Impact of dietary nitrogen reduction on electrolyte homeostasis in growing goats with a focus on renal electrolyte handling

THESIS

Submitted in partial fulfilment of the requirements for the degree

DOCTOR OF PHILOSOPHY

(PhD)

awarded by the University of Veterinary Medicine Hannover

by

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born in Oelde

Hannover, Germany 2013
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Date of final exam: 01.11.2013

Sponsorship: This PhD project was supported by the German Research Foundation (DFG) and by the H. Wilhelm Schaumann Foundation
„Aber für so was fehlen uns die Gelder.”

„Nun ja…da wäre noch das Ziegenlabor…“

aus: Männer, die auf Ziegen starren
Parts of this thesis have previously been published or communicated:

Publications:


Presentations on conferences:


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Abbreviations

BBM   brush border membrane
BBMV  brush border membrane vesicles
Ca    calcium
Calcidiol 25-hydroxy vitamin D
Calcitriol 1,25-dihydroxy vitamin D₃
cAMP  cyclic adenosine monophosphate
CaR   calcium sensing receptor
CTX   carboxyterminal cross-linked telopeptide of type I collagen
DTT   dithiothreitol
ERK1/2 extracellular signal regulated kinases 1 and 2
GIT   gastrointestinal tract
GOD/PAP glucose oxidase/phenol and aminophenazone
HE    hematoxylin and eosin
IGF-1 insulin-like growth factor 1
kDa   kilo Dalton
LDH   lactate dehydrogenase
LOX   lactate oxidase
mTOR  mammalian target of rapamycin
N     nitrogen
Na    sodium
NaPi IIa sodium-dependent phosphate transporter type IIa
NaPi IIc sodium-dependent phosphate transporter type IIc
NH₃   ammonia
P     phosphorus
PBST  phosphate buffered saline with tween
pERK1/2 phosphorylated extracellular signal regulated kinases 1 and 2
P₁    phosphate
PiT-2 sodium-dependent phosphate transporter type III
PKA   protein kinase A
PKC   protein kinase C
pmTOR phosphorylated mammalian target of rapamycin
PTH   parathyroid hormone
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<tr>
<td>PTHR</td>
<td>parathyroid hormone receptor</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecylsulfate polyacrylamide gelelectrophoresis</td>
</tr>
<tr>
<td>TRPV5</td>
<td>transient receptor potential channel subfamily vanilloid member 5</td>
</tr>
<tr>
<td>UT</td>
<td>urea transporter</td>
</tr>
<tr>
<td>UT-A</td>
<td>urea transporter isoform A</td>
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<td>UT-B</td>
<td>urea transporter isoform B</td>
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<tr>
<td>VDR</td>
<td>vitamin D receptor</td>
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Summary

For economical and ecological reasons, in the feeding of ruminant livestock precisely adjusted protein content is aspired. Ruminants have a unique ability to counterbalance low protein, and therefore low dietary nitrogen (N) intake by decreasing N excretion due to endogenous N recycling. Within this recycling process urea reabsorption in the kidneys by urea transporters (UTs) plays a central role. However, despite this endogenous N recycling, it was shown in growing goats that low N intake did not only impact N metabolism, but also electrolyte homeostasis (MUSCHER et al. 2011). This was reflected by decreased plasma concentrations of insulin-like growth factor-1 (IGF-1), 1,25-dihydroxy vitamin D₃ (calcitriol) and increased plasma concentrations of carboxyterminal cross-linked telopeptide of type I collagen (CTX). In the present PhD project, the adaptation of both N and electrolyte homeostasis to low dietary N intake was investigated in growing goats with special emphasis on examination of renal adaptation to low N intake. The response of renal N and electrolyte handling to the changing dietary conditions was investigated in four different studies.

In the first study the impact of low N intake on renal UT expression in growing goats was investigated. The results showed the mRNA expression of the UT isoform UT-A1 to be increased due to low N intake. This finding indicates an enhanced renal capacity for reabsorption of N and thus, a more efficient endogenous N recycling due to low N intake also in goats.

In the second study the involvement of renal calcium (Ca) and phosphate (Pi) transport in the adaptation of electrolyte homeostasis to low N intake was investigated. Therefore, renal expression of Ca and Pi transporters and of some of their regulatory proteins was measured. The results showed that the protein expression of the renal sodium (Na)-dependent Pi transporter type IIa (NaPi IIa) was increased upon low N intake. Accordingly, protein expression of the renal parathyroid hormone receptor (PTHR) and of the phosphorylated extracellular signal regulated kinases 1 and 2 (pERK1/2), which are known to reduce NaPi IIa protein amounts, was decreased. These findings suggest that not only adaptation of hormones, but also of renal Pi transport pathways occurred due to low N intake in growing goats.

In the third study the question was raised, whether the observed alterations in renal Pi transporter expression influenced electrolyte excretion in vivo. Therefore, balance trials were performed in goats fed low N content and daily intake and excretion of N, Ca and phosphorus (P) were measured. Also in these animals the protein expression of renal Ca and Pi transporters and their regulatory proteins was examined. Low N intake resulted in reduced
daily N excretion, but unchanged N retention. However, the excretion of Ca and P remained unchanged. On the level of renal protein expression, only the amount of PTHR was decreased, but neither NaPi IIa nor pERK1/2 protein amount was altered. Although adaptation of renal electrolyte handling appeared to differ from that in the second study, again lower plasma IGF-1 and a trend towards lower plasma calcitriol concentrations were observed. Furthermore, linear regression analyses indicated IGF-1, calcitriol and the renal Na-dependent P, transporter type IIc (NaPi IIc) to play a major role in the regulation of electrolyte handling in growing goats. These findings further emphasize a connection between low N intake and adaptive responses of electrolyte handling.

In the fourth study it was investigated whether the adaptive responses of electrolyte handling to low N intake, as observed in the previous experiments, were goat-specific. Thus, a comparative feeding experiment similar to that in goats was performed in rats, carefully considering species-specific peculiarities in the digestive physiology and metabolism. Rats were fed a diet reduced in N content and the same parameters as in the goat experiments were determined. For a comparative analysis all data were related to metabolic body size in rats and goats. The results showed that also in rats N excretion decreased due to low N intake. N retention in rats remained unchanged, despite a putative less effective N recycling, whereas in goats N retention was slightly, but not significantly decreased, when related to metabolic body size. Also the influence of low N intake on renal Ca excretion differed between the two species. Whilst in goats renal Ca excretion decreased in relation to metabolic body size, the urinary Ca concentration was increased in rats. Furthermore, in contrast to goats, the hormone concentrations and the expression of the detected renal proteins were not altered by low N intake in rats. Thus, the results showed that the adaptation of electrolyte handling to low N intake, as it was observed in the previous studies, was goat-specific. Further research is needed to elucidate the exact metabolic pathways leading to adaptation of electrolyte handling to low N intake.
Zusammenfassung


In der zweiten Studie wurde die Beteiligung des renalen Kalzium (Ca-) und Phosphat (Pₗ)-Transportes an der Adaptation des Elektrolythaushaltes an die reduzierte N-Aufnahme untersucht. Dazu wurde die Proteinxpression renaler Ca- und Pₗ-Transporter und die Expression einiger ihrer Regulationsproteine gemessen. Die Ergebnisse zeigten, dass die Proteinxpression des renalen Natrium (Na)-abhängigen Pₗ-Transporters Typ Ila (NaPi IIa) bei reduzierter N-Aufnahme erhöht war. Damit übereinstimmend war die Proteinxpression des renalen Parathormon-Rezeptors (PTHR) und der phosphorylierten extracellular signal regulated kinases 1 and 2 (pERK1/2), die die NaPi IIa Proteinmenge vermindern, erniedrigt.
Diese Ergebnisse zeigen, dass in wachsenden Ziegen neben hormonellen Veränderungen auch der renale Elektrolyttransport mit in die Adaptation an eine reduzierte N-Aufnahme einbezogen ist.

In der dritten Studie wurde untersucht, inwiefern sich diese Veränderungen der Proteinexpression im Bereich des renalen P\textsubscript{T}-Transportes auf die renale Elektrolytausscheidung \textit{in vivo} auswirken. Dazu wurden in einem Versuch mit Ziegen unter N-reduzierter Fütterung Bilanzversuche durchgeführt. Die tägliche Aufnahme und Ausscheidung von N, Ca und Phosphor (P) wurde gemessen. Des Weiteren wurde auch bei diesen Tieren die Proteinexpression der renalen Ca- und P\textsubscript{T}-Transporter und der zugehörigen Regulationsproteine untersucht. Die Ergebnisse zeigten, dass die tägliche N-Ausscheidung bei verminderten N-Aufnahme deutlich reduziert war, die N-Retention jedoch unverändert blieb. Auch die Ausscheidung von Ca und P war unverändert. Bezüglich der renalen Proteinexpression war bei dieser Tiergruppe allerdings lediglich die Expression des PTHR erniedrigt, während weder die Expression des NaPi IIa noch der pERK1/2 verändert war.


In der vierten Studie sollte festgestellt werden, ob die beobachteten Veränderungen aus den vorherigen Versuchen ziegenspezifische Adaptationsmechanismen an eine reduzierte N-Aufnahme darstellten. Für diese vergleichende Betrachtung wurde ein dem Ziegenversuch möglichst ähnlicher Fütterungsversuch an Ratten durchgeführt, wobei die nötigen spezies-spezifischen Fütterungsbedingungen beachtet wurden. Auch den Ratten wurde eine Diät mit reduziertem N-Gehalt gefüttert und die gleichen Parameter wie im Ziegenversuch wurden gemessen. Die Ergebnisse zeigten, dass auch bei den Ratten bei vermindriger N-Aufnahme die N-Ausscheidung erniedrigt war. Die N-Retention war trotz einer vermeintlich weniger starken N-Rezyklierung unverändert, während in Ziegen unter Bezug der Daten auf das metabolische Körpergewicht sogar eine tendenziell erniedrigte N-Retention beobachtet wurde. Auch die Beeinflussung der Ca-Erkretion durch die reduzierte N-Aufnahme unterschied sich zwischen beiden Tierarten. Während die renale Ca-Erkretion bei Ziegen in
Bezug auf das metabolische Körpergewicht erniedrigt war, war die Ca-Konzentration im Harn der Ratten erhöht. Auch eine Veränderung der untersuchten Hormone und der Expression der renalen Transportproteine wurde bei Ratten im Gegensatz zu den Ziegen nicht nachgewiesen. Die Ergebnisse zeigen daher, dass die Adaptation des Elektrolythaushaltes, wie sie in den vorhergehenden Versuchen beobachtet wurde, ziegenspezifisch war. Weitere Untersuchungen sind notwendig, um die genauen, zugrunde liegenden Regulationsmechanismen dieser Adaptation des Elektrolythaushaltes an eine reduzierte N-Aufnahme aufzuklären.
1 Introduction

1.1 Dietary nitrogen reduction – why?

In the feeding of ruminant livestock precisely adjusted protein, and thus nitrogen (N) content is aspired. On the one hand, the N content should be adjusted to the lowest possible level for reasons of minimizing costs and also minimizing pollution by N excretion. On the other hand, the N content should not be reduced to an extent, at which it has negative impact on the animals’ health and growth performance. Furthermore, in arid or semi-arid regions feeding high or at least adequate N containing diets can be virtually impossible due to the available feeding stuff in these climatic conditions. However, ruminants are considered being unique in their ability to economize N and therefore, in contrast to other mammals, they are able to deal with unfavourable nutritional conditions (MARINI and VAN AMBURGH 2003).

1.2 How ruminants economize N – rumino-hepatic urea-N recycling

Even in situations of N scarcity, ruminants are able to cope very efficiently with the residual amounts of N. This effective way of economizing N is based on ruminant-specific N recycling mechanisms, which are referred to as rumino-hepatic urea recycling (Figure 1). Rumen microbial protein serves as the main source of protein for the ruminant as host. Therefore, a constant N supply to the rumen is essential for maintaining microbial protein synthesis and thus, also for maintaining protein supply to the host. After protein degradation into amino acids and subsequent digestion, finally the toxic ammonia (NH₃) is produced. To a high proportion it is detoxified in the liver by synthesis of urea, which is then released into the blood stream and can be excreted mainly by the kidneys. However, when urea is shifted from the bloodstream to the salivary glands or directly into the rumen again, it can also be reused as source of N for rumen microbial protein synthesis. Therefore, in times of N scarcity, the excretion of valuable urea can be reduced to a minimum in ruminants. Reduction in renal urea excretion is mediated by increased urea reabsorption from the ultrafiltrate and by subsequent return of urea to the bloodstream. Renal reabsorption, and thereby excretion of urea are implemented by specific urea transporters (UTs). The structure, function and role of UTs for
urea recycling have been investigated in monogastric animals, in cattle and in parts in small ruminants, as discussed in the following.

Figure 1: Schematic overview of urea synthesis and urea recycling in ruminants. Protein in the gastrointestinal tract (GIT) is degraded into amino acids. Amino acids pass from the GIT into the blood stream and are metabolized, producing the waste product NH₃. The major proportion of NH₃ is detoxified by hepatic urea synthesis. Urea is returned to the plasma pool and can be transferred into the GIT again directly via transport across the rumen epithelium or via secretion with the saliva, serving as a new source of N for rumen microbial protein synthesis. Plasma urea is partially filtered by the kidneys and can either be excreted with the urine or can be reabsorbed into the blood stream again.

1.3 Urea transporters – a brief overview

The molecular structure of UTs was identified for the first time in Xenopus oocytes (YOU et al. 1993). Meanwhile, UT expression was detected in different tissues (for example kidney, liver, testis, intestine) and species (COURIAUD et al. 1996; FENTON et al. 2000; INOUE et al. 2004; SMITH et al. 2004). Furthermore, different UT isoforms encoded by the SLC14 genes are known (FENTON et al. 1999; BAGNASCO et al. 2001; NAKAYAMA et al. 2001). From the UT-A (SLC14A2) five protein and eight cDNA isoforms and from the UT-B (SLC14A1) two isoforms are known (for a review see SANDS 2002).

In the kidney urea transport is accomplished in medullary collecting ducts by UT-A isoforms, which are localized in the apical membranes of tubule epithelium and by UT-B, which is localized in the renal vasa recta. By recycling urea, both are also involved in regulating urine concentration. In ruminants, the UT-A and UT-B have been detected in renal medulla, papilla and pelvis of sheep (MARINI et al. 2004; ARTAGAVEYTIA et al. 2005).
By intestinal UTs, urea can be shifted into the enteric lumen, where it can be hydrolysed into NH₃ and carbon dioxide by urease activity of intestinal bacteria. Subsequently, NH₃ can either be used for microbial protein synthesis or it can be reabsorbed again. In human and rat the UT-B transporter was detected in colon (INOUE et al. 2004; SMITH et al. 2004; INOUE et al. 2005) and in human and mice also the UT-A has been characterized in the intestine (FENTON et al. 2004; STEWART et al. 2004). Also in ruminants, UT-A and UT-B have been detected in the gastrointestinal tract (GIT) (RITZHAUPT et al. 1997; RITZHAUPT et al. 1998; MARINI et al. 2004; STEWART et al. 2005; LUDDEN et al. 2009). Especially in ruminants, facilitated urea transport across intestinal but also ruminal epithelia could play a crucial role for ruminal N recycling mechanisms. The existence of UT-B in the rumen wall was documented in studies in cattle, sheep and goats (MARINI and VAN AMBURGH 2003; MARINI et al. 2004; STEWART et al. 2005; PETERS 2007; LUDDEN et al. 2009; ROJEN et al. 2011).

In addition, in vitro experiments revealed that urea flux across the ruminal wall was increased when goats were fed a diet with low N content (MUSCHER et al. 2010). More recently, these findings were also made in sheep (DORANALLI et al. 2011). Though, higher urea fluxes into the rumen could not be attributed to increased UT-B function here. Also in vivo studies documented that the proportion of blood-derived urea-N in the rumen increased in cows with low N intake (MARINI and VAN AMBURGH 2003). These findings underline the importance of maintaining urea flux into the rumen in the event of unfavourable dietary conditions, such as low N intake.

However, the exact regulation and interplay of renal and gastrointestinal UTs in ruminants, especially during low N intake, is not yet fully understood. It can be assumed that in times of dietary N shortage and accordingly lower plasma urea concentrations, N excretion is minimized by an increase in UT expression. Subsequently, urea reabsorption in the kidneys and urea transport into the rumen is advantageous to improve N availability for rumen microbes in times of dietary N shortage.

1.4 Dietary N intake and electrolyte homeostasis

An adequate supply of protein and amino acids guarantees maintenance of growth in all tissues. Any imbalance in N supply will therefore not only alter pathways related to N metabolism, but also pathways linked to body growth, like energy balance, lipid and glucose
metabolism and electrolyte homeostasis. Since phosphorous (P) and calcium (Ca) are essential for bone mineralization and phosphate (P<sub>i</sub>) is required for energy metabolism (ATP synthesis) and regulation of enzyme activity (phosphorylation), a close connection between N and electrolyte homeostasis seems quite compelling.

In humans and monogastric animals, it was shown that low N intake influenced the function and metabolism of key organs involved in regulating electrolyte homeostasis: the bones, the intestine and the kidneys. When consuming a low protein diet, bone growth, bone resorption and density were altered in rats and humans, but the results were ambiguous. From some studies it was concluded that consuming a high protein diet improved bone mass (DAWSON-HUGHES et al. 2004), from others that high protein intake increased bone resorption (KERSTETTER et al. 1999). Furthermore, in some studies intestinal Ca absorption decreased and in most studies also renal Ca excretion decreased upon low protein intake (ORWOLL et al. 1992; KERSTETTER et al. 1999; DAWSON-HUGHES et al. 2004). Recently varying N intake was shown to alter electrolyte homeostasis also in goats (MUSCHER and HUBER 2010). However, although several studies documented a connection between N intake and electrolyte handling, the causal relationship is still unknown and the exact signalling pathways remained unclear. It seems to be likely that not proteins themselves, but their metabolites, free amino acids, act as initial signals to alter further pathways. Amino acid sensing mechanisms have been found intra- and extracellularly (for review see CONIGRAVE et al. (2008)). Two amino acid sensing proteins were discussed as potential targets mediating the connection between N and electrolyte metabolism, the calcium sensing receptor (CaR) and the mammalian target of rapamycin (mTOR). The potential role of these two proteins regarding electrolyte homeostasis will be discussed later (chapter 7.3).

1.5 The regulatory network of electrolyte metabolism – a brief overview

Alongside amino acid sensing proteins, several other regulatory pathways of Ca and P<sub>i</sub> homeostasis are potentially influenced by low dietary N intake. Electrolyte handling underlies a tightly controlled system with involvement of different organs. Three organ systems are mainly responsible for maintaining balanced Ca and P<sub>i</sub> homeostasis: the bones, the intestine and the kidneys (Figure 2). The interplay of these organs particularly aims to maintain constant plasma Ca and P<sub>i</sub> concentrations. Thereby, electrolyte-related diseases like organ
calcifications or formation of stones, for example in the bladder, are prevented. In the bones, the intestine and the kidneys Ca and Pi absorption and reabsorption, and thereby also Ca and Pi excretion, are regulated to a great extent by hormonal influences. Parathyroid hormone (PTH) is synthesized in the parathyroid gland and it is released when plasma Ca concentration decreases. More recently it could be shown that the signal transduction between plasma Ca concentration and PTH release was mediated by the CaR localized in the parathyroid gland (BROWN et al. 1993). Increasing PTH plasma concentrations affect bone and renal Ca and Pi handling by binding to the respective PTH receptor (PTHR). In the bones this leads to increased release of Ca and subsequently of Pi. In the kidneys, Ca reabsorption from the ultrafiltrate increases, but Pi reabsorption decreases. In addition to factors, such as plasma Ca and Pi concentrations, also increasing plasma PTH concentrations lead to the activation of the renal 1α-hydroxylase. This enzyme catalyzes the transformation of 25-hydroxy vitamin D (calcidiol) to its biologically active form, 1,25-dihydroxy vitamin D₃ (calcitriol). Calcitriol is intracellularly bound to its receptor, the vitamin D receptor (VDR) and this complex can be transported into the nucleus, where it acts as transcription factor. Finally, an elevated calcitriol concentration leads to increased Ca and Pi release from the bones, similar to PTH action, and furthermore, to increased Ca and Pi reabsorption from the ultrafiltrate in the kidneys. In addition, in contrast to PTH, calcitriol also increases Ca and Pi absorption in the intestine. By the concerted action of these mechanisms both PTH and calcitriol increase and in turn normalize plasma Ca concentration. Obviously, for a balanced functioning of these mechanisms, Ca and Pi transport in the kidneys plays an essential role. Therefore, knowledge about renal Ca and Pi transport is summarized in the following.
1.6 Renal Ca and P\textsubscript{i} transport

As it is known from monogastric animals, in the renal distal convoluted tubule and in the connecting duct Ca reabsorption is mediated by the \textit{transient receptor potential channel subfamily vanilloid member 5} (TRPV5). TRPV5-mediated Ca reabsorption is influenced by both the presence of TRPV5 in the apical membrane and by its activity. Targeting to the apical membrane is modulated by the interplay of TRPV5 gene expression and clathrin- and dynamin-mediated internalization and recycling of the channel to the apical membrane. The activity of TRPV5 is mainly controlled by various factors including as klotho, tissue kallikrein, pH or different ion concentrations (for a review see DE GROOT et al. 2008). However, in the kidneys paracellular Ca transport in the proximal tubule and the thick
ascending limb of Henle’s loop was found to play a more central role than transcellular Ca transport (reviewed by HOENDEROP et al. (2005)).

Renal Pi reabsorption, however, is mainly mediated by Pi transporters. Pi reabsorption primarily occurs in the proximal convoluted tubule and plays a central role in controlling whole body Pi homeostasis. Hitherto, three renal sodium (Na)- dependent Pi cotransporters have been described. The \textit{Na-dependent Pi transporter type IIa} (NaPi IIa) is encoded by the \textit{SLC34A1} gene and is localized in the apical membranes of the proximal convoluted tubules. In addition, the \textit{Na-dependent Pi transporter type IIc} (NaPi IIc, \textit{SLC34A3}) and the \textit{Na-dependent Pi transporter type III} (PiT-2, \textit{SLC20A2}) mediate renal Pi transport and are expressed in the apical membranes of proximal convoluted tubules as well. The secondary-active Pi transport through the NaPi IIa, NaPi IIc and PiT-2 is driven by the force of the intracellular directed Na\(^+\) ion gradient. Electrogenic transport of Pi is mediated by NaPi IIa and PiT-2, whereas in contrast, NaPi IIc mediates electroneutral Pi transport (MURER et al. 2000; RAVERA et al. 2007; VIRKKI et al. 2007). The participation of these three transporters in total Pi reabsorption appeared to differ depending on factors like species and age. From rodent models it is suggested that mainly the NaPi IIa mediates renal Pi transport, whereas renal NaPi IIc-mediated Pi transport was found to be predominant in humans and in weaning rodents (BECK et al. 1998; SEGAWA et al. 2002; BERGWITZ et al. 2006; LORENZ-DEPIEREUX et al. 2006). The role of renal PiT-2 in whole body Pi homeostasis has not yet been described and remains subject to clarification.

Several factors regulate proximal tubular Pi reabsorption, such as hormones (PTH, growth factors, estrogen), acid base-load or plasma Pi concentration, whereby the different Pi transporters appeared to be differently influenced by these signals. However, most of the regulatory factors impact Pi reabsorption by altering the abundance of Pi transporters in the apical membranes (BIBER et al. 2009). The regulation of apical Pi transporter abundance is best known for the NaPi IIa and its downregulation by PTH (Figure 3). PTHR activation by PTH binding subsequently activates \textit{protein kinases A} and \textit{C} (PKA and PKC). Through involvement of phosphorylated \textit{extracellular signal regulated kinases 1 and 2} (pERK1/2) the NaPi IIa protein is removed from the membrane via endocytosis. Many different factors can influence this regulatory pathway, including stabilizing effects of different proteins on NaPi IIa in the membrane or intracellular signalling molecules, such as \textit{cyclic adenosine monophosphate} (cAMP). Recently it could be shown that NaPi IIa and PiT-2 were downregulated in an acute response to PTH, whereas NaPi IIc downregulation appeared to be
a long-term effect (PICARD et al. 2010). For a detailed review characterizing regulation of renal Pi transporters see BIBER et al. (2009).

Figure 3: Overview of sodium dependent phosphate transporter type IIa (NaPi IIa) downregulation by parathyroid hormone (PTH) via activation of protein kinases A and C (PKA, PKC) and extracellular signal regulated kinases (ERK)

1.7 Low N intake and electrolyte homeostasis – linked also in ruminants

Due to very efficient N recycling, as described in chapter 1.2, it can be hypothesized that in ruminants, consequences of low N intake for other organ systems is compensated to a certain extent in ruminants. Thus, related consequences for Ca and Pi homeostasis are hypothesized to be marginal in ruminant species. Nonetheless, first indications for a response of electrolyte homeostasis to low N intake were found in goats (MUSCHER et al. 2010; MUSCHER et al. 2011). This response included decreased plasma insulin like growth factor-1 (IGF-1) concentrations, a decrease of plasma calcitriol concentrations and an increase of plasma carboxyterminal cross-linked telopeptide of type I collagen (CTX) concentrations, the latter being a bone resorption marker. These findings confirm results of studies in humans and rodents (DAWSON-HUGHES et al. 2004; DUBOIS-FERRIERE et al. 2011). However, as mentioned before, the regulatory mechanisms, which led to an adaptive response of
electrolyte homeostasis due to low N intake, are neither known for monogastric animals nor for ruminants.

In ruminants, PTH did not affect renal Na-dependent \( P_i \) transport \textit{in vitro}, which was in contrast to studies in opossum kidney cells (PFISTER et al. 1997; SCHRÖDER et al. 2000). Furthermore, also calcitriol-mediated intestinal \( P_i \) transport has been suggested to be regulated different from that in monogastric species (SCHRÖDER et al. 1995). More recently it was shown that electrolyte homeostasis and its regulation differed even between ruminant species with respect to the regulation of intestinal Ca absorption (WILKENS et al. 2012). Therefore, the adaptive response of electrolyte handling to low N intake and its regulation is likely to be different from that in monogastric animals, possibly due to rumino-hepatic N recycling.

1.8 Hypothesis and aim of the present PhD project

It was shown that in growing goats low N intake did not only affect N, but also electrolyte homeostasis. It was hypothesized that renal Ca and \( P_i \) handling are included in the adaptive response of electrolyte homeostasis to low N intake.

Thus, the present PhD project aimed to investigate the adaptation of N, Ca and \( P_i \) homeostasis to low N intake with a focus on renal adaptive responses. Therefore, the impact of low N intake on hormonal regulation of electrolyte homeostasis, on renal urea, Ca and \( P_i \) transporter expression and on N, Ca and phosphorus (P) excretion \textit{in vivo} was examined. Furthermore, by comparing rats and goats, goat-specific adaptation mechanisms to low N intake were underlined. The study was divided into four subprojects to answer the following questions:

1. Do renal UTs contribute to the adaptation of N metabolism and N recycling to low N intake in goats?
2. Is renal electrolyte transport, especially renal \( P_i \) reabsorption, involved in the observed adaptive response of electrolyte homeostasis to low N intake in goats?
3. Does low N intake impact the renal daily electrolyte excretion \textit{in vivo}?
4. Are the observed adaptive responses of electrolyte homeostasis to low N intake specific to ruminants, possibly due to their ability to recycle N?
2 Background information on experimental setup

2.1 Animals

The results of the four subprojects (chapter 1.8) originate from different animal experiments. The first subproject was performed using samples from a total number of 12 goats. A detailed description of the animals is given in the 1st publication. For the second subproject samples obtained from 20 goats were analyzed. A detailed description of these animals was published by MUSCHER et al (2011). The third subproject was performed using a total number of 12 goats, as described more detailed in the 3rd publication. In the fourth subproject data obtained from a study in 16 rats were analyzed. In the 4th publication these data were compared to those from the goats of the third subproject.

Although the data of the present PhD project were obtained from different animal experiments, all trials were conducted according to the same criteria:

During growth N requirements are increased due to a great muscle protein accretion and a high basal energy rate. Hence, it is assumed that in young individuals restricted dietary N intake leads to more pronounced N recycling mechanisms than in adults. Therefore, all studies in the present PhD project were conducted on growing individuals. The experimental trials were started directly after weaning and acclimatization to solid feed. At the time of slaughtering the final body weight of all animals was within the ascending, linear range of the growth curve (data not shown), confirming that all animals were still within the growth phase.

Furthermore, it was summarized by SAINT-CRIQ et al. (2012) and BLAINE et al. (2011) that female sexual hormones can influence Ca and P, homeostasis and also energy metabolism due to milk production and specific female skeletal development. To exclude influences of female sexual hormones, all studies were conducted on male individuals.

Accordingly, all goat experiments were conducted on growing male Saanen-type goats, whereas the rat experiments were conducted on growing male Wistar rats.

2.2 Diets

In all goat experiments, the results of animals from two different feeding groups were compared to each other. In each experiment, one feeding group received a diet containing a moderately low, but not deficient N content. The animals were supposed to be able to cope
with the chosen degree of reduction in N content by urea recycling. The other group received the same diet supplemented with urea to achieve an N supply adequate for goats (GfE 2003). Both diets therefore only varied in their N-content and were isoenergetic.

Regarding the N content, the dietary conditions used for the rat experiment were chosen referring to the diets used in the goat experiment. One group received a diet containing an adequate N content (GV-SOLAS 2004). In the other rat diet the N content was reduced to the same extent as it was reduced in the goat experiment. Detailed components and composition of the diets for both species and a detailed discussion regarding the comparability of the rat and goat diets are given in the 3rd and 4th publication and in chapter 8.1.4.
3 1st publication

Expression of urea transporters is affected by dietary nitrogen restriction in goat kidney

S. Starke, A.S. Muscher, N. Hirschhausen, E. Pfeffer, G. Breves, K. Huber

Published in: Journal of Animal Science (2012) 90:3889-3897
doi: 10.2527/jas.2011-4262
Adaptive responses of calcium and phosphate homeostasis in goats to low nitrogen intake: renal aspects

S. Starke, K. Huber

Accepted for publication in: Journal of Animal Physiology and Animal Nutrition (2013)

doi: 10.1111/jpn.12144
5 3rd publication

Adaptation of electrolyte handling to low crude protein intake in growing goats and consequences for in vivo electrolyte excretion

S. Starke, C. Cox, K.-H. Südekum, K. Huber

Published in: Small Ruminant Research (2013) 114:90-96
doi: 10.1016/j.smallrumres.2013.06.008
Species-specific responses of N homeostasis and electrolyte handling to low N intake: A comparative physiological approach in a monogastric and a ruminant species

S. Starke, C. Cox, K.-H. Südekum, K. Huber

Published in: Journal of Comparative Physiology B (2013), Epub ahead of print
doi 10.1007/s00360-013-0785-9
7 Continuative investigations and unpublished data

The results of the four subprojects provide insights into adaptive responses of Ca and P\textsubscript{i} homeostasis and its regulation to low N intake in goats. However, the findings evoked several subsequent research questions.

It remains unclear how electrolyte homeostasis and especially renal Ca and P\textsubscript{i} handling is exactly regulated in ruminants. Hormonal effects by IGF-1 and calcitriol appeared to play a significant role within the regulation of electrolyte handling in goats (3\textsuperscript{rd} and 4\textsuperscript{th} publication). How far their modes of action differ from those in monogastric animals is not yet understood. However, with respect to the results of the 4\textsuperscript{th} publication, in ruminants renal Ca and P\textsubscript{i} transport and its regulation due to low N intake appeared to differ from those in monogastric animals. The participation of different Na-dependent P\textsubscript{i} transporters in renal P\textsubscript{i} reabsorption might contribute to the different renal adaptation of monogastric animals and ruminants to low N intake. However, the role of for example renal NaPi IIc or PiT-2-mediated P\textsubscript{i} reabsorption in ruminants remains subject to investigation. Also the interplay of Ca and P\textsubscript{i} handling in the kidneys with that in the intestine and in bones during low N intake in ruminants is poorly understood. Furthermore, the molecular link between N metabolism and Ca and P\textsubscript{i} handling remains unknown.

Therefore, continuing studies should include the elucidation of hormonal signals and of renal cellular structures controlling Ca and P\textsubscript{i} transport in monogastric animals and ruminants. The effects of PTH, calcitriol and IGF-1 on the activity of renal NaPi IIa, NaPi IIc, PiT-2 and TRPV5 in goats should be investigated more detailed. In addition, the renal VDR presents a possible target linking hormonal signals to subsequent renal cellular responses. Also the examination of the interplay between kidneys, gut and bones upon low N intake is of great interest due to the close regulatory interactions between these organs. In addition, the signalling between N homeostasis and electrolyte handling needs to be assessed more detailed.

Some of these parameters have already been investigated within the present PhD project and, at least in part, are still under investigation at present. Further experiments were carried out on

- the renal response of Ca and P\textsubscript{i} transport to low N intake by detecting the protein expression of renal PiT-2, TRPV5 and VDR
- the inter-organ crosstalk upon low N intake by measuring the content of Ca and P in chyme and bones
- the molecular link between N metabolism and renal Ca and P handling by detecting the protein expression of renal CaR, mTOR and phosphorylated mTOR (pmTOR)

Furthermore, the question arose how the renal response to particular stimuli, which are caused by dietary N reduction, can be investigated. These stimuli for example include variations in concentrations of hormones, electrolytes and urea, or for example variations in acid base-load. However, based on in vivo conditions it is nearly impossible to investigate the renal response to only one of these stimuli. A suitable method for examining renal responses to particular variables can be to work on isolated, perfused kidneys. Therefore, a preliminary protocol for this method was established within the present PhD project.

As far as possible, the continuative investigations were conducted in a comparative manner in rats and goats. The preliminary results of these experiments are demonstrated and discussed in the following.

### 7.1 Renal response of Ca and P\(_i\) transport to low N intake - PiT-2, TRPV5 and VDR protein expression

Until now, only a few studies have documented the contribution of PiT-2 to renal P\(_i\) reabsorption and to overall P\(_i\) homeostasis. In mice, renal P\(_i\) reabsorption was attributed to PiT-2 activity to a low extent (VILLA-BELLOST A et al. 2009). The PiT-2 was discussed to be a transporter involved in PTH-dependent P\(_i\) reabsorption (PICARD et al. 2010). The role of PiT-2 for renal P\(_i\) reabsorption in goats or other ruminants has not been elucidated to date. As discussed in the 2\(^{nd}\) publication, despite increased renal NaPi IIa protein amounts P\(_i\) uptake into brush border membrane vesicles (BBMV) did not increase in goats with low N intake. This discrepancy was likely to be linked to the activity of P\(_i\) transporters other than NaPi IIa. The PiT-2 can be hypothesized being one of these other transporters. Therefore, it is of special interest to find out whether the renal PiT-2 contributes to P\(_i\) reabsorption in ruminants and whether in the present study the protein expression of renal PiT-2 differed due to low N intake in rats and goats.

As described in the 4\(^{th}\) publication, renal Ca handling appeared to be regulated differently in rats and goats. However, it remains unknown whether these differences can be attributed to transcellular or paracellular Ca transport. In goats, the relation between transcellular and paracellular transport of Ca in the kidney and its regulation is unknown. To further investigate
the transcellular Ca transport via TRPV5 and its adaptation to low N intake in goats and rats, TRPV5 protein expression was determined in this study. Alterations in TRPV5 protein amounts might help clarifying the differences in renal Ca handling, which were observed in the fourth subproject. As described in chapter 1.5 renal Ca reabsorption can be regulated by calcitriol. Upon low N intake in goats, plasma calcitriol concentration tended to decrease. Therefore it seems reasonable that also the expression of its receptor, the VDR, might be altered. Thus, VDR protein was semiquantified in whole cell lysates and in nuclear extracts of renal cortical tissue in both rats and goats upon low N intake.

The detection and semiquantification of PiT-2, TRPV5 and VDR in caprine and rat kidney is still under investigation at present. Therefore, the current state concerning the establishment of the protocols for the use of the antibodies is given.

7.1.1 Materials and Methods

For detection of renal PiT-2, TRPV5 and VDR protein from caprine and rat renal cortices whole cell lysates, brush border membranes (BBM), whole membrane fractions and nuclear extracts were prepared. All preparation procedures are described in detail in the 4 publications.

Protein concentrations of all cell fractions were measured using a commercial protein assay (Bio-Rad) according to the Bradford method (BRADFORD 1976). Samples for detection of PiT-2 were treated with dithiothreitol (DTT) and samples for detection of TRPV5 and VDR with DTT and mercaptoethanol and with or without heat for denaturing the proteins. Samples of each fraction were separated by sodium dodecylsulfate polyacrylamide gelelectrophoresis (SDS-PAGE; 10% gel for PiT-2 detection, 8.1% gel for detection of all other proteins) and transferred onto nitrocellulose membranes. For detection of VDR in nuclear extracts, 15µg protein were loaded per lane, for detection of all other proteins and VDR in whole cell lysates, 30µg protein were loaded per lane. All nitrocellulose membranes were blocked with phosphate buffered saline with tween (PBST) containing 5% fat free milk for 2 h and were incubated at 4°C overnight with primary antibody for protein detection. The PiT-2 protein was detected in BBM, the antibody (anti human PiT-2, Alpha Diagnostics International, San Antonio, TX, USA) was diluted 1:100 in 1% fat free milk in PBST. The specificity of PiT-2 antibody was tested using a blocking peptide. The primary antibody was incubated with the fivefold amount of blocking peptide for 2 h at 37°C and for 4 h at 4°C. The membrane was incubated with the blocked antibody over night at 4°C. TRPV5 protein was
detected in BBM, the primary antibody (anti human TRPV5, AVIVA Systems Biology, San Diego, CA, USA) was diluted 1:1000 in 1% fat free milk in PBST. The VDR protein was detected in whole cell lysates and nuclear extracts. The primary antibody (anti human VDR, AVIVA Systems Biology) was diluted 1:1000 in 1% fat free milk in PBST. Immunoreactivity of the primary antibodies was detected using HRP-coupled secondary antibodies against rabbit (Cell Signaling, Danvers, MA, USA; dilution 1:4000). The band signals were measured using an enhanced chemiluminescence system (Perbio Science GmbH, Bonn, Germany). Semiquantitative analyses of the bands were performed using the software Image Lab (Bio-Rad, Munich, Germany).

7.1.2 Results and discussion

PiT-2 protein was detected as one prominent double band 70 kDa in size and two faint bands 40 and 100 kDa in size in rat kidney. In goat kidney four bands were detected 35, 40, 50, 70 in size and a double band 100 kDa in size (Figure 4). The expected size according to manufacturer was 72 kDa. Treatment of the antibody with blocking peptide resulted in disappearance of all bands in rat kidney and of the 35 kDa, 50 kDa, 70 kDa and 100 kDa bands in goat kidney (Figure 4). TRPV5 protein was detected as a single band 130 kDa in size in rat kidney and 3 bands 40, 70 and 170 kDa in size in caprine kidney (Figure 5). VDR protein was detected in whole cell lysates displayed by 3 bands in rat kidney about 35, 50 and 70 kDa in size, whereas in caprine kidney one prominent band about 50 kDa in size and faint bands 70 and 130 kDa in size were detected (Figure 6A). Detection of VDR protein in nuclear extracts was displayed by one prominent band of 70 kDa in size for both rats and goats, and one faint band 50 kDa in size in rat kidney (Figure 6B).

Further optimization of the protocol is needed to precisely detect PiT-2, TRPV5 and VDR. Afterwards, protein expression can be semiquantified in goats and rats to assess potential differences in protein expression between control and low N fed animals.
Figure 4: Detection of phosphate transporter type III (PiT-2) protein by Western Blot analysis in rat and goat kidney without (left) and with (right) treatment with blocking peptide (b.p.).

Figure 5: Detection of transient receptor potential channel vanilloid type 5 (TRPV5) protein by Western Blot analysis in rat and goat kidney. Samples were treated with mercaptoethanol (Merc.) or with dithiothreitol (DTT) and were heat denatured (+h) or not (-h).

Figure 6: (A) Detection of vitamin D receptor (VDR) protein in whole cell lysates and (B) in nuclear extracts by Western Blot analysis in rat and goat kidney. Samples were treated with mercaptoethanol (Merc.) or with dithiothreitol (DTT) and were heat denatured (+h) or not (-h).
7.2 Crosstalk between the organs - analyses of Ca and P content in bones and intestine upon low N intake

In addition to renal Ca and P handling, skeletal and intestinal Ca and P handling are likely to be influenced by low N intake. Adaptive responses of skeletal metabolism to low protein intake have been reported for monogastric animals and humans (see chapter 1.4), and also in growing goats plasma CTX concentrations increased upon low N intake (MUSCHER et al. 2011). However, these findings could not be confirmed by the present study, because neither in goats nor in rats plasma CTX concentrations were altered by low N intake (3rd and 4th publication). However, the investigation of only one single bone resorption marker does not yield conclusive data about bone turnover or alterations in bone formation. Therefore, alterations of Ca and P contents in bones by low N intake were investigated in this study. Due to technical reasons, only in rats skeletal Ca and P contents were determined and compared between the two feeding groups.

Adaptive responses of intestinal Ca transport to low protein intake have already been demonstrated in studies in rats and humans (ORWOLL et al. 1992; KERSTETTER et al. 1998; GAFFNEY-STOMBERG et al. 2010). Recently, also in goats adaptive responses of intestinal Ca and P fluxes and of intestinal Ca and P transporter expression to a diet reduced in N and Ca content were documented (MUSCHER et al. 2012). Furthermore, the existence of a direct gut-kidney-axis has been postulated by BERNDT et al. (2007). When rats were infused different P contents into the gut lumen a rapid increase in renal P excretion was observed. From these data it may be hypothesized that in the present study low N intake may have altered P content in chyme and thereby also influenced renal P reabsorption. To examine whether low N intake impacts the Ca or P content in the gut, chyme samples were taken from the jejunum and were analyzed for alterations of Ca and P content based on different N supply. Due to technical reasons, these samples could only be taken from goat, but not from rat jejunum.

7.2.1 Materials and methods

Directly after sacrificing, the right femur was removed from each animal and remaining muscles were eliminated. The Ca and P contents in rat femurs were measured in the Institute of Animal Nutrition, University of Veterinary Medicine, Hannover, Germany. In brief, dry matter and crude ash contents were determined and subsequently, acidic hydrolysis of the crude ash was performed. Ca content was measured by atomic absorption spectrometry and P
content was determined photometrically by the ammonium-vanadium-molybdate-method according to official methods established by the Association of German Agricultural Investigation and Research Institutions (VDLUFA 2007).

Chyme samples were taken from goat jejunum directly after slaughtering. The Ca and P contents were determined using the same methods as for detection of Ca and P in rat bones. For statistical analysis a comparison of Ca and P contents in bones and chyme between the two feeding groups was performed using Student’s t-test. Correlations of several variables of N, Ca and P handling with Ca and P contents in bones and chyme were tested by linear regression analyses. $P<0.05$ was set to be significant.

### 7.2.2 Results and discussion

The Ca and P contents in the rat bones did not differ between the two feeding groups. However, linear regression analyses showed that skeletal P content was positively correlated with plasma Pi concentrations and furthermore with renal NaPi IIc expression (Figure 7 A, B). In rats, plasma Ca, Pn, PTH, CTX, IGF-1 and calcitriol concentrations were not changed by low N intake. Therefore, unchanged skeletal Ca and P content were not unexpected. However, the weak correlation between renal NaPi IIc expression and skeletal P content may nonetheless indicate a link between skeletal and renal Pi handling. Hypothetically, increasing NaPi IIc expression enhances renal Pi reabsorption and results in increasing bone Pi content. This link between skeletal and renal Pi handling did apparently not involve the renal NaPi IIa expression (correlation NaPi IIa expression with skeletal P content: $P=0.25$). It appears to be reasonable that skeletal P content and renal NaPi IIc expression can be linked through plasma Pi concentration. Changes in skeletal Pi release or Pi incorporation can lead to changes in plasma Pi concentration. Plasma Pi concentration in turn regulates renal Pi reabsorption. Conversely, also altered renal Pi reabsorption can result in altered plasma Pi concentration and thus in altered skeletal Pi handling. However, the results show that plasma Pi concentration correlated with skeletal P content, but not with renal NaPi IIc protein expression (correlation NaPi IIc protein expression with plasma Pi concentration: $P=0.13$). Thus, the connection of skeletal P content and renal NaPi IIc expression did not appear to be modulated by plasma Pi concentrations. Several other variables, such as hormones or growth factors are known to regulate skeletal and as well renal electrolyte handling, as reviewed by RENKEMA et al. (2008). It can be hypothesized that during growth a greater demand of skeletal Pi incorporation results in increased levels of growth hormones. Increasing levels of growth
hormones in turn can lead to higher NaPi IIc expression and thus to increased renal Pi reabsorption. Besides growth hormones, another class of molecules called phosphatoninns was suggested to link skeletal and renal Pi homeostasis. The phosphatoninns are phosphaturic substances and comprise the fibroblast growth factor 23 (FGF 23), the secreted frizzled related protein 4 (sFRP-4), fibroblast growth factor 7 (FGF-7) and the matrix extracellular phosphor-glycoprotein (MEPE). All these phosphatoninns were shown to impact renal, skeletal and hormonal Pi homeostasis, as reviewed by LAROCHE and BOYER (2005) and by SHAIKH et al. (2008). Possibly, phosphatoninns are also involved in the adaptation of Pi homeostasis to low N intake in goats. However, further research is needed to investigate mechanisms of interactions between skeletal and renal Pi handling.

The Ca content in chyme of goats was not altered, but Pi content decreased with low N intake (Figure 8 A, B). In addition, Pi content of the chyme was negatively correlated with renal NaPi IIc expression and it was positively correlated with plasma IGF-1 concentrations. Also plasma IGF-1 concentration was negatively correlated with the renal NaPi IIc expression (Figure 9 A, B, C). It was documented by BERNDT et al. (2007) that an increase in Pi content in the intestine resulted in rapid increase of renal Pi excretion in rats. In accordance, our data showed a negative correlation between Pi content in chyme and renal NaPi IIc expression. This correlation indicates that also in goats increasing Pi content in chyme was associated with a renal response towards increasing Pi excretion. Furthermore, increasing Pi content in chyme was associated with increasing plasma IGF-1 concentration. Plasma IGF-1 concentrations in turn were negatively correlated with renal NaPi IIc expression. These associations might imply that increasing Pi content in chyme leads to a downregulation of renal NaPi IIc protein expression mediated by plasma IGF-1. Contrary to this assumption, IGF-1 was shown to increase NaPi II-mediated Pi transport in opossum kidney cells (JEHLE et al. 1998), likely due to increased stability of NaPi IIa in the membrane. However, whether IGF-1 impacts NaPi IIc in the same manner, and how IGF-1 influences Pi transport in ruminants, is not known so far.

To summarize, these results imply an interplay of the kidneys with bones and intestine regarding electrolyte handling. Mainly Pi handling and renal NaPi IIc expression seemed to be involved in this interplay. Further research is needed to clarify the regulatory mechanisms of this crosstalk between intestine, bones and kidneys and to clarify goat-specific metabolism in this context.
CONTINUATIVE INVESTIGATIONS AND UNPUBLISHED DATA

Figure 7: (A) Correlation of plasma phosphate (Pi) concentration with skeletal phosphorus (P) content ($r^2=0.3; P<0.05$) and (B) of sodium-dependent Pi transporter type IIc (NaPi IIc) protein expression with skeletal P content ($r^2=0.3; P<0.05$) in rats. Filled squares represent animals of the control group, blank circles represent animals of the low nitrogen (low N) fed group.

Figure 8: (A) Comparison of calcium (Ca) content and (B) phosphorus (P) content in chyme of goats between control and low nitrogen (low N) fed animals. The number in brackets represents the number of animals. * $P<0.05$

Figure 9: (A) Correlation of phosphorus (P) content in chyme with plasma insulin-like growth factor-1 (IGF-1) concentration ($r^2=0.6; P<0.01$), (B) sodium-dependent phosphate transporter type IIc (NaPi IIc) protein expression ($r^2=0.4; P<0.05$) and (C) of plasma IGF-1 concentration with NaPi IIc protein expression ($r^2=0.4; P<0.05$) in goats. Solid squares represent animals of the control group, blank circles represent animals fed low N.
7.3 The molecular link between N metabolism and renal Ca and $P_i$ handling – CaR and mTOR?

Until now the link between N intake and an adaptive response of the electrolyte homeostasis has not been identified. It is conceivable that hormonal signals can link N and electrolyte metabolism. Indeed, N intake was shown to impact the release of hormones, such as insulin or PTH and also of other variables like IGF-1. At least in goats IGF-1 was shown to play a central role within the adaptive response of renal electrolyte handling to low N intake. Due to its involvement in regulating growth, skeletal and as well renal metabolism, IGF-1 can be hypothesized being a hormone connecting N and electrolyte metabolism. However, in addition to hormonal signals, also free amino acids were assumed to be key signals within the interaction of protein and electrolyte metabolism (CONIGRAVE et al. 2008). After ingestion proteins are degraded to peptides or amino acids, and after digestion free amino acids can be released into the blood stream. Accordingly, cellular amino acid sensing mechanisms might play a crucial role in mediating signals obtained from protein intake to electrolyte handling. Amino acids can be sensed extra- but also intracellularly. One of the extracellular amino acid sensors, which have been identified so far, is the CaR. One of the intracellular amino acid sensors is a serine/threonine kinase named mTOR.

7.3.1 The CaR

The CaR consists of seven transmembrane domains, one N-terminal large extracellular domain comprising about 600 amino acids and a cytoplasmatic C terminus (BROWN et al. 1993; GARRETT et al. 1995; RICCARDI et al. 1995; RUAT et al. 1995). As reviewed by CONIGRAVE et al. (2008), the CaR has initially been cloned from bovine parathyroid cells and its expression has been verified in different tissues so far, also including kidney, thyroid C-cells and brain. The CaR possesses binding sites for amino acids and as well for Ca ions. For this reason it is predestinated as a molecular link between protein intake and electrolyte handling. Some groups of amino acids are preferentially bound, others with a lower sensitivity. The intracellular pathways, which are activated subsequently, appeared to depend on the activation of the CaR either by Ca or by amino acids. In general, it was found that signal transduction was mediated by G-protein-coupled processes, by activation of different protein kinases and by changes in intracellular Ca concentrations (VARRAULT et al. 1995; BROWN et al. 1996; RUAT et al. 1996; YE et al. 1996). In the parathyroid gland these mechanisms finally regulate gene expression and secretion of PTH (LEVI et al. 2006). The
CaR is also expressed in the kidneys and attenuating effects of PTH on P\textsubscript{i} transport (BA and FRIEDMAN 2004). Furthermore CaR activity mediated increased VDR expression and resultant decrease in plasma calcitriol concentrations (MAITI and BECKMAN 2007). Due to this action of the CaR on renal electrolyte handling, CaR protein expression was investigated in this study. Furthermore, it should be examined whether CaR protein expression was influenced by low N intake and might have contributed to changes in renal Ca and P\textsubscript{i} handling in goats.

### 7.3.2 The mTOR

The mTOR is a member of phosphoinositide 3-kinase-related kinases (PIKK) and was shown to act as serine/threonine kinase \textit{in vitro} (DENNIS et al. 2001). It is the target of rapamycin, an immunosuppressant factor. Rapamycin is used for several clinical treatments, taking advantage of the inhibiting action on mTOR activity. By the serine/threonine kinase activity mTOR can be autophosphorylated (BROWN et al. 1995) and thereby be activated. Activation of mTOR regulates both activation and inactivation of mRNA translation repressors and thus impacts cell and tissue growth by activation of protein synthesis. Signaling of mTOR includes various factors, such as hormones, growth factors, ATP, G proteins, mitogen-activated protein kinase (MAPK) pathways and several other intracellular signaling proteins (reviewed by HAY and SONENBERG 2004 and WANG and PROUD 2006). Furthermore, it was found that mTOR stimulated the activity of the intestinal Na\textsubscript{dependent} P\textsubscript{i} transporter type IIb (NaPi IIb) (SHOJAIEFARD and LANG 2006). More recently, this stimulating effect was also demonstrated for the renal NaPi IIa (KEMPE et al. 2010). Thus, mTOR might also be a key factor in mediating effects between protein metabolism and renal electrolyte handling. Therefore, renal mTOR protein expression was examined in this study.

To evaluate the impact of low N intake on protein expression of CaR and mTOR, Western Blot analyses were performed. Whereas for the CaR antibody the protocol still has to be modified, the protein expression of renal mTOR could be semiquantified and analyzed for rats and goats.
### 7.3.3 Materials and methods

For detection of renal mTOR, pmTOR and CaR protein from caprine and rat renal cortices whole cell lysates and whole membrane fractions were prepared. The preparations are described in the 4 publications.

Protein concentrations of all cell fractions were measured with a commercial protein assay (Bio-Rad) by a Bradford assay (BRADFORD 1976). Samples for detection of mTOR and pmTOR were treated with DTT for denaturation. For detection of CaR the samples were treated with DTT or, respectively, mercaptoethanol and were heat-denatured. Samples of each fraction were then separated by SDS-PAGE (8.1% gel). For detection of mTOR, pmTOR and CaR 30µg protein were loaded per lane. Afterwards, proteins were transferred onto nitrocellulose membranes. All nitrocellulose membranes were blocked with PBST containing 5% fat free milk for 2 h and were incubated at 4°C overnight with primary antibody for protein detection. The mTOR and pmTOR were detected in whole cell lysates. Primary antibodies (anti human mTOR/ pmTOR, Cell Signaling) were diluted 1: 500 (mTOR detection in goats), 1: 200 (pmTOR detection in goats), 1:400 (mTOR detection on rats) and 1:150 (pmTOR detection in rats). CaR protein was detected in whole membrane fractions and the primary antibody (anti rat CaR, Sigma-Aldrich, St Louis, MO, USA) was diluted 1:400 in 1% fat free milk in PBST overnight. Immunoreactivity of the primary antibodies was detected using HRP-coupled secondary antibodies against rabbit (detection of mTOR and pmTOR, dilution 1:4000) or against mouse (detection of CaR, dilution 1:100000). The band signals were measured using an enhanced chemiluminescence system (Perbio Science GmbH). Semiquantitative analyses of the bands were performed using the software Image Lab (Bio-Rad).

Statistical analyses were performed using GraphPad Prism version 4.0 software (San Diego, California, USA, www.graphpad.com). P-values <0.05 were set to be significant.

### 7.3.4 Results and Discussion

CaR protein detection showed 3 bands in rat kidney 40, 70 and 130 kDa in size, whereas in caprine kidney a single band 70 kDa in size was detected (Figure 10). The expected size according to the antibody manufacturer was 130 kDa. The bands 40 and 70 kDa in size might be cleavage products.

The mTOR protein was detected as one prominent band about 260 kDa in size in both rat and goat kidney. Further faint bands about 220 kDa in size occurred, which might represent
cleavage products (Figure 11 A, B). In both species only the prominent band was semiquantified. The pmTOR protein was detected as one single band about 250 kDa in size for both species (Fig. 11 A, B). For both mTOR and pmTOR, no difference in protein expression between the two feeding groups was detected in both species. The ratio between mTOR and pmTOR also remained unchanged by low N intake in both species, indicating that activation of mTOR by phosphorylation was not influenced by low N intake.

For the CaR antibody a new optimized protocol is needed to quantify and analyze protein content in both rats and goats.

The expression of mTOR and pmTOR was not altered by low N intake in both rats and goats. Also the ratio of pmTOR to mTOR did not vary with N intake in both species.
Therefore, it can be concluded that renal Ca and Pi transport was not regulated by variations of mTOR protein expression or its phosphorylation in rats and goats.

### 7.4 The isolated perfused kidney

The present PhD project aimed to elucidate the adaptation of renal Ca and Pi transport to low N intake. However, the kidneys participate in regulation of various different metabolic pathways and consequently themselves are closely integrated in a concerted interplay of different organ systems. Thus, many different humoral, endocrine and neuronal signals can alter kidney function and renal metabolism. Therefore, adaptive renal responses to low N intake, which were observed in this study, could often not be allocated to one initial signal. This complex regulation of kidney function in vivo impedes the investigation of renal responses to particular stimuli, such as PTH or calcitriol. For example, gaining knowledge about renal responses to variations for example in certain hormone concentrations would be of great interest, especially to further examine ruminant-specific kidney function. One methodological approach allowing exclusively the investigation of particular influences on kidney function is to work on isolated, perfused kidneys.

Working on isolated, perfused kidneys allows investigation of renal responses for example to hormones or other humoral factors, while maintaining all other experimental conditions. Thus, physiological goat-specific renal reactions to changing calcitriol, PTH, IGF-1, Ca or Pi concentrations could be examined. Furthermore, differences between the renal response of control and low N fed animals to these stimuli can be determined. Changes in renal metabolism can be detected by measuring metabolites in the renal venous outflow. Changes in renal function can be detected by measuring glomerular filtration rate and by measuring excretion of different substances in the ureteral outflow. By subsequent tissue preparation and in vitro analyses, such as Western Blotting, immunohistochemical staining or PCR, it is also possible to reveal changes on a molecular level. Therefore, the model of the isolated, perfused kidney could be a very suitable analytical method for gaining detailed insights into renal responses to low N intake in goats.

Research on dissociated kidneys has been described in literature for different species and with different stages of isolation from the complete organism (NIZET 1975). To achieve complete isolation and thereby to achieve complete control over all variables potentially influencing kidney function, an artificial perfusion by a mechanical pump is necessary.
According to STARLING and VERNEY (1925) this kind of kidney perfusion was firstly described by Löbell in 1848.

An aim of this study therefore was to establish a working protocol for the isolation and perfusion of kidneys.

### 7.4.1 Materials and methods

For the methodological establishment, kidneys from swine of the breed *Deutsche Landrasse*, aged about 5 months were used. Directly after stunning with a captive bolt gun and exsanguination the intestine was removed from the abdominal cavity and the kidney, the ureter and the afferent and efferent blood vessels (*Arteria* and *vena renalis*) were dissected (Figure 12A). The blood vessels were ligated with commercial surgical suture. A venous catheter was inserted in each vessel and fixed with a surgical suture. Afterwards, the kidney was completely removed from the abdominal cavity and was embedded in ice cold physiological NaCl solution. A small tube connector was fixed to the ureter for later collecting of ureteral outflows. Through the arterial catheter the kidney was rinsed with 20 mL of 0.002% heparin solution to remove clotted blood from the vascular network (Figure 12B) and to test vessels and catheters for patency. Afterwards the kidney was cautiously rinsed with 10 mL of iced physiological NaCl solution. The same procedure was performed with the second kidney. Both kidneys were then completely embedded in Ringer’s solution at 38°C. The tube connectors and catheters were fixed to commercial rubber tubes (Figure 12C). The arterial tube was connected via an automatic pump to 38°C-warmed Krebs-Henseleit buffer (KHB) solution (containing 117.0 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl₂, 1.2 mmol/L MgCl₂, 1.2 mmol/L NaH₂PO₄, 11.0 mmol/L glucose, 25.0 mmol/L NaHCO₃, gassed with carbogen (95% O₂ and 5% CO₂), pH adjusted to 7.4), which is widely accepted as standard perfusion medium for organs (BAILEY and ONG 1978). The venous and the ureter tube were fixed to sample cups. A schematic illustration of the experimental setup is given in Figure 13. During the connecting procedure attention was paid to the fact that no air bubbles were trapped in the tubes. Perfusion rate was set to 26 mL per minute. From both kidneys samples of the venous outflow were taken after 20, 40, 60, 90, 120, 150, 180, 210 and 240 minutes of perfusion. Ureter samples were collected after 60, 120 and 180 minutes. After a maximum of 5 hours, the perfusion was stopped and tissue samples for hematoxylin and eosin (HE)-staining and immunohistochemical staining were taken. In the outflow samples glucose content was measured using a commercial kit based on the GOD/PAP (glucose oxidase/
phenol + aminophenazone) method (mti diagnostics, Idstein, Germany). Lactate and lactate dehydrogenase (LDH) were measured using commercial kits for detection of lactate and LDH in blood according to the mono-LOX (lactate oxidase)-PAP method and to a kinetic UV test according to the International Federation of Clinical Chemistry (IFCC), respectively (mti diagnostics).

**Figure 12:** (A) Porcine kidney in situ, Arteria renalis (A.r.), Vena renalis (V.R.) and ureter (Ur.) dissected. (B) Perfusion of the kidney through the arterial catheter (art. Cat.) with heparin solution, outflow of clotted blood through the venous outflow (ven.out.). Ureteral catheter (ur.cat.) connected to ureter. (C) Left and right kidney in organ bath connected to arterial, venous and ureteral tubes.

**Figure 13:** Schematic overview of the experimental setup of the isolated perfused kidney. 1 water quench, 2 thermostat, 3 buffer solution, 4 carbogen fumigation, 5 automatic pump, 6 organ bath, 7 arterial tubes, 8 venous tubes, 9 ureteral tubes, 10 sample cups.
### 7.4.2 Results and discussion

After a few minutes of perfusion no blood residue was apparent in the venous outflow. Also peristaltic movement of the ureters could be observed and after 15-20 minutes of perfusion ultrafiltrate was released. After a maximum of 3 hours, edema formation in the perirenal capsule was apparent.

In the samples from venous and ureteral outflow glucose concentrations of about 10-11 mmol/L were measured. However, the renal ultrafiltrate of healthy animals is generally expected not to contain glucose possible explanation for the high glucose level measured in this study might be excessive glucose concentration in the perfusion buffer resulting in an exceeded renal threshold and in subsequent glucosuria. Indeed, standard values of plasma glucose in swine range between 3.9 and 6.5 mmol/L, but higher values about 11 mmol/L may occur under stress conditions (KRAFT and DÜRR 2005). Furthermore, an unphysiologically high perfusion rate would potentially result in increased pressure and consequentially in destruction of renal glomeruli. A glomerular destruction could be identified by histological staining of glomeruli. However, the samples taken for these investigations are yet to be analyzed.

Although the measurements of lactate and LDH in the samples were conducted according to the manufacturer’s protocol, measured values of the standards were too low (data not shown). Therefore, lactate and LDH could not be quantified in the samples. Since the original protocol was specified for plasma samples, measuring lactate and LDH concentrations in KHB solution might not work without modifying the standard protocols for perfusate samples.

In conclusion, a preliminary procedure of working on isolated, perfused kidneys was established. However, several single steps during the process need to be modified or optimized and the complete establishment of this methodological approach requires further investigation.
8 Discussion

8.1 Critical evaluation of the PhD project

8.1.1 First subproject: Impact of low N intake on renal UT expression

In the first subproject the expression and distribution of UTs in caprine kidney was examined. The expression of UT-A1 mRNA in the kidney was significantly increased in the animals fed low N. However, due to technical reasons, UT-A1 was only detected on mRNA, but not on protein level. Therefore it cannot be stated to what extent alterations in mRNA expression reflected alterations in protein expression and therefore in urea reabsorption. Posttranscriptional regulation of UTs, for instance protein folding, trafficking to the apical membrane or removal of UT protein from the apical membrane might occur independently of mRNA expression and might be influenced by low N intake. Thus, for all UTs, UT-A1, UT-A2 and UT-B a semiquantitative analysis of protein amounts present in the renal apical membranes would be desirable for future studies.

8.1.2 Second subproject: Adaptation of renal Ca and Pi handling to low N intake

The findings of the second subproject strongly suggest the kidneys to be involved in the adaptation of Ca and Pi homeostasis to low N intake. Changes in protein expression of key constituents of the renal Pi transport were found. However, from this study it remained unclear whether these observed molecular changes would also impact electrolyte excretion in vivo. Urine samples were taken from the bladder directly after slaughtering to measure urinary Ca and Pi, fractional excretion. However, the values obtained from these measurements likely were of limited validity, because electrolyte concentration in the bladder could have easily been influenced for example by differences in water uptake before slaughtering. Therefore, urine samples obtained from the bladder at one random time point do not provide sufficient evidence to the observed molecular changes for constant urinary Ca and Pi excretion in vivo.

Furthermore, in the second subproject, the animals were not fed individually and thus, feeding of accurate amounts of N, Ca and P for each animal could not be guaranteed. As a consequence, correlations between single variables of nutrient intake and excretion could not be performed. Therefore, in following studies it should be ensured that individual intake and
excretion of nutrients can be determined and that 24h-urine and faeces samples are taken. These demands could for example be met by performing balance trials in metabolic cages.

8.1.3 Third subproject: Impact of low N intake on Ca and Pi handling and excretion in vivo

When comparing repeated experiments from the second subproject, within the third subproject some discrepancies occurred. In the animals of this third subproject protein expression of the key proteins involved in renal Pi reabsorption was not altered by low dietary N intake, as it had been in the second subproject. One possible explanation for this is the fact that in general adaptation to changes in feeding or housing conditions may vary to a great extent between single individuals in goats. An individual capacity for adaptation to low N intake might have resulted in high standard deviation within each feeding group and thus in missing statistical significances in the third subproject. Furthermore, although the same breed of goats was used in both experiments, the animals originated from different breeding lines in the two trials. Therefore, genetic differences are possible factors leading to differences in adaptation to low N intake. In addition, it should be taken into account that in the second subproject a number of 10 animals per group was available, whereas the third subproject was performed with only 6 animals per group. Combined with a high individual adaptability and with variances regarding the genetic background this small number of animals possibly might have contributed to missing statistical significances between the two feeding groups for some of the measured parameters.

Furthermore, the experimental design slightly varied between the two experiments. As implicated above, only in the third subproject feed intake could be controlled individually. Therefore, in the second subproject also an equal energy intake between the two feeding groups was not warranted. This fact is raising the possibility that differences in feed or energy intake may at least in part have led to differences in adaptation of electrolyte handling to low N intake between the two feeding groups in the second subproject. With exactly adjusted individual feed and energy intake in the third subproject these differences could not be detected.

Altogether, it is not possible to assess one particular reason for the differences observed between the results of the two subprojects. From these findings it is concluded that in future experiments with goats an exact adjustment of experimental conditions is necessary to strengthen reproducibility.
8.1.4 Fourth subproject: Impact of low N intake on Ca and Pi handling and excretion in vivo – comparative approach between rats and goats

In the fourth subproject, adaptive responses of N, Ca and P handling based on low N intake were compared between goats and rats, representing a ruminant and a non-ruminant species, respectively. As discussed in the 4th publication, completely identical experimental conditions for the two species cannot be achieved due to large physiological differences in nutritional requirements. Therefore, some differences in the feeding conditions occurred, as discussed in the manuscript. However, it should be considered that the purpose of this study was not to compare adaptation of two species to exactly identical diets, but rather to compare adaptive responses of the two species to dietary N restriction with maintenance of all other species-specific nutritive aspects. Obviously, the data gained from this study are not transferable to other ruminant or monogastric species without limitations. As discussed above, goats appear to be unique in their adaptation to different nutritional or housing conditions. Thus, adaptive metabolic responses in goats do not necessarily have to be similar to those in other ruminants, such as cattle or sheep. Differences in electrolyte handling between goats and sheep have already been characterized (WILKENS et al. 2012). Due to characteristics in physiology and anatomy of rats, also the transfer of the obtained data to other monogastric species has to be assessed cautiously. Nonetheless, specificity of metabolic pathways in ruminants can only be examined by comparison to metabolic pathways in monogastric animals. A lack of data exists concerning such direct comparisons between ruminants and monogastric animals. Therefore, to identify species-specific physiological pathways it would be important to conduct further trials comparing different species under preferably similar conditions, but taking the animals’ physiological requirements into account.

8.2 Adaptation of N, Ca and P handling to low N intake – main findings and discussion

In the present PhD project the adaptation of N, Ca and P, homeostasis to low N intake was investigated with a focus on renal adaptive responses. The hypothesis that renal Ca and P, handling are involved in the adaptive response of electrolyte homeostasis to low N intake was confirmed. The project included investigations on adaptation of renal urea, Ca and P, transport, adaptation of electrolyte excretion in vivo and on adaptation of hormonal regulation
of electrolyte homeostasis. Finally, the specificity of the observed adaptive responses for caprine metabolism was examined by comparing the results to those of a similar study in rats.

The initial questions underlying the four subprojects were investigated in the 4 publications (chapter 3-6) and could be answered as follows:

1. Do renal UTs contribute to the adaptation of N metabolism and N recycling to low N intake in goats?

The adaptation of renal urea transport to low N intake was proven by detecting renal UT-A1, UT-A2 and UT-B mRNA and renal UT-A2 and UT-B protein expression (1st publication). Also the localization of renal UTs in caprine kidney was identified. UT-A2 expression was detected in caprine renal medulla, whereas UT-B expression was detected in medulla and papilla. Furthermore, it could be shown that renal UT-A1 mRNA expression was increased by low N intake. Therefore, the capacity for saving N by renal urea recycling was concluded to be elevated due to low N intake. The adaptation of renal N saving mechanisms is suggested to result in more effective usage of N under these dietary conditions.

Studies aiming to quantify the amounts of recycled urea and of its reuse for microbial protein synthesis emerged over the last years. In cows it was shown that absolute amounts of recycled urea were not altered by low N intake (KRISTENSEN et al. 2010), but it was demonstrated in young goats that the efficiency of rumino-hepatic N recycling was increased due to dietary N reduction (COX 2013). However, the exact amount of urea, which is recycled in the kidneys and subsequently contributes to rumino-hepatic N recycling upon low N intake, still remains subject to investigation.

Furthermore, the specific involvement of the different UT isoforms in renal urea recycling in goats is not fully understood. The regulation of UTs by low N intake appears to differ between goats and monogastric animals (1st publication, INOUE et al. 2005), as also discussed in the 1st publication. These differences provide evidence that urea transport upon low N intake may be regulated species-specifically. Further research on the adaptation of UTs to low N intake in monogastric animals and ruminants is needed to confirm this hypothesis.
2. Is renal electrolyte transport, especially renal Pi reabsorption, included in the observed adaptive response of electrolyte homeostasis to low N intake in goats?

Renal NaPi IIa, PTHR and pERK1/2 protein expression was detected in caprine kidney and was shown to be altered by low N intake (2nd publication). The abundance of NaPi IIa protein in renal apical membranes increased, whereas PTHR and pERK1/2 protein amounts decreased. It can be concluded that on molecular level renal Pi transport was upregulated PTHR-dependently due to low N intake in growing goats.

However, it remains to be discussed why these findings were not reflected by functional studies. The Pi uptake into isolated BBMV was not altered by low N intake. This discrepancy may be attributed to the methodological approach of BBMV studies, as discussed in the 2nd publication more detailed. Furthermore, Pi transporters other than the NaPi IIa may have contributed to Pi reabsorption in the functional studies and may have led to the ambiguous results. Thus, in following experiments also the protein expression of the renal NaPi IIc (3rd publication) and of the renal PiT-2 (chapter 7.1) upon low N intake was investigated. The analysis of PiT-2 expression is still under investigation at present. However, it was shown that the renal NaPi IIc protein expression was not altered by low N intake. Nonetheless, the renal NaPi IIc appeared to play a more important role for Ca and Pi handling upon low N intake than the renal NaPi IIa, as also discussed in the 3rd publication. Further investigations are needed to clarify the significance of renal NaPi IIa, NaPi IIc and PiT-2 activity for Pi reabsorption in growing goats, especially upon low N intake.

The results of the 2nd publication furthermore suggested the NaPi IIa being regulated PTHR-dependently, as it is known from monogastric animals. However, according to SCHRÖDER et al (1995, 2000) the effect of PTH on Ca and Pi handling in goats may differ from that in monogastric animals. No assay was available for PTH measurement in goat plasma in the second subproject. Therefore, the effect of low N intake on plasma PTH concentrations and thus, possible effects of plasma PTH on renal Pi transport could not be examined. PTH-dependent regulation of renal Pi transport in goats therefore remains to be investigated. The isolated, perfused kidneys could be taken as suitable experimental approach for this investigation.
3. Does low N intake impact the renal daily electrolyte excretion \textit{in vivo}?

To further assess the consequences of the adaptation of renal urea and Pi transporter expression, in an additional study on goats fed low N content, daily \textit{in vivo} excretion of N, Ca and P were examined (3\textsuperscript{rd} publication). As expected, daily N excretion decreased due to low N intake, whereas N retention remained unaffected. Unexpectedly, low N intake did not affect daily Ca and P excretion and retention \textit{in vivo}. However, in this new group of animals only renal PTHR, but neither NaPi IIa nor pERK1/2 protein expression was altered. Possible reasons for this discrepancy between the two studies are discussed in chapter 8.1.3. From the results of this third subproject it cannot be stated, whether an increase in renal NaPi IIa expression, as observed in the second subproject, necessarily results in decreased daily P\textsubscript{i} excretion in growing goats.

Although daily Ca and P excretion were not affected by low N intake, an adaptive response of hormonal regulation of electrolyte homeostasis to low N intake was observed. It was displayed by decreased plasma IGF-1 and a trend towards decreased plasma calcitriol concentrations. At least IGF-1 was shown to increase Na-dependent P\textsubscript{i} transport in opossum kidney cells (CAVERZASIO and BONJOUR 1989). However, despite decreased plasma IGF-1 concentrations in the goats NaPi IIa and NaPi IIc expression remained unchanged. Whether the enhancing effect of IGF-1 on Na-dependent P\textsubscript{i} transport also exists in ruminants and may even influence daily renal P\textsubscript{i} excretion, remains to be examined. Also for assessing IGF-1 effects on renal Pi reabsorption the isolated, perfused kidneys could be taken as suitable experimental approach.

4. Are the observed adaptive responses of electrolyte homeostasis to low N intake specific to ruminants, possibly due to their ability to recycle N?

It was supposed that due to effective N recycling possible effects of low N intake on \textit{in vivo} electrolyte excretion and on renal P\textsubscript{i} transporter expression are attenuated in goats or completely differ from those in monogastric animals. Thus, the adaptive responses of Ca and P\textsubscript{i} handling to low N intake as described in the 2\textsuperscript{nd} and 3\textsuperscript{rd} publication were suggested to be goat-specific. It was assumed that effects of low N intake on renal Ca and P\textsubscript{i} handling were more accentuated in monogastric animals with a putative less effective N recycling. However, a comparative study between both species (4\textsuperscript{th} publication) revealed that the effects of low N intake on Ca and P\textsubscript{i} homeostasis were not more accentuated in rats. Similar to the goat
In addition, related to metabolic body size, urinary Ca excretion decreased only in goats, whereas in rats the urinary Ca concentration increased. These findings underline a goat-specific adaptive response of Ca handling to low N intake. The reduced urinary Ca excretion in goats might be based on increased renal Ca reabsorption. However, the analysis of renal TRPV5 protein expression is still under investigation at present (chapter 7.1). Low dietary N intake combined with reduced dietary Ca intake was shown to increase intestinal Ca fluxes in growing goats (MUSCHER et al. 2011). Also in that study plasma IGF-1 concentration decreased and plasma calcitriol concentration tended to decrease. Thus, enhanced Ca reabsorption in goats upon low N intake can be speculated to be attributed to IGF-1 or calcitriol action. Also the differences between Ca handling in rats and goats upon low N intake may be based on the different adaptation of IGF-1 and calcitriol to the dietary changes. However, to assess the underlying mechanisms of this different response to low N intake, Ca handling upon low N intake needs to be investigated more detailed.

In conclusion, the findings of the fourth subproject indicate a goat-specific response of electrolyte handling to low N intake.
Summary and closing remarks

In the present PhD project the adaptive response of N, Ca and P\textsubscript{i} handling to low dietary N intake in growing goats was investigated. A focus was set on renal adaptive responses to the dietary changes. The consequences of low N intake for hormonal regulation of Ca and P\textsubscript{i} homeostasis, for renal Ca and P\textsubscript{i} transporter expression and for Ca and P excretion \textit{in vivo} were examined. Furthermore, specificity of the observed alterations for caprine metabolism was highlighted by conducting comparative studies in rats.

The results showed that N, Ca and P\textsubscript{i} handling were adapted to low N intake, including adaptation of plasma IGF-1 and calcitriol concentrations on the one hand and of urea, Ca and P\textsubscript{i} transport in the kidneys on the other hand. However, low N intake did not affect daily Ca and P\textsubscript{i} excretion \textit{in vivo}. In addition, it was shown that N, Ca and P\textsubscript{i} handling responded differently to low N intake in goats and rats, representing a ruminant and a non-ruminant species. These results confirm the existence of a goat-specific adaptation of N metabolism and of hormonal and renal electrolyte handling to low N intake, possibly based on rumino-hepatic N recycling.

The biological meaning of the adaptation of Ca and P\textsubscript{i} homeostasis to low N intake in ruminants is not known so far. It can be hypothesized that reduced N intake results in reduced growth, including reduced skeletal growth, protein turnover and changes in energy rates. All these metabolic changes in turn can lead to changes in requirements of Ca and P\textsubscript{i}. Therefore, an adjustment of renal Ca and P\textsubscript{i} reabsorption due to low N intake appears being reasonable. However, further investigations are needed to exactly clarify the metabolic pathways connecting N and electrolyte homeostasis in ruminants.

With respect to practical relevance of the results of this PhD project it can be concluded that feeding lower protein content than actually considered to be adequate decreased N emission without impairment of body weight gain, but Ca and P emission were not affected. However, an adaptive response of overall electrolyte homeostasis was triggered by feeding a low N diet. Whether this response might lead to diseases based on electrolyte imbalances as a long-term effect, or if it remains without health impairments still needs to be determined. Investigations on this topic could be of special interest in goats kept in arid or semi-arid regions or in goat breeds which are preferably kept in such areas and which might exhibit very pronounced adaptive responses.
With respect to scientific significance the data allowed gaining first insights into a complex system of adaptive processes caused by low N intake in goats. However, the exact mechanisms of urea recycling and of Ca and Pi handling in goats still need to be examined with respect to specificity of ruminant metabolism. Within this PhD project particularly renal Pi transport was investigated. In future studies also renal Ca transport should be examined in detail, especially because urinary Ca excretion was pointed out to be regulated differently in goats and rats. Furthermore, as already indicated in chapter 7.2, for a detailed understanding of renal electrolyte transport and its regulation the interplay between kidneys and other organs controlling electrolyte homeostasis is essential. To gain a deeper understanding of ruminant-specific adaptive responses of electrolyte handling to low N intake studies comparing ruminant and non-ruminant species can be useful.
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Acknowledgement

I would particularly like to thank…

…Professor Dr. Korinna Huber for her excellent supervision and very good cooperation at any time. I also wish to thank her for the great working atmosphere, for her untiring willingness for discussion, for always being willing to listen and for best support during preparation of manuscripts and publications. Not least, I wish to thank her for allowing lots of freedom and personality.

Furthermore, I wish to thank…

…Professor Dr. Karl-Heinz Südekum for many inspiring conversations, excellent supervision, his support at any time and for many “sunny greetings” per mail.

…Professor Dr. Marion Hewicker-Trautwein for good supervision and always taking time

…Kathrin Hansen and Susanne Hoppe for excellent technical support and help at any time.

…all members of the Institute of Animal Science, University of Bonn, who supported me during the animal experiments.

…Professor Dr. Gerhard Breves and all members of the Department of Physiology, who supported me, for their help and for the nice working atmosphere.

Special thanks are directed to…

…Maria Guschlbauer, Karen Tappenbeck and Ákos Kenez for being the best colleagues I can imagine and for their heartiness, continuous support and helpfulness. They all have become more than just colleagues.
…Yvonne Armbrecht for unremittingly coming up with new, brilliant ideas, for many honest words and for just as much non-verbal communication.

…my parents and Martin for their support, help and belief in me at any time.