goats fed an N- and/or Ca-reduced diet (mean values with their pooled SEM; number of animals: 6 to 7)

Item	N ⁺ /Ca ⁺	N ⁻ /Ca ⁺	N ⁺ /Ca ⁻	N ⁻ /Ca ⁻	SEM	P values of 2-way ANOVA		
						N reduction	Ca reduction	Interaction
Albumin (g/L)	30.8 ^a	31.4 ^a	35.2 ^a	36.7 ^a	3.17	0.52	0.006	0.77
Creatinine (μ mol/L)	54.8 ^a	80.3 ^b	49.5 ^a	76.9 ^b	9.85	<0.001	0.39	0.85
Osmolarity (mOsmol)	295 ^a	286 ^a	289 ^a	289 ^a	5.45	0.13	0.49	0.12
Ca (mM) ¹	2.40 ^{a,c}	2.19 ^{b,d}	2.35 ^{a,c,d}	2.11 ^b	0.09	<0.001	0.15	0.68
P _i (mM) ¹	2.30 ^{a,b}	2.37 ^{a,b}	1.86 ^a	2.72 ^b	0.43	0.04	0.84	0.08
T3 (nM) ¹	2.29 ^a	2.43 ^a	2.25 ^a	1.83 ^a	0.42	0.53	0.14	0.19
Urea (mM) ¹	7.10 ^a	0.83 ^b	6.29 ^a	1.60 ^b	0.58	<0.001	0.94	0.01

^{a,b,c,d}Mean values within a row with dissimilar superscript letters were significantly different; Tukey's multiple comparisons test ($P < 0.05$).

¹Results were previously published in Elfers et al. (2015).

Urine osmolarity decreased significantly due to a singular N-reduced diet and a concomitant N- and Ca-reduced diet. The FE of Ca was not affected by any dietary treatment, whereas the FE of inorganic P_i increased significantly due to an N-reduced diet and increased even more when dietary Ca supply was reduced.

Semiquantification of Na^+/K^+ -ATPase Activity

The activity of the Na^+/K^+ -ATPase in renal cortical tissue is presented in Fig. 1. The activity was significantly diminished by both dietary N ($P < 0.001$) and Ca reduction independently ($P = 0.05$).

Semiquantification of TRPV5, $CaBP_{D28K}$, NCX1, NaPi IIa, Na^+/K^+ -ATPase, CaR, and PTHR mRNA Expression

The mean integrity number of isolated RNA from renal cortical tissue used for qPCR was 9.3 (data not shown). All mRNA expression data are summarized in Table 5.

The expression of TRPV5 was not significantly affected by dietary N reduction and increased by trend due to the dietary Ca reduction. Expression of $CaBP_{D28K}$ was significantly reduced due to

the N-reduced diet, whereas a dietary Ca reduction increased its expression significantly. The N-reduced fed goats had a significantly reduced NCX1 mRNA expression, whereas in Ca reduced fed goats NCX1 expression was significantly increased. The NaPi IIa expression remained unaffected by any dietary intervention, whereas dietary N reduction led to a reduction in Na^+/K^+ -ATPase mRNA expression by trend. The expression of CaR remained unaltered due to the applied feeding regimes. The N-reduced fed goats showed a significantly reduced PTHR mRNA expression, whereas in Ca-reduced fed goats the expression was unaffected.

Semiquantification of TRPV5, $CaBP_{D28K}$, NCX1, NaPi IIa, Na^+/K^+ -ATPase, CaR, and PTHR Protein Expression

Data on protein expression are shown in Table 6. Protein expression of TRPV5 was detected at approximately 80 kDa. The N-reduced fed goats had a significantly reduced TRPV5 protein expression in renal cortical tissue, whereas in Ca-reduced fed goats, the expression of TRPV5 was not modified. No linear relationship was found between TRPV5 mRNA expression and its corresponding protein (data

Table 4. Urine osmolarity and fractional excretion of calcium (FE Ca) and phosphate (FE P_i) of young goats fed an N- and/or Ca-reduced diet (mean values with their pooled SEM; number of animals: 6 to 7)

Item	N ⁺ /Ca ⁺	N ⁻ /Ca ⁺	N ⁺ /Ca ⁻	N ⁻ /Ca ⁻ ¹	SEM	P values of 2-way ANOVA		
						N reduction	Ca reduction	Interaction
Osmolarity (mOsmol)	1,534 ^a	550 ^b	1,151 ^a	425 ^b	276	<0.0001	0.08	0.36
FE Ca (%)	0.52 ^a	0.66 ^a	0.67 ^a	0.79 ^a	0.24	0.28	0.26	0.95
FE P_i (%)	0.42 ^a	5.37 ^a	0.68 ^a	8.31 ^a	4.08	0.006	0.44	0.52

^{a,b}Mean values within a row with dissimilar superscript letters were significantly different; Tukey's multiple comparisons test ($P < 0.05$).

¹For 1 animal, no urine was present in the bladder.

Figure 1. Modulation of renal sodium-potassium adenosine triphosphatase (Na^+/K^+ -ATPase) activity by dietary N and/or Ca reduction. Means $\pm$ SEM; significant effects between the individual groups revealed by Tukey's multiple comparisons test after 2-way ANOVA marked with asterisk; * $P < 0.05$.

Table 5. Relative amounts of TRPV5, CaBP_{D28K}, NCX1, NaPi IIa, Na⁺/K⁺-ATPase, CaR, and PTHR mRNA expression normalized to 18S in the kidney cortex of goats fed an N- and/or Ca-reduced diet (mean values with their pooled SEM; number of animals: 6 to 7)¹

Item	N ⁺ /Ca ⁺	N ⁻ /Ca ⁺	N ⁺ /Ca ⁻	N ⁻ /Ca ⁻	SEM	<i>P</i> values of 2-way ANOVA		
						N reduction	Ca reduction	Interaction
<i>n</i>	7	6	6	7				
TRPV5	3.20e-06 ^a	4.31e-06 ^a	4.33e-06 ^a	6.39e-06 ^a	2.00e-06	0.10	0.09	0.61
CaBP _{D28K}	2.63e-04 ^a	2.23e-04 ^a	3.66e-04 ^b	2.32e-04 ^{a,2}	5.26e-05	0.003	0.05	0.09
NCX1	4.68e-06 ^{a,b}	2.52e-06 ^c	6.60e-06 ^a	3.56e-06 ^{b,c}	1.05e-06	<0.001	0.01	0.41
NaPi IIa	1.89e-04 ^a	1.87e-04 ^a	1.66e-04 ^a	2.08e-04 ^a	8.27e-05	0.64	0.98	0.61
Na ⁺ /K ⁺ -ATPase	1.28e-03 ^a	9.28e-04 ^a	1.42e-03 ^a	1.22e-03 ^a	3.02e-04	0.08	0.16	0.61
CaR	4.36e-05 ^a	4.39e-04 ^a	5.68e-05 ^a	4.01e-05 ^a	1.71e-05	0.35	0.59	0.33
PTHr	7.10e-03 ^a	5.37e-03 ^a	8.34e-03 ^a	5.05e-03 ^a	1.92e-03	0.02	0.64	0.43

^{a,b,c}Mean values within a row with dissimilar superscript letters were significantly different; Tukey's multiple comparisons test ($P < 0.05$).

¹TRPV5 = transient receptor potential cation channel subfamily V member 5; CaBP_{D28K} = Ca-binding protein calbindin-D28K; NCX1 = sodium-Ca exchanger 1; NaPi IIa, SLC34A1 = sodium-phosphate cotransporter type IIa; Na⁺/K⁺-ATPase = Na⁺/K⁺ adenosine triphosphatase; CaR = Ca-sensing receptor; PTHR = parathyroid hormone receptor.

²One animal excluded to gain Gaussian distribution.

Table 6. Relative amounts of TRPV5, CaBP_{D28K}, NCX1, CaR, NaPi IIa, Na⁺/K⁺-ATPase, CaR, and PTHR protein expression in the kidney cortex of goats fed an N- and/or Ca-reduced diet (mean values with their pooled SEM; number of animals: 6 to 7)¹

Item	N ⁺ /Ca ⁺	N ⁻ /Ca ⁺	N ⁺ /Ca ⁻	N ⁻ /Ca ⁻	SEM	<i>P</i> values of 2-way ANOVA		
						N reduction	Ca reduction	Interaction
<i>n</i>	7	6	6	7				
TRPV5 protein/β-actin protein ratio	7.25 ^a	5.79 ^a	8.03 ^a	3.40 ^a	2.66	0.03	0.55	0.24
CaBP _{D28K} protein/GAPDH protein ratio	0.66 ^{a,b}	0.35 ^b	0.79 ^a	0.49 ^{a,b}	0.16	0.002	0.14	0.82
NCX1 protein/β-actin protein ratio	0.37 ^a	0.20 ^a	0.38 ^a	0.18 ^a	0.14	0.02	0.93	0.82
NaPi IIa protein/β-actin protein ratio	0.78 ^a	1.08 ^a	0.86 ^a	1.04 ^a	0.17	0.009	0.81	0.46
Na ⁺ /K ⁺ -ATPase protein abundance, INT·mm ²	1,708,431 ^a	1,400,909 ^a	1,599,762 ^a	1,435,482 ^a	172,388	0.01	0.67	0.42
CaR protein/β-actin protein ratio	0.30 ^a	0.38 ^a	0.34 ^a	0.33 ^a	0.08	0.44	0.81	0.31
PTHr protein abundance, INT·mm ²	1,120,845 ^a	994,397 ^a	1,126,211 ^a	859,378 ^a	168,750	0.03	0.45	0.41

^{a,b}Mean values within a row with dissimilar superscript letters were significantly different; Tukey's multiple comparisons test ($P < 0.05$).

¹TRPV5 = transient receptor potential cation channel subfamily V member 5; CaBP_{D28K} = Ca-binding protein calbindin-D28K; NCX1 = sodium-Ca exchanger 1; NaPi IIa, SLC34A1 = sodium-phosphate cotransporter type IIa; Na⁺/K⁺-ATPase = Na⁺/K⁺ adenosine triphosphatase; CaR = Ca-sensing receptor; PTHR = parathyroid hormone receptor.

²INT·mm, pixel intensity·millimeters.

not shown). The expression of CaBP_{D28K} protein was detected in the caprine renal cortex as a band of approximately 26 to 34 kDa. The expression was significantly reduced due to the N-reduced feed, whereas a dietary Ca reduction caused no changes. A significant positive correlation between mRNA and protein expression of CaBP_{D28K} was shown by Pearson's correlation ($P = 0.003$; Fig. 2). Protein expression of NCX1 was detected at approximately 116 kDa. The protein expression of NCX1 was significantly

decreased due to the N-reduced diet, whereas a dietary Ca reduction led to no changes in NCX1 protein expression. A significant positive correlation between mRNA and protein expression was shown for NCX1 ($P = 0.03$; Fig. 3). Protein expression of NaPi IIa was detected as a band of approximately 80 to 90 kDa. Dietary N reduction led to a significant increase in NaPi IIa protein expression that was not affected by a dietary Ca reduction. No linear relationship was shown for NaPi IIa mRNA and protein expression (data

Figure 2. Relationship between Ca-binding protein calbindin-D28K (CaBP_{D28K}) mRNA and CaBP_{D28K} protein ($r = 0.56$, $P = 0.003$). The level of significance in accordance with Pearson's correlation coefficient was set at $P = 0.05$.

Figure 4. Relationship between sodium-potassium adenosine triphosphatase (Na⁺/K⁺-ATPase) mRNA and Na⁺/K⁺-ATPase protein ($r = 0.57$, $P = 0.002$). The level of significance in accordance with Pearson's correlation coefficient was set at $P = 0.05$.

Figure 3. Relationship between sodium-Ca exchanger 1 (NCX1) mRNA and NCX1 protein ($r = 0.44$, $P = 0.03$). The level of significance in accordance with Pearson's correlation coefficient was set at $P = 0.05$.

Figure 5. Relationship between parathyroid hormone receptor (PTHr) mRNA and PTHR protein ($r = 0.43$, $P = 0.03$). The level of significance in accordance with Pearson's correlation coefficient was set at $P = 0.05$.

not shown). The Na⁺/K⁺-ATPase protein expression was detected as a band of approximately 110 kDa and was significantly diminished due to the singular N-reduced diet and concomitant N- and Ca-reduced diet. A significant positive correlation between Na⁺/K⁺-ATPase mRNA and corresponding protein expression was shown ($P = 0.002$; Fig. 4). The protein expression of CaR was detected as a band of approximately 120 kDa and remained unchanged due to the applied feeding regimes. A linear relationship between CaR mRNA and its protein could not be shown (data not shown). Protein expression of PTHR was detected as a band of approximately 80 to 90 kDa. The N-reduced fed goats showed a significantly reduced PTHR protein expression, whereas in animals fed a Ca-reduced diet, the expression of PTHR remained unaltered. A significant positive correlation between mRNA and protein expression of the PTHR was shown by Pearson's correlation ($P = 0.03$; Fig. 5).

DISCUSSION

The aim of the present study was to evaluate the impact of an N- and/or Ca-reduced diet on renal Ca and P_i transporting protein expression in young goats. It could be shown that these feeding regimes influenced the Ca and P_i transporting protein expression in the kidney of young ruminants and therefore contributed to changes in mineral homeostasis.

A limitation of this study was a difference in final BW of the goats receiving the N-reduced diets. This finding indicates that an adequate N supply is necessary to generate optimal conditions for growth and development of young ruminants. Nonetheless, energy supply was sufficient for all groups due to the recommendations of the GfE. Furthermore, daily weight gain and concentrations of plasma T3 showed no alternation due to the different diets, indicating sufficient energy intake in all groups. Moreover, all animals grew and gained

weight throughout the study, and concentrations of growth hormone did not differ between the feeding groups (data not shown). It seems that the animals receiving the reduced N diets grow slower and therefore need more time than the animals receiving a sufficient amount of N to finally gain the same weight and height.

The apical located TRPV5 channel is essential for the renal Ca reabsorption in ruminants as in monogastric animals (Hoenderop et al., 2003; Herm et al., 2015). The decrease in TRPV5 protein expression during dietary N reduction could be explained by the concomitant reduction in serum calcitriol concentrations during this dietary treatment (Elfers et al., 2015). Although in our study the mRNA expression of TRPV5 remained unchanged, indicating translational changes, in humans and mice, the existence of vitamin D-responsive elements (VDREs) in the promoter region of the TRPV5 gene was shown (Hoenderop et al., 2001; Mensenkamp et al., 2007). Moreover, in rats, a low vitamin D diet led to an initial decrease in serum calcitriol, diminished Ca concentrations and decreased renal TRPV5 mRNA and protein expression (Hoenderop et al., 2001). The treatment of these animals with calcitriol caused an increase in TRPV5 mRNA and protein expression comparable with the expression levels of the control animals (Hoenderop et al., 2001). Therefore, the decrease in TRPV5 protein expression in goats fed an N-reduced diet could be a calcitriol-dependent modulation that is mediated through VDREs. Apart from the indirect modulation in a calcitriol-dependent manner, TRPV5 was found to be regulated on extracellular enzymatic activation too (Gkika et al., 2006). Extracellular tissue kallikrein stimulated TRPV5-mediated Ca reabsorption and reduced urinary Ca excretion by accumulating and stabilizing the channel (Gkika et al., 2006).

Besides the modulation of TRPV5, calcitriol modulated the intracellular expression of CaBP_{D28K} RNA that was decreased in the groups fed an N-reduced diet and increased in the goats receiving a reduced Ca amount. The CaBP_{D28K} buffered the influx of Ca intracellularly to prevent high toxic Ca levels by binding it on its 4 docking sites. The bound Ca diffused to the basolateral membrane where the NCX1 extruded Ca into the blood in exchange for Na⁺ (Hoenderop et al., 2002). In rats, it was shown that a VDRE exists in the promoter region of the CaBP_{D28K} gene (Gill and Christakos, 1993). Therefore, it is assumed that CaBP_{D28K} was regulated through a VDRE in a calcitriol-dependent manner in goats as well. Further coherence

between CaBP_{D28K} and decreased TRPV5 protein expression can be discussed. In mice, an inactivation of the TRPV5 gene led to diminished CaBP_{D28K} and NCX1 mRNA expression even in the presence of increased calcitriol levels, suggesting a regulatory mechanism controlled primarily by TRPV5 (Hoenderop et al., 2003). It was presumed that TRPV5 or the Ca influx through this channel controls the transcription of other Ca transporting genes, including CaBP_{D28K} and NCX1 (Hoenderop et al., 2003). Therefore, it can be assumed that in our study, in addition to the diminished calcitriol concentrations, the reduction in TRPV5 protein expression led to a decrease in CaBP_{D28K} protein expression.

In cell cultures from humans and rabbits, it was found that low Ca concentrations, after treatment with a cell-permeant chelator that was a highly selective for Ca, CaBP_{D28K}, shifted to the apical membrane and interacted with TRPV5 (Lambers et al., 2006). Protein expression levels of TRPV5 were not altered and therefore, it was assumed that an increase in Ca uptake was due to the CaBP_{D28K} buffering close to the TRPV5 channel (Lambers et al., 2006). Dietary Ca reduction increased CaBP_{D28K} RNA expression significantly, indicating a compensatory modulation due to high calcitriol levels. Surprisingly, in 7-mo-old female goats, a dietary Ca reduction led to reduced renal CaBP_{D28K} RNA and protein expression although calcitriol levels were enhanced (Herm et al., 2015). It was assumed that not only calcitriol regulates CaBP_{D28K} mRNA and protein expression levels, but also additional factors like enhanced parathyroid hormone (PTH) or changes in Ca concentrations in the glomerular filtrate might affect its mRNA and protein expression levels as well (Herm et al., 2015). A possible explanation for the surprising results might be the age of the animals, as the state of physical constitution and growth is very important in relation to physiological and functional development in young animals. The goats of the previous study were aged 7 mo in contrast to the animals used in our study which were 7 wk old when they received the experimental diet for the first time. At an age of 7 mo, the forestomach system is almost completely developed and the animals become sexually mature. The sex might play an additional role in the older goats because due to their ability to lactate, female goats might be more sensitive to changes in the Ca supply and therefore have greater capacities for compensation. Nevertheless, it remains unclear why CaBP_{D28K} RNA and protein expression levels were

not modulated in a calcitriol-dependent manner in the older female goats.

Due to the N-reduced diet and as a result of lower Ca and calcitriol concentrations, the basolateral located NCX1 was decreased although no VDRE for this protein is described at present in monogastric species (Herm et al., 2015). This could be due to a missing binding site for a coactivator protein for the heterodimer. In general, calcitriol binds to the VDR and the receptor heterodimerizes with retinoid X receptors. It remains unclear how the effect of calcitriol is mediated in goats. A possible explanation for the diminished NCX1 mRNA and protein expression in the N-reduced fed animals might be an interaction with TRPV5, as described previously for CaBP_{D28K} and TRPV5 (Hoenderop et al., 2003). Furthermore, the NCX1 mRNA and protein expression levels might be modulated in a direct way by low plasma Ca concentrations because no VDRE is known.

Dietary Ca reduction and as a result elevated calcitriol levels (Elfers et al., 2015) did not lead to changes in NCX1 protein expression, but to a significant increase in NCX1 mRNA expression. As no VDRE for NCX1 is known, the Ca-sensitive enhancement in NCX1 RNA expression was assumed to apply to the compensatory upregulation of CaBP_{D28K} mRNA expression (Hoenderop et al., 2002).

In the N- and Ca-reduced fed goats, the Na⁺/K⁺-ATPase activity was significantly decreased. This pump provides, among others, the driving force for Na⁺ coupled P_i transport via NaPi IIa and Ca transport through NCX1. It is possible that reduced pump activity of Na⁺/K⁺-ATPase contributes to low plasma Ca levels in N-reduced fed goats. Nevertheless, Na⁺/K⁺-ATPase activity only affected transcellular Ca transport and had no impact on paracellular pathways of Ca absorption that was not measured. A modulation of the renal paracellular Ca transport during a reduced N intake cannot be excluded because the FE of Ca was not adapted although renal transcellular Ca transporting proteins were decreased.

In the kidney of monogastric species, the CaR is essential for inhibiting reabsorption of Ca, K⁺, Na⁺, and water, which depends on the particular function of the activated segment of the tubule (Vezzoli et al., 2009). The CaR modulates the signaling pathways that were used by the tubulocytes to activate mineral or water reabsorption (Vezzoli et al., 2009). Furthermore, the CaR regulates claudins, which are tight-junction membrane proteins that function as pores and barriers in the

ascending limb and are important for the paracellular reabsorption of Ca (Yu, 2015). Besides this, the CaR controls Ca homeostasis by regulating the release of PTH from parathyroid glands. It possesses 2 binding sites for Ca and for amino acids (Conigrave et al., 2000). The CaR was supposed to be the link between protein metabolism and Ca homeostasis in humans and monogastric animals, as tissues expressing the CaR operate in sensing and normalizing Ca and adjusting the serum levels of P_i as well as in supporting the development, growth, maintenance, and turnover of the skeleton (Conigrave et al., 2008). In rats, it was shown that the CaR was colocalized with NaPi IIa in the proximal tubules (Riccardi et al., 2000). In the present study, the CaR mRNA and protein expression levels remained unchanged although the protein expression of NaPi IIa was increased by reduced dietary N intake. A translational modification of NaPi IIa protein expression was assumed because no linear relationship was found between NaPi IIa mRNA and its protein expression. Enhanced NaPi IIa protein expression as a compensatory mechanism led to stable P_i levels in the singular N-reduced fed animals and even caused an increase in P_i levels in the group receiving a concomitant N and Ca reduction. The FE of P_i increased during dietary N reduction. It is interesting to note that phosphaturia is preceded by an increase in plasma P_i. The observed phosphaturia would require a downregulation or inhibition of NaPi IIa, but the opposite occurred during dietary N reduction. Therefore, differential regulation of other proximal tubular P_i transporters such as the Na⁺-dependent P_i transporter NaPi IIc may be responsible for our observations. In a previous study, it was shown that a dietary Ca depletion did not have significant effects on Na-dependent P_i transport capacities and affinities in goats (Schröder et al., 2000). In monogastric animals, it was shown that NaPi IIa was regulated in a calcitriol-dependent manner (Taketani et al., 1998). Moreover, in humans and monogastric animals, a PTH-dependent regulation of NaPi IIa was shown (Hammerman et al., 1980; Sneddon et al., 2004). In the present study, PTHR mRNA and protein expression decreased under an N-reduced diet. Coherence between a low-protein diet and reduced plasma PTH concentration was shown in humans and monogastric animals (Kerstetter et al., 1997; Dubois-Ferrière et al., 2011). The PTH increases Ca absorption and simultaneously stimulates the excretion of P_i in the kidney. Due to the lack of a suitable PTH assay for goats, cyclic adenosine monophosphate (cAMP) was measured (Elfers et al., 2016) as

an indirect parameter for PTH as done in human studies (Kaminsky et al., 1970). As cAMP levels showed no modulation during this feeding regime (data not shown), it was assumed that the parathyroid glands were insensitive to reduced Ca levels during a solitary N-reduced diet in young goats.

In a former study in goats, the glomerular filtration rate (GFR) dropped when dietary N was restricted as a consequence of renal mechanisms to save urea (Eriksson and Valtonen, 1982). A slowdown in the urinary flow rate by absorbing more water would result in enhanced urea concentration in the tubular fluid and therefore an increased urea absorption rate independently of plasma urea concentrations (Eriksson and Valtonen, 1982). Therefore, it was assumed that increased plasma creatinine concentrations in the N-reduced fed animals of our study might be due to a reduction in GFR. All creatinine levels remained within the clinical standards for goats (Matthews, 1999), and therefore no renal damage was expected. Furthermore, plasma albumin concentration showed no differences, revealing that the applied diets did not lead to hemoconcentration or hemodilution.

Urine osmolality is mainly determined by Na^+ and urea concentrations and therefore decreased in the groups receiving a reduced N diet. In a previous study, it was shown that due to an N-reduced feeding, renal urea transporters (UT) of the isoform UT-A1 increased significantly leading to an increased renal urea reabsorption (Starke et al., 2012). The reabsorbed urea could be returned to the forestomach system by blood to serve as a source of N for protein synthesis (Starke et al., 2012). Renal absorption of urea is important for water absorption and urine concentration (Elfers et al., 2014) and might therefore be causal for the decreased urine osmolality in the goats fed an N diet too. In addition to increased expression levels of the UT due to N-reduced feed, young goats showed greater levels of renal aquaporin 1 and 2 expression and increased plasma concentrations of vasopressin (Elfers et al., 2014). Increased water absorption in the kidneys might be beneficial for ruminants in case of reduced N intake due to enhanced urea concentrations in the tubular fluid leading to greater absorption rates of urea (Elfers et al., 2014). The interaction of aquaporin water channels and UT in the kidney may occur to maintain the availability of urea in case of reduced N intake (Elfers et al., 2014).

In summary, during a reduced dietary N intake and the resulting decreased calcitriol levels, NaPi IIa protein expression and plasma P_i concentrations were elevated, indicating that P_i homeostasis

is modulated in a calcitriol-independent manner in young ruminants.

The expression of TRPV5 protein, $\text{CaBP}_{\text{D28K}}$, PTHR, and NCX1 mRNA and protein were diminished in the N-reduced fed goats not able to compensate low intestinal Ca absorption, followed by low levels of blood Ca. In Ca-reduced fed goats with the resulting stimulated calcitriol levels, the expressions of Ca transporting proteins were not adapted. This indicates that renal Ca reabsorption in ruminants runs at a high level allowing no further increase when Ca intake is diminished (Herm et al., 2015). Hence, despite the rumino-hepatic cycle and young ruminants' ability to recycle N effectively, these animals are not able to maintain their Ca homeostasis during an N-reduced diet.

With a wider physiological perspective, these findings might be of importance for dairy cows as well. A lack of calcitriol production is an important factor in dairy cows susceptible to milk fever after parturition (Goff et al., 1989). Diminished calcitriol synthesis (Wilkins et al. 2018), leading to reduced levels of blood Ca by downregulating renal Ca transporting proteins. Reduced intake of feed and protein around parturition might be comparable to the conditions for the young goats receiving an N-reduced feeding in the present study. Therefore, to ensure an adequate feeding regime for ruminants, especially in the early stages of life, a sufficient dietary N supply has to be provided. Further investigations are needed to comprehend the unknown modulation mechanism of the calcitriol synthesis occurring during a reduced dietary N intake.

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