Potassium deficiency decreases the capacity for urea synthesis and markedly increases ammonia in rats

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Running head: Potassium deficiency compromises urea synthesis and increases plasma ammonia

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ABSTRACT

Background and Aims: Potassium deficiency decreases gene expression, protein synthesis, and growth. The urea cycle maintains body nitrogen homeostasis including removal of toxic ammonia. Hyperammonemia is an obligatory trait of liver failure, increasing the risk for hepatic encephalopathy, and hypokalemia is reported to increase ammonia. We aimed to clarify the effects of experimental hypokalemia on the *in vivo* capacity of the urea cycle, on the genes of the enzymes involved, and on ammonia concentrations.

Method: Female Wistar rats were fed a potassium free diet for 13 days. Half of the rats were then potassium repleted. Both groups were compared to pair- and free-fed controls. The following were measured: *in vivo* capacity of urea-nitrogen synthesis (CUNS); gene expression (mRNA) of urea cycle enzymes; plasma potassium, sodium, and ammonia; intracellular potassium, sodium, and magnesium in liver, kidney, and muscle tissues, and liver sodium/potassium pumps. Liver histology was assessed.

***Results*:** The diet induced hypokalemia of 1.9±0.4 mmol/L. Compared to pair-fed controls, the *in vivo* CUNS was reduced by 34% ($p<0.01$), gene expression of argininosuccinate synthetase 1 (ASS1) was decreased by 33% ($p<0.05$), and plasma ammonia concentrations were eightfold elevated ($p<0.001$). Kidney and muscle tissue potassium contents were markedly decreased, but unchanged in liver tissue. Protein expressions of liver sodium/potassium pumps were unchanged. Repletion of potassium reverted all the changes.

***Conclusion*:** Hypokalemia decreased the capacity for urea synthesis via gene effects. The intervention led to marked hyperammonemia, quantitatively explainable by the compromised urea cycle. Our findings motivate clinical studies of patients with liver disease.

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Key words: potassium deficiency; urea synthesis; hyperammonemia; rats

New and Noteworthy: Our study provides novel findings of experimental hypokalemia reducing urea cycle functionality and thereby severely increasing plasma ammonia. This is pathophysiologically interesting because plasma ammonia increases during hypokalemia by a hitherto unknown mechanism which may be particular important in relation to the unexplained link between hypokalemia and hepatic encephalopathy.

INTRODUCTION

Hypokalemia is the most common electrolyte disorder occurring in 10-20% of all hospitalized patients (29, 33). More than 98% of the total body potassium is located intracellularly, and the ion is vital for various cell functions. Numerous experiments have proven potassium depletion to decrease gene expression and protein synthesis, thereby reducing growth, enzyme concentrations and enzyme activities in general. Notably, the underlying mechanism has never been clarified (3, 10-12). Whether, in line with these findings, the functionality of the urea cycle is reduced during hypokalemia because of decreased gene expressions of the enzymes involved has, to our knowledge, never been studied.

This specific issue is relevant because plasma ammonia increases considerably during hypokalemia (4, 8). This indicates that urea cycle functionality is reduced, but other mechanisms may also be of importance. Decreased protein synthesis during hypokalemia, especially in skeletal muscles (11), may increase the amount of free amino acids, as well as impair ammonia scavenging in the muscles, thus contributing to elevations in blood nitrogen content (45). In addition, potassium depletion in rats, dogs, and humans stimulates renal ammoniagenesis, leading to increased ammonia excretion in the urine and ammonia release into the systemic circulation, but it is unknown to what extent the kidneys are the source of the hyperammonemia (1, 4, 8, 16, 39). In potassium depleted rats, blood ammonia levels obtained from the renal vein, aorta and hepatic vein were all increased compared to control rats. Importantly, aortic and hepatic venous ammonia levels were comparable in the potassium depleted rats, indicating that the liver did not increase its clearance of ammonia (8).

The urea cycle machinery, which is predominantly located in zone one of the liver acinus, is the major irreversible nitrogen scavenger processing ammonia nitrogen (N) into urea (41, 43). Under normal circumstances, the system can handle large amounts of excess N, and is far from saturated. However, in patients with liver failure, urea synthesis is impaired with the risk of developing hyperammonemia and hepatic encephalopathy (HE), and indeed, hypokalemia appears to aggravate HE in liver cirrhosis (4, 16, 17, 46).

The rate of urea formation by the urea cycle is controlled and limited by the first and the third cycle enzymes, carbamoyl phosphate synthetase 1 (CPS1) and argininosuccinate synthetase 1 (ASS1), respectively. CPS1 is the flux-generating enzyme, and ASS1 is the rate-limiting enzyme as it has the lowest V_{max} among the cycle enzymes. The urea synthesis rate is mainly determined by the blood concentration of its substrate, amino nitrogen, and the substrate-product relationship is regulated by a number of hormones and interleukins (36-38, 41, 42). Furthermore, urea production is dependent on the functional liver mass (21).

It is potentially harmful to experimentally induce hypokalemia in humans, so we used a rat dietary potassium depletion model that resembles clinical cases of hypokalemia.

The aim of this study was to investigate the effects of potassium depletion in rats on the *in vivo* capacity of the urea-nitrogen synthesis (CUNS), on gene expression of the five urea cycle enzymes, and on plasma ammonia concentrations. Also, we measured intracellular sodium and potassium contents in liver, kidney, and muscle tissues, intracellular magnesium contents in liver tissue, and hepatic sodium-potassium pump expression.

METHODS

Animals

Seventy-nine female Wistar rats (Janvier Labs, Le Genest-Saint-Isle, France; body weight 160-180 g) were housed at 20±2^{o}C with a 12-hour on/off light cycle. Three rats were housed in each cage. Food and water intake were recorded every day for every cage, and all rats were weighed 3 times a week. The experimental protocol was approved by the Danish Experimental Animal Inspectorate (no. 2017-15-0201-01250).

Dietary interventions and study design

All rats received standard diet for the first week to acclimatize. Rats were then randomized into 5 groups, *Figure 1*. The first three groups were allocated to a depletion study. Potassium (K^+) depleted (KD) rats, group 1, received a potassium free diet (C1037, Altromin, Lage, Germany) and distilled water (*n=17)*, the pair-fed control (PFCon) rats, group 2, received the same amount of food as consumed by the depleted rats the previous 24 hours (*n=15)*, and the free-fed control (FFCon) rats, group 3, were fed ad libitum (*n=15*). These three groups were kept on their feeding regime for 13 days. All rats received the same diet, and normal serum potassium was secured in control rats by adding KCl to distilled water in a concentration of 250 mmol/L (Sigma-Aldrich, Darmstadt, Germany, CAS No.: 7447-40-7), which corresponds to the content in standard chow. All rats had free access to water. The remaining two groups were placed in a repletion study where they were kept on their feeding regime for 20 days. In the potassium repleted (KD-R) rats, group 4, potassium free diet was given for the first 13 days, and in the remaining 7 days, KCl was added to the drinking water to replete body potassium levels (KD-R) (*n=16*). Pair-fed control rats (PFCon-R), group 5, were fed potassium free diet and distilled water with KCl (250 mmol/L) to follow the potassium modified rats through all 20 days (*n=16*).

Half of the rats in each group were used for *in vivo* measurement of CUNS to study the primary outcome (i.e. changes in CUNS), and the other half for blood and tissue sampling to study secondary outcomes (i.e. gene and protein expressions of urea cycle enzymes, biochemistry including plasma ammonia, concentration of electrolytes in tissues, urea regulatory hormones, and liver histology). The rats fasted the night before the surgical procedures, which were performed between 8 am and 2 pm in a designated room. Based on a pilot study, a sample size of nine in each group was calculated with a power of 80% at a significance level of 0.05 to identify a 50% change in CUNS.

Capacity of Urea Nitrogen Synthesis (CUNS)

Following anesthesia, the rats were nephrectomized performed to avoid urinary excretion. A catheter (Neoflon 0.6 mm) was inserted into the femoral vein for infusion of alanine which was administered according to body weight as a bolus of 0.7-0.8 ml of an 1124 mmol/L solution followed by a constant infusion of 2.3-3.0 ml/h of a 224 mmol/L solution for 60 min. During infusion, blood samples were drawn from the retrobulbary plexus using heparinized micropipettes. After a 20-min equilibration period, blood was sampled (100 µL) for 40 min at 10-min intervals for blood urea and total AAN determination. The alanine infusion was aimed at obtaining a steady-state blood ∝-amino-nitrogen (AAN) concentration between 7.3 mmol/L and 11.6 mmol/L, in which interval urea synthesis approximates its maximum in rats (22). This concentration interval was not achieved in one KD rat, three PFCon rats, three FFCon rats, two KD-R rats, and two PFCon-R rats in which CUNS could not be calculated.

Blood urea was measured by the urease-Berthelot method(13) and AAN by the dinitrofluorobenzene method (18). CUNS was then calculated as the body accumulation of urea corrected for intestinal hydrolysis as previously described (22):

$$CUNS = \frac{\mathrm{d}c_u}{\mathrm{d}t} * 0.63 \times BW * \frac{1}{1 - 0.14}$$

where ($\mathrm{d}c_u/\mathrm{d}t$) is the slope of the linear regression analysis of blood urea on time during steady state, 0.63 × BW (body weight) is the distribution volume of urea, and $1/(1-0.14)$ is the correction factor for intestinal hydrolysis and recycling of ammonia into urea.

In a pilot study, we measured the gut hydrolysis and distribution volume of urea in potassium deficient rats. The values were similar to the standard values used in this study (data not shown).

Blood and tissue analyses

Blood was collected from the retrobulbary plexus using heparinized micropipettes. Blood glucose was measured immediately using a blood glucose meter and test strips from Contour XT (Bayer, Zürich, Switzerland, ref: 84030970 and 84030881). Plasma analyses included potassium, sodium, alanine transaminase (ALT), creatinine, albumin, and ammonia (NH_4^+). Blood for ammonia determination was constantly kept cold, centrifuged within 15 minutes (3000 g, 5 minutes, 4°C) and then frozen on dry ice. Additional blood samples were stored at -80°C for later analyses of glucagon,

IGF-1, corticosterone and insulin using specific ELISA kits. Serum glucagon concentrations were assessed with a specific rat ELISA kit (Mercodia, Uppsala, Sweden, ref: 10-1271-01), serum insulin with an ultra-sensitive rat insulin immunoassay kit (Biorbyt, Cambridge, UK, Cat. No.: orb54820), serum IGF-1 by a specific rat DuoSet ELISA kit (R&D systems, Denmark, Cat. No. DY791), and serum corticosterone with a specific ELISA kit (DRG International, Springfield, NJ, ref: EIA-5186). Tissue samples were obtained from the right lateral lobe of the liver for histology and analyses of electrolytes, mRNA levels of urea cycle enzymes, and protein expression of sodium/potassium pumps (Na,K-ATPase); and from the left kidney and gastrocnemius muscle for analyses of electrolytes. Organs were weighed and slices were immediately snap-frozen in liquid N_2 and stored at -80^{o}C until further analysis.

Approximately 25 mg of liver, kidney and muscle tissues were analyzed for intracellular sodium and potassium contents expressed as µmol/g wet weight. Additionally, liver tissue from KD and PFCon rats was analyzed for intracellular magnesium content expressed as µg/g wet weight. In brief, tissue specimens were blotted on dry filter paper, followed by wet weight determination and homogenization in 0.3 M trichloroacetic acid. After centrifugation and dilution, sodium, potassium, and magnesium contents were directly measured by flame photometry (Radiometer, Copenhagen, Denmark).

The mRNA expression of the urea cycle enzymes was analyzed using qPCR. Liver tissue stored at -80^{o}C was homogenized in TriReagent (Sigma-Aldrich, Ref. No.: 93289). RNA was extracted with chloroform, the aqueous phase was mixed with ethanol, and then the RNeasy® Mini Kit (Qiagen, Manchester, UK, Cat. No.: 74104) was used for purification following the manufacture's guidelines. RNA concentration and purity were determined using the NanoDrop™ 2000 (Thermo Scientific™, Paisley, UK) spectrophotometer. cDNA was retrotranscribed from 1 ug RNA using a QuantiTect Reverse Transcription Kit (Qiagen, Germantown, MD, Cat. No.: 205310). qRT-PCR was performed using hydrolysis probes (Integrated DNA Technologies, Leuven, Belgium) with TaqMan Fast Advanced Mastermix (Thermo Scientific™, Cat. No.: 4444556) on a 7500 FAST termocycler (Applied Biosystems, Thermo Scientific™). The five urea cycle enzymes analysed were CPS1, ornithin transcarbamylase (OTC), ASS1, argininosuccinate lyase (ASL), and arginase (ARG). Relative gene expression for each gene of interest was normalized using the geometric mean of the relative expression of four reference genes (TBP, PPIA, HMBS, HPRT1). Assays for the qPCR analysis were obtained from Integrated DNA Technologies.

Western Blot analyses were performed on liver tissue homogenized in RIPA Lysis and Extraction Buffer (Thermo Scientific™, Cat. No.: 89900) containing protease and phosphatase inhibitors (Sigma-Aldrich). Protein concentrations were determined with BCA Protein Assay Kit (Pierce™, Thermo Scientific™, Cat. No.: 23225). The protein extractions were mixed with ddH_2O, LDS Sample Buffer, and Sample Reducing Agent in calculated amounts (Bolt™, Thermo Scientific™, Cat. No.: B0007 and B0009). Samples were run on Bis-Tris 4-12% gels (polyacrylamide) using a Mini Gel Tank (Bolt™, Thermo Scientific™, Cat. No.: NW04125BOX and A25977). Proteins were transferred to Trans-Blot® Turbo™ PVDF membranes with the Turbo™ Transfer System (Bio-Rad, Copenhagen, Denmark, #1704157). Sufficient protein transfer was checked with Ponceau S solution (Abcam, Cambridge, UK, ab1473593). The membrane was blocked in 5% skimmed milk in TBS-tween20. Membranes were separately incubated with one loading control (GAPDH, Abcam, Cambridge, UK, ab9485, dilution 1:2.500) and an antibody to Na,K-ATPase (Santa Cruz Biotechnology, TX, sc-21712, dilution 1:2.500). The antibodies were diluted in 5% BSA in TBS-tween20. Secondary antibody (Abcam, ab6721, dilution 1:5000) was diluted in 5% skimmed milk in TBS-tween20. Detection of antibody binding was performed with ImageLab and Clarity™ Western ECL Substrate (Bio-Rad). ImageLab was used to quantify expressions of the loading control and the Na,K-ATPase. For the latter, bands detected the ∝-subunit, β-subunit and total complex, and the expression of each band was multiplied to calculate the total density of Na,K-ATPase. Normalization was done by dividing the density of the Na,K-ATPase by the density of the loading control.

Liver histology was assessed by the study hepatic pathologist in routine hematoxylin and eosin stained sections of study livers.

Statistical analyses

Continuous parametric data were analyzed using one-way ANOVA. Normality was checked by QQ-plots and variance homogeneity with Bartlett's test. If the assumptions of normality and/or equal variance were not met, data were log transformed to obtain normality. If this was not achieved, the non-parametric Kruskal-Wallis test was used. When the ANOVA or Kruskal-Wallis test were significant ($p<0.05$), pairwise comparison was done using a Student's t-test or Mann-Whitney U test. Normally distributed data are presented as mean±SD, log-transformed data as median (95% CI), and non-parametric data as median (IQR, Q_1-Q_3). Study data were collected and managed using REDCap electronic data capture tools. Statistical analyses were made in STATA version 15.0.

RESULTS

Animal characteristics

At study start (Day 0) rats weighed on average 194±10 g in all five groups, *Figure 2*. From Day 0 to the day of sacrifice (Day 13), the KD rats lost 0.4±7.7 g, while their PFCon rats gained 12.3±10.4 g, and the FFCon rats gained 35.2±8.3 g ($p<0.001$, KD vs. both control groups). When potassium repletion was induced, the KD-R rats gained 13.9±4.3 g within the first 2 days, while their PFCon-R controls gained 9.4±5.0 g ($p<0.05$). On the day of sacrifice (Day 20), there was no difference in BW between those two groups ($p=0.14$), *Figure 2*. During the feeding regime, a cage with three KD rats consumed on average 23% less food (40.4±5.8 g/day) than the FFCon rats (52.3±5.4 g/day) ($p<0.01$), but drank 33% more water than these controls (103±20 ml/day vs. 69±9 ml/day; $p<0.01$). No adverse events were recorded during the study period.

Biochemistry

Potassium depletion induced severe hypokalemia of 1.9±0.4 mmol/L, *Table 1*. The intervention also lowered plasma Na^+ ($p<0.001$) and increased plasma creatinine ($p<0.001$), ALT ($p<0.05$) and fasting blood glucose ($p<0.05$) compared to PFCons. There was no difference in plasma albumin among the 3 groups in the depletion study. However, plasma albumin in KD rats was significantly lower compared to KD-R rats ($p<0.001$), *Table 1*.

Electrolytes in liver, kidney and skeletal muscle tissue

Potassium depletion reduced potassium content in the kidney and skeletal muscles, but not in the liver. Liver magnesium content was similar in KD and PFCon rats. Sodium content was elevated in the skeletal muscles, but not in the kidney or liver, compared to PFCons, *Table 2.* There were no differences in the expression of the Na,K-ATPase protein between the five groups, *Table 2.*

CUNS

Potassium depletion reduced the *in vivo* CUNS by 34% compared to the PFCons (3.1±0.6 vs 4.7±1.3 µmol/min/100g, $p<0.01$). Repletion of potassium normalized CUNS (5.5±1.3 µmol/min/100g). There was no difference in CUNS between the other four groups, *Figure 3*.

Blood AAN concentration (mmol/L) increased by approximately 15% in KD rats compared to all four groups ($p<0.05$ for all). Baseline blood urea concentration was 15% lower in KD rats compared to PFCons ($p=0.16$), *Table 1*.

Hepatic gene expression

Potassium depletion reduced CPS1 mRNA levels by 20% compared to PFCons ($p=0.10$) and by 28% compared to FFCons ($p<0.01$). The ASS1 mRNA levels were decreased by 33% compared to both PFCons and FFCons ($p<0.05$, both), *Figure 4*. ASL mRNA was decreased by 52% in the KD rats compared to the FFCons ($p<0.001$) but did not differ compared to PFCons ($p=0.2$), whereas ARG mRNA did not differ in any of the groups, *Table 3*.

Plasma ammonia

Potassium depletion induced marked hyperammonemia of 235 (95%CI: 194;287) μmol/L, *Table 1 & Figure 5*. Plasma ammonia was at least 5 times higher in the KD group compared to all the other groups ($p<0.001$), and potassium repletion normalized ammonia levels.

Urea regulatory hormones

Potassium depletion did not change the concentration of insulin, corticosterone, IGF-1, or glucagon in the KD rats compared to the PFCon rats. However, IGF-1 concentrations were decreased in the KD and PFCon groups compared to the other three groups, and insulin and glucagon were reduced in the groups of the depletion study compared to the groups of the repletion study, *Table 4*.

Histological evaluation of rat livers

Routinely stained liver sections from the study rats showed normal architecture, *Figure 6*. There was variable, slight hepatocyte steatosis in animals from all study groups. Livers were otherwise morphologically unremarkable, without inflammation of note, and without evidence of more marked cellular degeneration or necrosis in hepatocytes in any of the acinar zones.

DISCUSSION

The main finding of this study was that the experimental hypokalemia decreased hepatic ureagenesis via reduced gene expression of central urea cycle enzymes. The experimental intervention also induced severe hyperammonemia. Kidney and skeletal muscle potassium declined, whereas liver intracellular potassium remained normal. Potassium repletion restored all these changes.

As expected, the potassium free diet induced severe hypokalemia, which reduced daily food intake and induced growth retardation. The inclusion of pair-fed control animals provides evidence that the observed growth arrest in KD rats can be ascribed to potassium deficiency rather than to reduced energy and protein consumption. For reasons that are unclear, liver and kidney weights (particularly kidney) were increased relative to BW in KD animals. These effects of hypokalemia are in accordance with a number of earlier studies on hypokalemia confirming the validity of the experimental model (11, 14, 15, 20). We used female rats because of their slower growth rate, limiting effects of growth stage. Thus, we did not look for possible gender effects, but the effects of hypokalemia have been studied in both male and female rats with comparable results irrespective of sex (11, 14, 15, 20).

The potassium depletion reduced the capacity for urea nitrogen synthesis by 34%, which we ascribe to the reduced mRNA expressions of urea cycle enzymes. Thus, the gene expression of ASS1, the rate-limiting enzyme of the urea cycle, was lowered by 33%, and there was a trend towards a lower expression of CPS1, the flux-generating enzyme of the urea cycle. We have earlier described such parallels between reduced gene expression and impaired *in vivo* capacity for urea synthesis (19). The decreased cycle capacity implies that less nitrogen can be processed to its irreversibly excreted form as urea. Accordingly, blood concentrations of the overall cycle substrate, AAN, increased significantly in KD rats (3, 11).

There was a striking eightfold increase in plasma ammonia. This hyperammonemia may have resulted from either reduced removal of ammonia or increased production, or a combination. Reduced removal by the urea cycle may explain the hyperammonemia when considering the following stochiometric calculation. Normally, the aqueous phase of a rat is 60%, which constitutes 120 g of water in an animal with a BW of 200 g. The ammonia concentration in the PFCon rats was 31.1 µmol/L corresponding to a total ammonia pool of 3.7 µmol. CUNS was 4.7 µmol/min/100 g = 9.4 µmol/min/rat with a BW of 200 g. Consequently, the relatively small pool of systemic ammonia can

be removed within approximately 20 seconds. When CUNS is reduced by 34%, as was the case in the KD rats, one-third less of the total ammonia pool will be removed per minute, accounting for 1.2 µmol. The ammonia concentration of 235 µmol/L in the KD rats gives a total pool of 28.2 µmol. The excess amount of 1.2 µmol not removed per minute can amount to 28.2 µmol within 28.2/1.2 = 23.5 minutes. This implies that the magnitude and speed of the irreversible ammonia turnover is very sensitive to changes in CUNS and that the decrease in CUNS observed could be the sole explanation for the hyperammonemia.

However, it should be acknowledged that other molecular mechanisms than the urea cycle are involved in keeping serum ammonia within a normal range. Excess ammonia not removed by periportal hepatocytes via urea synthesis is fused with alpha-ketoglutarate to form glutamine facilitated by glutamine synthetase in the perivenular hepatocytes (45). A previous study showed increased glutamine synthetase activity in the liver of hypokalaemic rats, but this did not prevent hyperammonemia as the plasma ammonia concentration was comparable to levels in our study (8).

Hypokalemia may also stimulate increased production of ammonia. The kidneys play a role in systemic ammonia release (32, 40) and hypokalemia results in release of renal ammonia into the systemic circulation and urine of experimental animals, and of potassium depleted patients, both with or without cirrhosis (4, 8, 16, 39, 44). In any case, even with an increased renal ammonia output to the systemic circulation, we would expect excess ammonia to be cleared by a normal functioning liver with its abundant urea synthesis capacity without a major increase in plasma ammonia. The reduced capacity of urea synthesis by hypokalemia impairs the liver's ability to adjust to changing requirements for nitrogen homeostasis, and this impairment may play a vital role in producing hyperammonemia.

Study livers showed no morphological evidence of significant hepatocyte damage. There was some mild steatosis, but this was seen to a similar degree in both test and control rats from all study groups. Thus, there was no evidence that hypokalemia-mediated liver damage might have contributed to the hyperammonemia or other changes observed.

A number of known regulators of urea cycle activity could theoretically be modulated by hypokalemia. Thus, IGF-I and to some extent insulin are down-regulators, and glucagon and corticosterone are up-regulators of urea synthesis. However, concentrations of these hormones were similar in the KD and PFCon rats. Thus, the reduction in CUNS in KD animals cannot readily be explained by changes in these hormones.

As discussed below, liver potassium was preserved, so other intracellular mechanisms might lead to the decreased urea cycle capacity. Dehydration has been shown to reduce urea synthesis capacity (26). We measured diuresis and concentration of urine electrolytes and creatinine in a subgroup of rats (data not shown). The KD rats had a higher urine output compared to PFCon rats, mirroring their higher *ad libitum* water intake because of the thirst effect of low potassium (5). The 24-hour urine excretions of sodium and creatinine were similar in the groups, so we take them to maintain spontaneous normohydration. Thus, we assume that cellular dehydration was not the mechanism of our findings.

Potassium content was preserved in the liver tissue of KD rats as shown previously (20, 23, 24, 28). We found a trend towards an increased amount of liver Na,K-ATPase. Aligned with earlier studies revealing increased gene expression and activity of the Na,K-pumps, these results may suggest that there is an adaptive mechanism to preserve intracellular liver potassium (25, 34, 35). In contrast, in skeletal muscle cells, hypokalemia reduces the number of sodium-potassium pumps (7, 28), consistent with the low contents of potassium and high sodium contents found in this study.

Our data combined with earlier findings suggest that hypokalemia increases production and reduces removal of ammonia. The association between hypokalemia and hyperammonemia may be important in clinical settings, as hyperammonia has been linked to brain dysfunction, especially in hepatic encephalopathy but also in other inflammatory brain diseases, such as Alzheimers disease (2, 6, 9, 27, 30, 31). Our primary study focus was to investigate by which mechanisms hypokalemia and hyperammonemia are linked, and we did not evaluate whether the potassium depleted rats showed signs of cerebral involvement. This is a limitation of our study that in the light of the striking hyperammonemia needs to be pursued in future studies.

Our potassium depletion model quantitatively mimics a frequent human condition, i.e. the gradually developed hypokalemia. We therefore expect our results to be to some extent transferable to human pathophysiology. Probably, hypokalemia in humans also leads to a rise in ammonia, but we do not have extended clinical evidence of it being as marked as in our study rats. The whole-body ammonia dynamics seems to differ between the two species, and the rats usually have much higher plasma ammonia than humans. A reversible reduction in urea synthesis capacity may not be a problem as long as the process is not already restricted by other conditions or diseases, such as acute or chronic liver disease. This should be pursued in future human studies.

Conclusion

In this study, we show for the first time that hypokalemia reduces the capacity for urea synthesis, via reduction in expression of key urea cycle enzymes, resulting in a marked hyperammonemia. We demonstrate that this phenomenon is reversible after potassium repletion. The association between hypokalemia and hyperammonemia may have considerable clinical significance, as ammonia has been linked to brain dysfunction, especially in the form of hepatic encephalopathy in patients with liver failure.

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FIGURE LEGENDS

FIGURE 1: Study design. Three groups were allocated to a 13-day depletion study: group 1) Potassium depleted rats (KD) had free access to potassium free diet and distilled water (*n=8+9*), group 2) pair-fed control rats (PFCon) received the same amount of potassium free diet as consumed by group 1 the previous 24 hours and distilled water with KCl (*n=6+9*), group 3) free-fed control rats (FFCon) had free access to potassium free diet and distilled water with KCl (*n=6+9*). Two groups were allocated to a 20-day repletion study: group 4) Potassium repleted rats (KD-R) received potassium free diet and distilled water for 13 days and then KCl was added to the water for the remaining 7 days (*n=7+9*), group 5) pair-fed control rats (PFCon-R) received the same amount of potassium free diet as consumed by group 4 the previous 24 hours and distilled water with KCl (*n=7+9*). At the end of the feeding regimes, the rats were randomized to undergo *in vivo* determination of CUNS or collection of blood and tissue samples.

FIGURE 2: Mean body weight (g) during feeding regime in potassium deficient rats (KD, *n=17*), pair-fed control rats (PFCon, *n=15*), free-fed control rats (FFCon, *n=15*), potassium repleted rats (KD-R, *n=16*) and pair-fed control rats to the KD-R (PFCon-R, *n=16*). Weights were recorded 3 times a week during feeding regime and at the day of sacrifice (Day 13 for KD, PFCon and FFCon and Day 20 for KD-R and PFCon-R). The line represents the last weighing at the day of sacrifice for KD, PFCon and Con and the first day of repletion for KD-R and PFCon-R. Potassium depletion caused growth retardation and weight loss, and repletion immediately increased and normalized body weight.

FIGURE 3: Capacity of Urea Nitrogen Synthesis in potassium deficient rats (KD), pair-fed control rats (PFCon), free-fed control rats (FFCon), potassium repleted rats (KD-R) and pair-fed control rats to the KD-R (PFCon-R). Dots represents each rat and whiskers indicate mean and SD. KD rats have a significantly lower mean CUNS compared to all 4 groups. * = $p<0.05$, ** = $p<0.01$, *** $p=<0.001$ compared to KD rats.

FIGURE 4: mRNA quantities of urea cycle enzymes normalized to reference genes.
A) mRNA levels of carbamoyl phosphate synthetase 1 (CPS1). Bars represents median with IQR. B) mRNA levels of argininosuccinate synthetase (ASS1) Bars represents mean with SD. Potassium deficient rats (KD, *n=9*), pair-fed control rats (PFCon, *n=9*), free-fed control rats (FFCon, *n=9*), potassium repleted rats (KD-R, *n=9*) and pair-fed control rats to the KD-R (PFCon-R, *n=8*). Asterisks indicates a significant difference (* = $p<0.05$, ** = $p<0.01$) compared to KD rats.

FIGURE 5: Plasma concentrations of ammonia in potassium deficient rats (KD), pair-fed control rats (PFCon), free-fed control rats (FFCon), potassium repleted rats (KD-R) and pair-fed control rats to the KD-R (PFCon-R). Dots represent each rat and whiskers indicate median with 95% confidence interval.

FIGURE 6: Histological evaluation. Liver sections from a potassium depleted (KD) rat (A) and a pair-fed control (PFCon) rat (B) showing similar histological pictures. The architecture in both livers is preserved with portal tracts on the left and hepatic veins on the right. Liver cells in both rats show varying, light, non-specific, steatosis, but are otherwise unremarkable. There is no necrosis or inflammation of note. (Hematoxylin and eosin staining).

TABLES

Table 1

	KD	PFCon	FFCon	KD-R	PFCon-R	One-way ANOVA p-value	Kruskal Wallis p-value
Potassium (mmol/L)	1.9 (±0.4)	3.6 (±0.2)^{c}	3.4 (±0.3)^{c}	3.8 (±0.2)^{c}	3.9 (±0.2)^{c}	<0.001*	
Sodium (mmol/L)	141.0 (±1.3)	143.7 (±1.0)^{c}	143.8 (±0.8)^{c}	142.9 (±1,7)^{a}	142.9 (±0,6)^{b}	<0.001*	
Ammonia (µmol/L)	235.1 (194;287)	28.5 (26;32)^{c} $\oint$	31.2 (27;36)^{c} $\oint$	40.4 (33;45)^{c}	33.1 (30;37)^{c}	<0.001*	
Creatinine (µmol/L)	40 (36;49)	33 (30;33) ^{c}	30 (27;33)^{c}	30 (27;33)^{c}	30 (27;29)^{c}	<0.001*	
Albumin (g/L)	14.7 (±0.9) $\oint$	15.2 (±1.2) $\oint$	15.4 (±1) $\oint$	16.6 (±1.0)^{c}	16.6 (±1.3)^{b}	<0.01*	
ALT (U/L)	37 (35-57)	31 (30-33)^{a} $\oint$	33 (29-34)	37 (34-42)	37 (34-40)		<0.01*
Glucose (mmol/L)	6.7 (±1.0)	5.6 (±0.8)^{a}	5.7 (±1.3)	6.9 (±1.3)	6.6 (±1.0)	<0.05*	
Baseline alfa-amino-nitrogen (mmol/L)	6.6 (±0.6)	5.6 (±0.5)^{a}	5.7 (±0.6)^{a}	5.6 (±0.5)^{b}	5.5 (±0.5)^{b}	<0.01*	
Baseline blood urea (mmol/L)	5.1 (±1.4)	6.0 (±1.0)	5.5 (±0.6)	6.5 (±1.2)	6.0 (±0.7)	0.1	

Table 1: Plasma concentrations.

Plasma concentrations of potassium, sodium, ammonia, creatinine, albumin, and alanine aminotransferase (ALT) and blood glucose concentration, blood alfa-amino-nitrogen and blood urea in potassium deficient rats (KD, *n=9*), pair-fed control rats (PFCon, *n=9*), free-fed control rats (FFCon, *n=9*), potassium repleted rats (KD-R, *n=9*) and pair-fed control rats to the KD-R (PFCon-R, *n=9*). Values are mean±SD, median (95%CI) or median (IQR, Q_1-Q_3). ^{a} = $p < 0.05$, ^{b} = $p < 0.01$, ^{c} = $p < 0.001$ compared to KD rats. $\oint$ = significantly different from KD-R rats ($p<0.05$). * = a significant difference.

Table 2

		KD	PFCon	FFCon	KD-R	PFCon-R	One-way ANOVA p-value	Kruskal-Wallis/ Mann-Whitney p-value
Liver	Na^+ (µmol/g)	32.0 (±2.2)	30.1 (±3.0)	29.9 (±1.5)[a]	27.4 (±3.1)[b]	27.3 (±3.2)[b]	<0.01*	
	K^+ (µmol/g)	89.9 (88.7-92.9)	89.1 (86.9-92.3)	92.1 (89.5-94.0)	89.5 (86.4-95.2)	90.7 (88.4-91.9)		0.6
	Mg^{2+} (µg/g)	250 (250-250)	260 (250-260)					0.3
	Na,K-ATPase	0.54 (±0.19)	0.49 (±0.11)	0.58 (±0.14)	0.44 (±0.13)	0.42 (±0.14)	0.13	
Kidney	Na^+ (µmol/g)	47.6 (±3.3)	48.6 (±5.4)	45.0 (±6.7)	48.6 (±6.7)	47.3 (±3.4)	0.6	
	K^+ (µmol/g)	60.4 (±5.4)	72.7 (±3.8)[c]	73.2 (±7.0)[c]	70.0 (±4.2)[c]	70.3 (±4.3)[c]	<0.001*	
Muscle	Na^+ (µmol/g)	36.6 (33.1;44.7)	24.5 (22.2;27.1)[c]	22.2 (20.1;24.5)[c]	22.2 (20.1;27.1)[c]	24.5 (22.2;30.0)[b]	<0.001*	
	K^+ (µmol/g)	73.0 (71.4-85.7)	99.5 (92.5-104.5)[c]	103.6 (101.7-107.8)[c]	105.3 (100.9-107.0)[c]	103.1 (101.0-109.5)[c]		<0.001*

Table 2: Electrolyte contents in liver, kidney, and muscle tissues and hepatic protein expression of Na,K-ATPase

Sodium (Na^+) and potassium (K^+) contents in liver, kidney and muscle tissues and magnesium (Mg^{2+}) content in liver tissue expressed as concentration per wet weight, and normalized relative hepatic protein expression of Na,K-ATPase in potassium deficient rats (KD, *n=9*), pair-fed control rats (PFCon, *n=9*), free-fed control rats (FFCon, *n=9*), potassium repleted rats (KD-R, *n=9*) and pair-fed control rats to the KD-R (PFCon-R, *n=9*). Values are mean±SD, median (95%CI) or median (IQR, Q_1-Q_3). [a] = $p < 0.05$, [b] = $p < 0.01$, [c] = $p < 0.001$ compared to KD rats. * = a significant difference.

Table 3

mRNA	**KD**	**PFCon**	**FFCon**	**KD-R**	**PFCon-R**	**One-way ANOVA p-value**	**Kruskal-Wallis p-value**
CPS1	0.84 (0.8;0.9)	1.05 (0.9;1.4)	1.17 (1.0;1.4)^{b}	0.96 (0.7;1.0)	1.04 (0.8;1.3)		0.08
OTC	0.84 (±0.2)	0.91 (±0.3)	0.93 (±0.2)	1.36 (±0.3)^{b}	1.27 (±0.4)^{a}	<0.001*	
ASS1	0.73 (±0.2)	1.09 (±0.3)^{a}	1.04 (±0.4)^{a}	1.22 (±0.3)^{b}	1.23 (±0.4)^{b}	<0.05*	
ASL	0.75 (0.6;0.8)	1.07 (0.8;1.1)	1.44 (1.0;2.0)^{c}	1.22 (1.0;1.4)^{b}	1.04 (0.8;1.2)		<0.01*
ARG	1.11 (±0.2)	1.20 (±0.3)	0.90 (±0.4)	0.97 (±0.3)	1.04 (±0.2)	0.32	

Table 3: Hepatic gene expressions.

Normalized relative mRNA levels of the five urea cycle enzymes in potassium deficient rats (KD, *n=9*), pair-fed control rats (PFCon, *n=9*), free-fed control rats (FFCon, *n=9*), potassium repleted rats (KD-R, *n=9*) and pair-fed control rats to the KD-R (PFCon-R, *n=8*). Values are mean±SD or median (95%CI). ^{a} = $p < 0.05$, ^{b} = $p < 0.01$, ^{c} = $p < 0.001$ compared to KD rats. * = a significant difference.

Table 4

	KD	PFCon	FFCon	KD-R	PFCon-R	One-way ANOVA p-value	Kruskal Wallis p-value
Insulin (µg/L)	0.10 (0.02-0.30)	0.34 (0.07-0.69)	0.45 (0.09-1.18)	0.65 (0.41-1.26)[a]	1.30 (1.11-1.50)[b]		<0.01*
Corticosterone (µg/L)	0.19 (±0.10)	0.13 (±0.06)	0.16 (±0.06)	0.18 (±0.03)	0.16 (±0.06)	0.57	
IGF-I (µg/L)	766 (714-799)	782 (735-804)	1029 (990-1084)[c]	993 (926-1051)[c]	1036 (841-1112)[a]		<0.001*
Glucagon (pmol/L)	2.7 (1.7-3.0)	1.63 (1.0-2.8)	4.5 (2.9-6.9)	7.2 (6.7-10.5)[c]	7.1 (4.4-8.0)[c]		<0.001*

Table 4: Hormones regulating urea synthesis.

Concentrations of insulin, corticosterone, insuline-like growth factor I (IGF-I) and glucagon in potassium deficient rats (KD, *n=9*), pair-fed control rats (PFCon, *n=9*), free-fed control rats (FFCon, *n=9*), potassium repleted rats (KD-R, *n=9*) and pair-fed control rats to the KD-R (PFCon-R, *n=9*). Values are mean±SD or median (IQR, Q_1-Q_3). [a] = $p < 0.05$, [b] = $p < 0.01$, [c] = $p < 0.001$ compared to KD rats. * = a significant difference.