

Modulation of electrolyte homeostasis by dietary nitrogen intake in growing goats

Alexandra S. Muscher^{1*}, Marion Piechotta², Gerhard Breves¹ and Korinna Huber¹

¹Department of Physiology, University of Veterinary Medicine Hannover, Bischofsholer Damm 15/102, 30173 Hannover, Germany

²Clinic for Cattle, University of Veterinary Medicine Hannover, Hannover, Germany

(Received 22 September 2010 – Revised 18 November 2010 – Accepted 23 November 2010 – First published online 29 March 2011)

Abstract

In goats, the combination of dietary N and Ca reduction caused hypocalcaemia and further changes in Ca homeostasis. The aim of the present study was to characterise the effects of dietary N reduction under normocalcaemia on mineral and bone metabolism in young goats. Young male goats of the Saanen breed were fed a diet reduced in N (8%) for about 7 weeks (ten animals per group) and were compared with goats fed with an adequate N (14%) diet. When N intake was reduced in young goats, plasma urea concentrations as well as renal elimination of urea were reduced. This was inversely related to creatinine in plasma and urine, which increased during a dietary N reduction as a function of reduced renal activity to save urea during N scarcity. During this decrease in renal function, associated with declined insulin-like growth factor 1 concentrations, a reduction in calcidiol and calcitriol concentrations could be observed. Meanwhile, carboxyterminal cross-linked telopeptide of type I collagen values and activity of total alkaline phosphatase were both elevated, indicating some bone remodelling processes taking place during a reduced N diet in young goats. The concentrations of inorganic phosphate (P_i) and total Ca were changed in several body fluids, indicating that P_i and Ca homeostasis was perturbed in goats fed a reduced N diet. Therefore, more research is needed to find the balance between reduction of environmental N pollution by reducing dietary N in ruminant feeding and maintaining the animal's health.

Key words: Goats: Nitrogen: Calcitriol: Calcium and phosphate homeostasis

In ruminant feeding, one aim is to reduce environmental pollutants, including emissions of N generally and NH₃ and N₂O especially. While the contribution of manure causes high eutrophication of aquatic environments, NH₃ affects air quality and the presence of N₂O increases global greenhouse gas concentrations. Recently, it has been shown that in lactating dairy cows, the total dietary N intake determines the total N excretion as manure⁽¹⁾. Therefore, there is an opportunity for decreasing N pollution in ruminants by reducing dietary N intake as long as reducing N does not decrease animal health and productivity. Exacerbating the known changes in Ca homeostasis by dietary N and Ca reduction⁽²⁾ could abolish the positive effects of N reduction on environmental pollution. Since ruminants are unique in their ability to save N by endogenous recycling mechanisms, especially at times of protein scarcity^(3,4), it is expected that these efficient recycling mechanisms help to maintain the animal's health in times of dietary N reduction.

In monogastric animals and humans, metabolic responses to variation in dietary protein supply provided evidence that protein metabolism was closely related to electrolyte homeostasis. Alterations in plasma 25-hydroxyvitamin D₃ (calcidiol) and urinary excretion of Ca and inorganic phosphate (P_i) in dietary protein-restricted fed rats confirmed that hypothesis⁽⁵⁾. Additionally, a reduction of intestinal Ca absorption occurs in monogastric animals and humans with a low-protein diet^(5,6). During the reduction of intestinal Ca absorption in humans, an abrupt rise in serum parathyroid hormone (PTH) concentration has been observed^(6,7). A low-protein diet reduces plasma amino acid concentrations^(8,9), which induces higher secretion of PTH from parathyroid glands⁽¹⁰⁾. *In vitro* studies have shown that in isolated bovine parathyroid gland cells, changes in amino acid concentrations were able to modulate PTH secretion⁽¹¹⁾. In kidneys, high PTH concentrations induced internalisation of Na-dependent P_i transporters (NaP_i II; SLC34A1, renal NaP_i IIa) located in proximal tubules,

Abbreviations: BBMV, brush-border membrane vesicle; CaR, Ca-sensing receptor; CTX, carboxyterminal cross-linked telopeptide of type I collagen; FE, fractional excretion; GFR, glomerular filtration rate; GH, growth hormone; IGF-1, insulin-like growth factor 1; NaP_i II, Na-dependent inorganic phosphate transporter; P_i, inorganic phosphate; PTH, parathyroid hormone.

* **Corresponding author:** Dr A. S. Muscher, fax +49 511 856 7687, email alexandra.muscher@tiho-hannover.de

resulting in higher renal P_i excretion⁽¹²⁾. Simultaneously, PTH stimulated renal synthesis of 1,25-dihydroxyvitamin D_3 (calcitriol), which elevates the intestinal absorption of Ca and P_i ⁽¹³⁾. In bones, an increase in PTH concentrations activated the mobilisation of Ca and P_i , which causes a reduction in bone density and increases the risk of osteoporosis⁽¹⁴⁾.

During periods of protein deficiency, it is expected that in goats, because of their ability to recycle N from the blood and saliva into the gastrointestinal tract, the described interaction between protein and electrolyte metabolism in monogastric animals would be less expressed, and therefore the possible negative effect for the animal's health would be less pronounced.

The first findings showed that when growing goats were fed a diet reduced in N and Ca, hypocalcaemia and further changes in Ca homeostasis occurred⁽²⁾. However, it could not be distinguished to what extent the dietary N reduction elicited these changes. Therefore, the purpose of the present study was to determine the effect of dietary N reduction under normocalcaemia on the relationship between N and electrolyte metabolism in young goats. For this reason, parameters of Ca and P_i homeostasis were determined in goats fed a reduced N diet. In addition, calcitriol and calcidiol concentrations were measured to evaluate whether this relationship is hormonally regulated. Finally, we investigated whether a reduced N diet affects the intestinal absorption of P_i focusing on Na^+ -dependent P_i transport across the apical membrane using brush-border membrane vesicles (BBMV) and examining the protein amount of Na^+ -dependent P_i transporters (SLC34A2, intestinal NaP_i IIB).

Materials and methods

Animals and feeding regimen

The protocol of the animal treatment was approved, and its conduct was supervised by the Animal Welfare Commissioner of the University of Veterinary Medicine Hannover (Hannover, Germany) according to the German Animal Welfare Law.

A total of twenty male White Saanen goats, about 3 months of age and weighing approximately 24.0 (SE 1.0) kg live weight, were subdivided into two different feeding regimens with an adequate or a reduced N supply. Each group of ten animals was housed separately. Water was available at all times. The amounts of all feeds offered and refused was monitored for estimating intake 'per animal' based on the intake amounts from pooled group mean values.

The reduction of N supply was maintained for about 7 weeks. The feed content of DM, crude ash, crude fat and crude protein was determined by the standard procedure of VDLUFA (Der Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten; Weender analysis)⁽¹⁵⁾. The amount of acid-detergent and neutral-detergent fibres was measured by a method described by Van Soest *et al.*⁽¹⁶⁾. The components and composition of the two diets are presented in Table 1. Both diets were isoenergetic, containing 11.35 kJ metabolisable energy/kg diet.

Table 1. Components and composition of the pelleted concentrate diets*

	Control (g/kg)	Reduced N (g/kg)
Components		
Beet pulp	425	425
Tapioca	455	455
Soyabean meal	60	60
Soyabean oil	10	10
Mineral–vitamin mix†	10	10
NaH_2PO_4	10	10
Urea	30	–
Sipernat 22S‡	–	30
Composition		
DM	896	896
Crude ash	44	65
Crude protein	144	81
Acid-detergent fibre	112.3	74.2
Neutral-detergent fibre	218.4	199.3
Crude fat	18	22
Urea	31	ND
Ca	9.72	9.61
P	4.32	4.37
Vitamin D_3	ND	ND
DCAD (mEq/kg DM)	271	271

ND, not detected; DCAD, dietary cation–anion differences.

* Composition expressed as fed.

† Per kg mineral–vitamin mix: 180 g Ca, 60 g P, 100 g Na, 30 g Mg, 500 000 IU (525 μ mol/l) vitamin A, 80 000 IU vitamin D_3 (4992 nmol/l), 300 mg vitamin E (697 μ mol/l), 4200 mg Zn, 900 mg Mn, 16 mg Co, 20 mg I and 44 mg Se. Values are determined by Weender analysis.

‡ Sipernat type 22S (Evonik Industries AG, Essen, Germany) is a fine particle silica with high oil absorption capacity. It is widely used as a flow regulator, anti-caking and dusting agent, especially in the food and feed industry.

Sampling

Plasma and saliva samples were obtained shortly before the goats were killed. These samples were stored at -20°C for subsequent analysis. At the end of the study (4–5 months of age), the animals were slaughtered after captive bolt stunning by exsanguination. Samples of ruminal fluid, abomasal fluid and urine from the bladder were taken. Mid-jejunal segments were removed within 3–5 min after slaughter. After being rinsed with ice-cold saline (0.9% NaCl, w/v), the mucosa of the jejunum was stripped off. After freezing in liquid N_2 , the jejunal samples were stored at -80°C until further structural and functional analyses.

Biochemical determinations

Haematocrit was determined in total blood samples. An aliquot of each blood sample was placed in a microhaematocrit tube and spun to constant packed cell volume at 14926 g for 5 min at room temperature. Plasma and urine urea concentrations were measured by using a commercial kit (R-Biopharm, Darmstadt, Germany). Creatinine concentration in plasma and urine was analysed by a standard enzymatic method, the creatinine *p*-amino-phenazone method⁽¹⁷⁾. Total plasma protein concentration was measured with a commercial Coomassie blue protein assay (Bio-Rad, Munich, Germany) using bovine plasma γ -globulin as a standard protein. Plasma albumin concentrations were detected by a standard dye-binding technique using bromocresol green

(Hengler Analytik, Steinbach, Germany). Concentrations of P_i and total Ca were measured colorimetrically in plasma, saliva, urine, abomasal and ruminal fluids by standard spectrometric techniques^(18,19). The amount of calcidiol was determined by a competitive enzyme-linked immunosorbent assay (Immundiagnostik AG, Bensheim, Germany). The declared inter- and intra-assay CV of the kit were <13.2 and <10.7%. Calcitriol concentrations were measured by a commercial radioreceptor assay (Immundiagnostik AG, Bensheim, Germany). For this process, the plasma was cleaned by Extrelut extraction. The intra-assay CV were <15 and <10% for samples with calcitriol concentrations of 10 and 60 pg/ml, respectively. The inter-assay CV were <20 and <15% for these two concentrations. The detection limit of this assay was 2 pg/ml. The calcitriol assay systems had already been used in other studies to detect caprine hormone concentrations^(20–22). cAMP concentrations were determined in plasma and urine by a commercial competitive binding assay (Immundiagnostik AG, Bensheim, Germany). The inter- and intra-assay CV of the kit were <8.8 and <7.6%. The concentration of plasma carboxyterminal cross-linked telopeptide of type I collagen (CTX) was determined using the commercial kit Serum CrossLaps[®] ELISA (Immunodiagnostic Systems, Frankfurt, Germany). The cross-reactivity for goats had been tested by the company⁽²³⁾. The inter- and intra-assay CV of the kit were <10 and <6%. Total alkaline phosphatase (*EC* 3.1.3.1) activity was determined in plasma samples by measuring the increase in absorbance (405 nm) due to the formation of *p*-nitrophenol from *p*-nitrophenyl phosphate ester⁽²⁴⁾. For the determination of total plasma insulin-like growth factor 1 (IGF-1), an ACTIVE Insulin-Like Growth Factor-I coated-tube IRMA Kit (DSL-5600; Diagnostic Systems Laboratories, Inc., Webster, TX, USA) was used. IGF-1 was separated from its binding proteins by an acid-ethanol extraction procedure, and IGF-1 concentrations were determined with a two-site immunoradiometric assay. Intra- and inter-assay CV were 1.5–3.5, and 1.5–8.5%, respectively. The concentration of growth hormone (GH) was measured by an enzyme-linked immunosorbent assay, as described previously^(25,26), with the following modifications: a rabbit antibody against ovine GH (anti-ovine GH-3, 1:20, AFP-0802210, obtained from the National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney, and Dr Parlow) was distributed in all wells of a ninety-six-well microplate previously coated with anti-rabbit γ -globulin antiserum (D Schams; TU-Munich, Weihenstephan, Germany). After incubation for 24 h at room temperature and decantation of the antibody solution, 100 μ l of chicken serum diluted in assay buffer (1%) was added to each well. Then, 15 μ l of a standard GH solution (0.78–100 ng/ml, bovine GH, AFP-9984C, obtained from the National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney, and Dr Parlow) dissolved in assay buffer or plasma samples were added, and the plate was incubated for 24 h at room temperature. After washing the plate, biotin-labelled GH was distributed in all wells and then incubated for 3 h at room temperature. Streptavidin solution (Sigma Aldrich, St Louis, MO, USA) was added and optical

density was measured, and concentrations were calculated using Magellan software (Magellan 3.11, Dortmund, Germany). Intra- and inter-assay CV were 9.8 and 12.6%, respectively. The lowest detection limit was 1.0 ng/ml, and the effective dose 50 in this assay system was 7.6 ng/ml.

Calculation of fractional excretion

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

$$\text{FE urea} = \frac{\text{creatinine}_{\text{plasma}} (\text{mM}) \times \text{urea}_{\text{urine}} (\text{mM})}{\text{creatinine}_{\text{urine}} (\text{mM}) \times \text{urea}_{\text{plasma}} (\text{mM})} \times 100 (\%)$$

The fractional excretion of cAMP, P_i and total Ca was calculated according to the same equation, but with respective cAMP, P_i and total Ca concentrations.

Isolation of intestinal brush-border membrane vesicles, transport studies and Western blot analysis

For preparation, transport studies and Western blot analysis of small-intestinal BBMVs have already been described in Muscher *et al.*⁽²²⁾. Briefly, BBMVs were prepared according to the Mg^{2+} precipitation method, with two precipitation steps. For the uptake of $^{32}P/P_i$, BBMVs were incubated at 21°C with the uptake medium containing non-labelled P_i and α - ^{32}P (Hartmann, Braunschweig, Germany; 0.037 MBq/incubation vessel). Concentration-dependent P_i uptakes were performed over a range of 0.01–1.0 mM- P_i . Extravesicular incubation buffer contained 100 mM-mannitol, 10 mM-HEPES–Tris, pH 7.4, and 100 mM-NaCl. The stop solution contained 150 mM-KCl, 1 mM- KH_2PO_4 and 10 mM-HEPES–Tris, pH 7.4. The Na^+ dependency of P_i transport was established by incubating BBMVs in solutions in which KCl replaced NaCl equimolarly. BBMVs were washed with stop solution on a 0.65 μ m cellulose nitrate filter. The ^{32}P activity of each filter was counted using a Packard Tri-Carb 2500TR scintillation counter. Kinetic parameters V_{max} (nmol \times 10 s P_i /mg protein) and K_m (mmol P_i /l) were calculated from the Michaelis–Menten kinetic equation of P_i uptake into the BBMVs.

Immunoblot assays using brush-border membrane preparations were performed as described previously^(22,27). In brief, brush-border membrane protein (50 μ g/lane) was separated by 8.5% SDS–PAGE. Separated proteins were transferred onto nitrocellulose membranes and blocked with 10% fat-free milk powder. Immunodetection of electrotransferred protein was performed according to standard procedures. The NaP_i IIb antibody was used at 1:2000 dilution. Specificity of the murine antibody for goat NaP_i IIb protein was validated by blocking the antibody with the respective antigenic peptide. Immunoreactive proteins were visualised using the enhanced chemiluminescence system (Perbio Science GmbH, Bonn, Germany) according to the manufacturer's protocol. The internal standard β -actin was used to semi-quantify relative protein expression amounts. Bands were analysed semi-quantitatively using Quantity One software (Bio-Rad).

Table 2. Daily feed, DM, nitrogen, calcium and phosphorus intake, initial and final body weight and daily weight gains on a per animal basis, as estimated from group mean values over the entire experimental period (about 7 weeks)

(Mean values with their pooled standard errors)

	Control	Reduced N	SE	<i>P</i>
Feed intake (g/d)	740	713	14.7	0.07
DM intake (g/d)	817	792	13.9	0.07
N (g/d)	17.9	10.2	0.29	<0.0001
Ca (g/d)	7.7	7.4	0.15	0.02
P (g/d)	3.33	3.25	0.07	0.20
Initial body weight (kg)	24.2	23.8	1.93	0.86
Final body weight (kg)	33.9	31.8	1.98	0.31
Body weight gain (kg/d)	0.18	0.14	0.02	0.09

Statistical analyses

Results are expressed as means with their pooled standard errors, with *n* number of animals. Significance of differences was tested by unpaired Student's *t* test, if appropriate (software GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com). In all cases, *P* values <0.05 were set to be significant, and a tendency was assumed when *P*<0.1. To test for a linear relationship between two variables, a simple correlation analysis with Pearson's correlation coefficient was calculated.

Results

Daily weight gain, body weight, daily feed intake, intake of DM, nitrogen, calcium and phosphorus

The animals were clinically healthy throughout the study. The daily weight gain of the goats receiving a reduced N diet was tendentially lower than in the control animals (Table 2). However, the mean body weight of all goats at the beginning and at the end of the study was not affected by dietary treatment (Table 2). Mean daily feed, DM, N, Ca and P intake for each

animal was estimated from group mean values, and parts of these results have already been published in Muscher *et al.*⁽²⁸⁾ (Table 2).

Blood parameters

The concentration of urea in plasma decreased significantly by a reduced N feeding regimen, while plasma creatinine concentrations increased simultaneously (Table 3). Both parameters were inversely related ($r = 0.604$, $P < 0.01$). Haematocrit, total plasma protein and albumin content remained unchanged throughout the complete experiment, indicating that there was neither haemoconcentration nor haemodilution with the various diets (Table 3). The concentrations of plasma P_i and total Ca (Table 4) were not affected by a reduction of dietary N supply in young goats. Plasma concentrations of calcidiol and calcitriol were significantly reduced in the animals fed a reduced N diet (Table 3). There were no significant differences between control and N-reduced fed goats in the plasma concentrations of cAMP (Table 3). However, a linear correlation between plasma cAMP concentrations and calcidiol could be detected in the goats ($r = 0.642$, $P < 0.01$). The amount of plasma CTX, as the bone resorption marker, and total alkaline phosphatase was both significantly elevated in the goats of the N-reduced N group.

The reduction of dietary N content was associated with a decrease in plasma IGF-1 concentration in these animals, while concentrations of GH were not affected (Table 3). Additionally, the concentrations of IGF-1 and plasma urea were positively related ($r = 0.475$, $P < 0.05$).

Concentrations of inorganic phosphate and total calcium in saliva, ruminal and abomasal fluids

The P_i concentrations of parotid saliva, ruminal and abomasal fluids were all significantly reduced in goats fed a reduced N diet, whereas plasma P_i concentrations were not affected

Table 3. Haematocrit values of young goats fed a reduced nitrogen diet*(Mean values with their pooled standard errors and number of animals (*n*))

	<i>n</i>	Control	Reduced N	SE	<i>P</i>
Haematocrit (%)	10	29.4	31.5	1.99	0.31
Plasma					
Urea (mM)	10	5.8	1.49	0.41	<0.001
Creatinine (μM)	10	83.78	95.51	3.63	0.005
Total protein (mg/ml)	10	51.24	54.57	3.52	0.36
Albumin (g/l)	10	33.27	32.61	1.27	0.61
Calcidiol (nM)	9	21.46	12.46	3.98	0.038
Calcitriol (pg/ml)	9	40.50	27.86	4.54	0.013
cAMP (pmol/ml)	10	28.4	13.57	11.84	0.23
CrossLaps (ng/ml)	9	0.876	1.259	0.12	0.007
Total alkaline phosphatase (μkat/l)	8	5.65	7.80	0.99	0.048
Growth hormone (ng/ml)	10	5.52	5.32	1.75	0.91
Insulin-like growth factor 1 (ng/ml)	10	311.5	156.1	44.9	0.003
Urine					
Urea (mM)	7–10	319.5	27.2	55.0	<0.001
Creatinine (mM)	7–10	9921	21 400	2924	0.001
cAMP (pmol/ml)	7–10	3208	4233	768.3	0.20

* Concentrations of urea, creatinine, total protein, albumin, calcidiol, calcitriol, cAMP, CrossLaps, total alkaline phosphatase, growth hormone and insulin-like growth factor 1 in the plasma and urine of goats as affected by a reduced N diet.

Table 4. Effects of a reduced nitrogen diet on calcium and inorganic phosphate (P_i) concentrations in plasma, saliva, ruminal fluid, abomasal fluid and fractional excretion (FE) in young goats(Mean values with their pooled standard errors and number of animals (n))

	n	Control	Reduced N	SE	P
Plasma Ca (mM)	10	2.52	2.55	0.10	0.81
Plasma P_i (mM)	10	2.18	1.86	0.24	0.20
Saliva Ca (mM)	10	0.197	0.266	0.02	0.004
Saliva P_i (mM)	10	30.77	22.62	1.64	<0.001
Ruminal fluid Ca (mM)	10	0.98	1.36	0.25	0.14
Ruminal fluid P_i (mM)	10	30.68	23.37	2.74	0.016
Abomasal fluid Ca (mM)	9–10	10.85	7.42	1.09	0.006
Abomasal fluid P_i (mM)	9–10	30.47	20.48	2.47	0.001
FE Ca (%)	7–10	0.80	3.85	1.14	0.018
FE P_i (%)	7–10	0.86	2.09	0.36	0.21

(Table 4). The decline in P_i concentrations in saliva (r 0.684, P <0.001), ruminal (r 0.503, P <0.05) and abomasal fluids (r 0.715, P <0.001) was positively correlated with decreasing plasma urea concentrations.

Salivary total Ca concentrations were significantly elevated at the end of the experiment (Table 4). Ruminal concentrations of total Ca were not affected, whereas total Ca concentrations in the abomasal fluid were significantly diminished in the goats with the reduced N diet (Table 4).

Effects of the reduced nitrogen diet on the renal excretion of urea, total calcium, inorganic phosphate and adenosine 3',5'-cyclic monophosphate

A reduced dietary N supply was associated with a decrease in the FE of urea (control: 48.4%, reduced N: 9.34 (SE 6.13)%) and an increase in total urine Ca, while FE of P_i (Table 4) and cAMP (control: 232.7%, reduced N: 262.6 (SE 127.7)%) remained unaffected.

Na^+ -dependent inorganic phosphate transport and type IIb Na^+ -inorganic phosphate cotransporter expression in the small intestine

Transport capacity (V_{max} ; control: 1.38 nmol/mg protein per 10 s, reduced N: 1.48 (SE 0.33) nmol/mg protein per 10 s; P =0.76) and transporter affinity (K_m ; control: 0.074 mM, reduced N: 0.063 (SE 0.01) mM; P =0.29) of Na^+ -dependent P_i transport into mid-jejunal BBMV were not affected by a reduced dietary N supply in young goats. However, the protein expression (90 kDa) of NaP_i IIb normalised to β -actin in the mid-jejunum was tendentially reduced under a reduced N diet (control: 2.29, reduced N: 1.24 (SE 0.51); P =0.08).

Discussion

The present study demonstrates that the electrolyte homeostasis of young goats was influenced by a reduced N diet despite efficient N recycling mechanisms. These effects of a reduced N diet might be most marked during growth; therefore, a growing goat model was chosen. A slightly reduced daily weight gain indicates that dietary N reduction (8%) may show the

limitation of compensation of N metabolism. Isoenergetic diets were used to detect the effect of N reduction as the single variable on electrolyte homeostasis.

Plasma urea concentrations were determined as a significant indicator of the extent of dietary N supply. The reduction of dietary N by about 44% caused a marked decrease in plasma urea concentrations. The order of magnitude of this effect is in accordance with data reported for other goats and sheep⁽²⁹⁾. The FE of urea in the kidney fell to a very low concentration as one of the N-saving mechanisms to facilitate the recycling of urea for microbial protein synthesis in the rumen. In previous studies in goats, this renal reduction of urea excretion rate could be demonstrated as a consequence of renal mechanisms to conserve urea during N deprivation, whereas glomerular filtration rate (GFR) dropped by about 60% when N intake was reduced^(30,31). A decrease in GFR could be caused by a reduction in IGF-1 concentrations, which were able to modulate GFR in rats⁽³²⁾. Such a decline in GFR was indicated by an increase in plasma creatinine concentration in the goats fed a reduced N diet in the present study. However, as indicated by the simultaneous increase in urinary creatinine concentrations, it is also likely that creatinine was increasingly released from muscle due to a higher muscle turnover. A higher muscle protein turnover would provide more N for urea synthesis in the N-reduced fed goats to improve N supply to ruminal micro-organisms. Furthermore, a reduction in urine volume, as has been observed in ongoing balance studies in N-reduced fed goats, could contribute to high creatinine concentrations in urine.

Besides a decrease in GFR, reduced IGF-1 concentrations could also cause a decrease in the renal blood flow as in monogastric animals⁽³³⁾. Both parameters, a reduced GFR and a decrease in renal blood flow, could diminish substrate delivery to the renal 1- α hydroxylase, which would cause less calcitriol formation in the kidney⁽³⁴⁾. Confirmingly, a correlation between GFR and plasma calcitriol concentrations was shown in humans⁽³⁵⁾. Another reason could be that reduced IGF-1 concentrations affect the activity of the renal 1- α hydroxylase as in humans⁽³⁶⁾. A reduction in calcitriol concentrations could be already shown in goats fed a reduced N and Ca diet⁽²⁾ as well as in rats with a low-protein diet⁽⁵⁾. In monogastric animals, changes in calcitriol concentrations

were able to regulate the intestinal absorption of P_i by the modulation of intestinal P_i transporter function and expression amounts^(37,38). However, the decrease in calcitriol concentrations in the young goats caused only a tendential reduction in the intestinal transport protein NaP_i IIb, which was not reflected in changes in the intestinal absorption of P_i .

Low calcitriol concentrations in plasma are known to decrease bone mineralisation⁽³⁹⁾. In this experiment, the bone resorption marker CTX in goats fed a reduced N diet was increased, indicating a higher bone resorption in these animals; this could be shown in goats fed a reduced N and Ca diet, too⁽²⁾. Hypothetically, higher bone resorption could be caused by changes in PTH concentrations because of the intense intertwining of these regulating hormones in Ca and P_i homeostasis⁽⁴⁰⁾. Humans consuming a low-protein diet expressed an increase in serum PTH⁽¹⁰⁾. These elevated PTH concentrations were able to activate Ca and P_i mobilisation from bones. Hence, a link between protein and electrolyte metabolism was postulated for monogastric animals and humans⁽⁴¹⁾. A possible candidate for such a link could be the Ca-sensing receptor (CaR), which is expressed in many organs^(42,43). The CaR seemed to be able to respond to altered Ca and also to changes in amino acid concentrations due to its binding sites for both⁽⁴¹⁾. This property of the CaR may be the molecular basis for the crosstalk between protein and Ca and P_i metabolism. Therefore, it is assumed that a low-protein diet was able to stimulate PTH synthesis and secretion via the CaR. Another possibility could be that as in rats, the reduced calcitriol concentrations inhibited less of the CaR, which results in higher PTH secretion by the parathyroid glands⁽⁴⁴⁾. Further investigation of the expression and activity of the CaR in young goats fed a reduced N diet is needed.

The elevated activity of total alkaline phosphatase in plasma in the goats of the N-reduced group could be a result of the decreased calcitriol concentrations, which caused perturbed mineralisation of the bones. However, total alkaline phosphatase was not a specific marker of bone formation because its activity in plasma was also contributed to other sources than the bone⁽⁴⁵⁾.

The concentration of cAMP in plasma and urine, which were described in the literature as indirect parameters of PTH activation^(46,47) in humans and monogastric animals, could not confirm that the higher bone resorption detected in the goats fed a reduced N diet was PTH-dependent.

Alterations in amounts of other humoral factors could also be another explanation for the change in bone resorption induced by dietary N reduction. In particular, changes in IGF-1 concentrations were associated with unchanged GH concentrations in the growing N-reduced fed goats. In humans, a positive correlation between protein intake and bone growth factor IGF-1 was observed, while concentrations of CTX decreased at the same time⁽⁴⁸⁾. Therefore, the effects of N reduction on mineral and bone metabolism observed in the present study may be mediated in part by reduced IGF-1 concentrations. Calcidiol synthesis in the liver might also be affected by reduced IGF-1 concentrations because, in humans, a positive correlation between these two hormones was detected⁽⁴⁹⁾.

Besides the effects on bone mineralisation, the influence of a dietary N reduction on electrolyte homeostasis was expressed by alterations of P_i and Ca concentrations in body fluids of young goats (Table 4). Changes in P_i homeostasis included a decrease of P_i in saliva starting at the 28th day of the experiment (Fig. 1; Table 4) and low P_i concentrations in ruminal and abomasal fluids (Table 4). Plasma P_i concentrations and extent of urinary P_i excretion were not affected as in goats fed a reduced N and Ca diet⁽²⁾ (Fig. 1; Table 4). The concentrations of P_i in saliva, ruminal and abomasal fluids were all positively correlated with plasma urea concentrations. Since neither the rumen epithelium nor the abomasal mucosa is able to absorb P_i , explaining lower concentrations in these fluids, the modulation of P_i concentrations must be mediated by the capacity of salivary glands to concentrate and secrete P_i into the rumen. In general, it is well known that salivary P_i is adjusted to plasma P_i concentration without any regulation by hormones or other factors⁽⁵⁰⁾. During a reduced N feeding, this tight adjustment seemed to be uncoupled by mechanisms not known so far. Caprine salivary gland P_i transporter was assessed to be a P_i transporter similar to those which are members of the SLC34 transporter family (NaP_i IIb-like)⁽⁵¹⁾. NaP_i IIb protein is ubiquitously expressed in different organs (e.g. small intestine) and tissues⁽⁵²⁾. In monogastric animals, intestinal NaP_i IIb was regulated by calcitriol, which stimulated NaP_i IIb expression as well as Na^+ -dependent P_i transport⁽⁵³⁾. Hypothetically, the caprine Na^+ -dependent P_i transporter might be decreased in its function by the lack of calcitriol stimulation due to low calcitriol concentrations in N-reduced goats.

In the present study, Ca homeostasis was also affected in growing goats by a reduced N intake. While in plasma and ruminal fluids, total Ca concentrations were not affected, which was in contrast to goats fed a reduced N and Ca diet where plasma Ca concentrations were reduced⁽²⁾, an increase in salivary Ca was found (Table 4). Even though salivary total Ca concentrations were elevated at the end of the experiment, the physiological meaning of the increased Ca concentrations

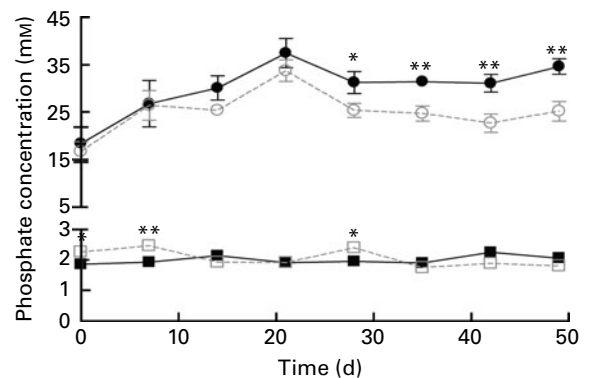


Fig. 1. Concentrations of inorganic phosphate (P_i) in the plasma and saliva of goats fed a reduced N diet for about 7 weeks. Values are means, with standard errors represented by vertical bars (n 10). Mean values were significant for the effect of a dietary reduced N level: * P <0.05 and ** P <0.01. —●—, P_i saliva control; ---○---, P_i saliva reduced N; —■—, P_i plasma control; ---□---, P_i plasma reduced N.

in saliva was not clear. Low abomasal Ca concentrations may be associated with an enhanced ruminal active Ca absorption, which was described for goats and sheep⁽⁵⁴⁾. The reason for this activated ruminal absorption of Ca during a reduced N diet cannot be explained yet.

The hypercalciuria of the goats receiving a N-reduced diet was not associated with a reduction in plasma total Ca concentrations. In goats fed a reduced N and Ca diet, a reduction in plasma total and ionised Ca concentrations could be observed, caused by a lower Ca intake, which resulted in hypocalciuria⁽²⁾. In the present study, a higher Ca release from bones could be assessed, as indicated by the increase in the bone resorption marker CTX. The majority of Ca is transported in the plasma bound to protein. However, a reduction in binding capacity could be excluded because plasma protein and albumin concentrations did not differ between the two feeding groups. The excess of Ca was finally excreted by the kidneys resulting in a higher FE of Ca. Hypothetically, this could be mediated by lower calcitriol concentrations due to a down-regulation of calcitriol-dependent epithelial Ca channels (transient receptor potential cation channel, subfamily V, member 5) as in rats⁽⁵⁵⁾.

In summary, the present study shows that a reduced N diet in young goats led to decreased calcitriol, calcidiol as well as IGF-1 concentrations and increased bone resorption marker CTX. Furthermore, P_i and Ca homeostasis was perturbed in the N-reduced fed goats despite N recycling mechanisms. In conclusion, an interaction between dietary N and electrolyte metabolism occurred in young goats as in monogastric animals. The dietary requirements of N for young goats must be investigated further regarding the aim to decrease N pollution, especially with a focus on mineral and bone metabolism.

Acknowledgements

The present study was supported by the German Research Foundation. The authors thank K. Hansen, K. Hustedt and B. Leppich for their excellent technical assistance. The NaP_i IIb antibody was kindly provided by Dr J. Biber and Professor Dr H. Murer, Institute of Physiology, University of Zurich-Irchel, Zurich, Switzerland. A. S. M., G. B. and K. H. designed the experiments. A. S. M. performed the animal feeding trials, biochemical analyses, molecular analyses and statistical analyses. M. P. performed the IGF-1 and GH measurements. A. S. M. and K. H. wrote the manuscript. All authors discussed the results and commented on the manuscript. There are no conflicts of interest to declare.

References

1. Castillo AR, Kebreab E, Beever DE, *et al.* (2001) The effect of protein supplementation on nitrogen utilization in lactating dairy cows fed grass silage diets. *J Anim Sci* **79**, 247–253.
2. Muscher A & Huber K (2010) Effects of a reduced nitrogen diet on calcitriol levels and calcium metabolism in growing goats. *J Steroid Biochem Mol Biol* **121**, 304–307.
3. Houpt TR (1959) Utilization of blood urea in ruminants. *Am J Physiol* **197**, 115–120.
4. Harmeyer J & Martens H (1980) Aspects of urea metabolism in ruminants with reference to the goat. *J Dairy Sci* **63**, 1707–1728.
5. Orwoll E, Ware M, Stribrska L, *et al.* (1992) Effects of dietary protein deficiency on mineral metabolism and bone mineral density. *Am J Clin Nutr* **56**, 314–319.
6. Kerstetter JE, O'Brien KO & Insogna KL (1998) Dietary protein affects intestinal calcium absorption. *Am J Clin Nutr* **68**, 859–865.
7. Kerstetter JE, O'Brien KO & Insogna KL (2003) Low protein intake: the impact on calcium and bone homeostasis in humans. *J Nutr* **133**, 855S–861S.
8. Peng YS, Meliza LL, Vavich MG, *et al.* (1974) Changes in food intake and nitrogen metabolism of rats while adapting to a low or high protein diet. *J Nutr* **104**, 1008–1017.
9. Fujita Y, Yoshimura Y & Inoue G (1978) Effect of low-protein diets on free amino acids in plasma of young men: effect of protein quality with maintenance or excess energy intake. *J Nutr Sci Vitaminol (Tokyo)* **24**, 297–309.
10. Kerstetter JE, Caseria DM, Mitnick ME, *et al.* (1997) Increased circulating concentrations of parathyroid hormone in healthy, young women consuming a protein-restricted diet. *Am J Clin Nutr* **66**, 1188–1196.
11. Conigrave AD, Mun HC, Delbridge L, *et al.* (2004) L-Amino acids regulate parathyroid hormone secretion. *J Biol Chem* **279**, 38151–38159.
12. Murer H, Forster I, Hernando N, *et al.* (1999) Posttranscriptional regulation of the proximal tubule NaPi-II transporter in response to PTH and dietary P(i). *Am J Physiol* **277**, F676–F684.
13. Brown AJ, Dusso A & Slatopolsky E (1999) Vitamin D. *Am J Physiol* **277**, F157–F175.
14. Sahota O, Masud T, San P, *et al.* (1999) Vitamin D insufficiency increases bone turnover markers and enhances bone loss at the hip in patients with established vertebral osteoporosis. *Clin Endocrinol (Oxf)* **51**, 217–221.
15. Naumann C & Basler R (1976) *Verband deutscher landwirtschaftlicher Untersuchungs- und Forschungsanstalten. Methodenbuch Band III. Die chemische Untersuchung von Futtermitteln. Mit Ergänzungslieferungen 1983, 1988, 1993, 1997 (Association of German Agricultural Testing and Research Institutes. Methods Book Volume III. The Chemical Analysis of Feed. With Additional Deliveries in 1983, 1988, 1993, 1997)*. Darmstadt: VDLUFA-Verlag.
16. Van Soest PJ, Roberston JB & Lewis BA (1991) Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci* **74**, 3583–3597.
17. Jung K, Wesslau C, Priem F, *et al.* (1987) Specific creatinine determination in laboratory animals using the new enzymatic test kit "Creatinine-PAP". *J Clin Chem Clin Biochem* **25**, 357–361.
18. Sarkar BC & Chauhan UP (1967) A new method for determining micro quantities of calcium in biological materials. *Anal Biochem* **20**, 155–166.
19. Kruse-Jarres J (1979) *Klinische Chemie, spezielle klinisch-chemische Analytik (Clinical Chemistry, Special Clinical-Chemical Analysis)*. Stuttgart: Fischer-Verlag.
20. Schröder B & Breves G (1996) Mechanisms of phosphate uptake into brush-border membrane vesicles from goat jejuna. *J Comp Physiol B* **166**, 230–240.
21. Widiyono I, Huber K, Failing K, *et al.* (1998) Renal phosphate excretion in goats. *Zentralbl Veterinarmed A* **45**, 145–153.

22. Muscher A, Hattendorf J, Pfeffer E, *et al.* (2008) Hormonal regulation of phosphate homeostasis in goats during transition to rumination. *J Comp Physiol B* **178**, 585–596.
23. Chavassieux P, Garnero P, Duboeuf F, *et al.* (2001) Effects of a new selective estrogen receptor modulator (MDL 103,323) on cancellous and cortical bone in ovariectomized ewes: a biochemical, histomorphometric, and densitometric study. *J Bone Miner Res* **16**, 89–96.
24. Kawade M (1964) An automatic method for determination of alkaline phosphatase in serum using *p*-nitrophenylphosphate as substrate. *Mie Med J* **14**, 41–46.
25. Kawashima C, Sakaguchi M, Szuki T, *et al.* (2007) Metabolic profiles in ovulatory and anovulatory primiparous dairy cows during the first follicular wave postpartum. *J Reprod Dev* **53**, 113–120.
26. Roh SG, Matsunaga N, Miyamoto A, *et al.* (1997) Competitive enzyme immunoassay for bovine growth hormone. *Endocr J* **44**, 195–198.
27. Huber K, Walter C, Schröder B, *et al.* (2002) Phosphate transport in the duodenum and jejunum of goats and its adaptation by dietary phosphate and calcium. *Am J Physiol Regul Integr Comp Physiol* **283**, R296–R302.
28. Muscher A, Schröder B, Breves G, *et al.* (2010) Dietary nitrogen reduction enhances urea transport across goat rumen epithelium. *J Anim Sci* **88**, 3390–3398.
29. Schröder B, Schöneberger M, Rodehutschord M, *et al.* (2003) Dietary protein reduction in sheep and goats: different effects on L-alanine and L-leucine transport across the brush-border membrane of jejunal enterocytes. *J Comp Physiol B* **173**, 511–518.
30. Eriksson L & Valtonen M (1982) Renal urea handling in goats fed high and low protein diets. *J Dairy Sci* **65**, 385–389.
31. Valtonen MH, Uusi-Rauva A & Eriksson L (1982) The effect of protein deprivation on the validity of creatinine and urea in evaluation of renal function. An experimental study in the goat. *Scand J Clin Lab Invest* **42**, 507–512.
32. Hirschberg R & Kopple JD (1989) Effects of growth hormone and IGF-I on renal function. *Kidney Int Suppl* **27**, S20–S26.
33. Rabkin R & Schaefer F (2004) New concepts: growth hormone, insulin-like growth factor-I and the kidney. *Growth Horm IGF Res* **14**, 270–276.
34. Schroeder NJ & Cunningham J (2000) What's new in vitamin D for the nephrologist? *Nephrol Dial Transplant* **15**, 460–466.
35. Prince RL, Hutchison BG, Kent JC, *et al.* (1988) Calcitriol deficiency with retained synthetic reserve in chronic renal failure. *Kidney Int* **33**, 722–728.
36. Bianda T, Hussain MA, Glatz Y, *et al.* (1997) Effects of short-term insulin-like growth factor-I or growth hormone treatment on bone turnover, renal phosphate reabsorption and 1,25 dihydroxyvitamin D₃ production in healthy man. *J Intern Med* **241**, 143–150.
37. Yagci A, Werner A, Murer H, *et al.* (1992) Effect of rabbit duodenal mRNA on phosphate transport in *Xenopus laevis* oocytes: dependence on 1,25-dihydroxy-vitamin-D₃. *Pflugers Arch* **422**, 211–216.
38. Xu H, Bai L, Collins JF, *et al.* (2002) Age-dependent regulation of rat intestinal type IIb sodium-phosphate cotransporter by 1,25-(OH)₂ vitamin D(3). *Am J Physiol Cell Physiol* **282**, C487–C493.
39. Dusso AS, Brown AJ & Slatopolsky E (2005) Vitamin D. *Am J Physiol Renal Physiol* **289**, F8–F28.
40. Bergwitz C & Juppner H (2010) Regulation of phosphate homeostasis by PTH, vitamin D, and FGF23. *Annu Rev Med* **61**, 91–104.
41. Conigrave AD, Mun HC & Lok HC (2007) Aromatic L-amino acids activate the calcium-sensing receptor. *J Nutr* **137**, 1524S–1527S, discussion 1548S.
42. Brown EM (2000) G protein-coupled, extracellular Ca²⁺ (Ca²⁺(o))-sensing receptor enables Ca²⁺(o) to function as a versatile extracellular first messenger. *Cell Biochem Biophys* **33**, 63–95.
43. Goodman WG (2004) Calcium-sensing receptors. *Semin Nephrol* **24**, 17–24.
44. Brown AJ, Zhong M, Finch J, *et al.* (1996) Rat calcium-sensing receptor is regulated by vitamin D but not by calcium. *Am J Physiol* **270**, F454–F460.
45. Moss DW (1982) Alkaline phosphatase isoenzymes. *Clin Chem* **28**, 2007–2016.
46. Drezner MK, Neelon FA, Curtis HB, *et al.* (1976) Renal cyclic adenosine monophosphate: an accurate index of parathyroid function. *Metabolism* **25**, 1103–1112.
47. Chase LR & Aurbach GD (1967) Parathyroid function and the renal excretion of 3′5′-adenylic acid. *Proc Natl Acad Sci U S A* **58**, 518–525.
48. Dawson-Hughes B, Harris SS, Rasmussen H, *et al.* (2004) Effect of dietary protein supplements on calcium excretion in healthy older men and women. *J Clin Endocrinol Metab* **89**, 1169–1173.
49. Gomez JM, Maravall FJ, Gomez N, *et al.* (2004) Relationship between 25-(OH)D₃, the IGF-I system, leptin, anthropometric and body composition variables in a healthy, randomly selected population. *Horm Metab Res* **36**, 48–53.
50. Scott D & Beastall G (1978) The effects of intravenous phosphate loading on salivary phosphate secretion and plasma parathyroid hormone levels in the sheep. *Q J Exp Physiol Cogn Med Sci* **63**, 147–156.
51. Huber K, Roesler U, Muscher A, *et al.* (2003) Ontogenesis of epithelial phosphate transport systems in goats. *Am J Physiol Regul Integr Comp Physiol* **284**, R413–R421.
52. Murer H, Forster I & Biber J (2004) The sodium phosphate cotransporter family SLC34. *Pflugers Arch* **447**, 763–767.
53. Hattenhauer O, Traebert M, Murer H, *et al.* (1999) Regulation of small intestinal Na-P(i) type IIb cotransporter by dietary phosphate intake. *Am J Physiol* **277**, G756–G762.
54. Schröder B, Rittmann I, Pfeffer E, *et al.* (1997) *In vitro* studies on calcium absorption from the gastrointestinal tract in small ruminants. *J Comp Physiol B* **167**, 43–51.
55. Hoenderop JG, Muller D, Van Der Kemp AW, *et al.* (2001) Calcitriol controls the epithelial calcium channel in kidney. *J Am Soc Nephrol* **12**, 1342–1349.