Prompt Rise in Urinary Ammonium Excretion Suffices To Mitigate Metabolic Acidosis in an Experimental Animal Model of Severe Normovolemic Hemodilution

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Summary

Recently, we have established a model of severe stepwise normovolemic hemodilution to a hematocrit of 10 % in rats employing three different colloidal volume replacement solutions (Voluven, Volulyte and Gelafundin) that are routinely used in clinical practice at present. We did not see severe dilutional acidosis as to be expected, but a decline in urinary pH. We here looked on further mechanisms of renal acid excretion during normovolemic hemodilution. Bicarbonate, which had been removed during normovolemic hemodilution, was calculated with the help of the Henderson-Hasselbalch equation. The urinary amount of ammonium as well as phosphate was determined in residual probes. The absolute amount of free protons in urine was obtained from the pH of the respective samples. The amount of protons generated during normovolemic hemodilution was approximately 0.6 mmol. During experimental time (5.5 h), distinct urinary ammonium excretion occurred (Voluven 0.52 mmol, Volulyte 0.39 mmol and Gelafundin 0.77 mmol). Proton excretion *via* the phosphate buffer constituted 0.04 mmol in every experimental group. Excretion of free protons was in the range of 10^{-6} mmol. The present data prove that the prompt rise in urinary ammonium excretion is also valid for acute metabolic acidosis originating from severe normovolemic hemodilution.

Key words

Acute metabolic acidosis • Ammonium excretion • Acid-base homeostasis • Normovolemic hemodilution • Renal acid excretion

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Introduction

Metabolic acidosis is defined as a condition in which plasma bicarbonate is reduced to a degree that equals a base excess below -2 mEq/l (Astrup *et al.* 1960, Kraut and Madias 2012). Severe manifestation of this type of acid-base disturbance is associated with increased morbidity and mortality, and therefore with poor clinical outcome (Gunnerson *et al.* 2006, Kraut and Madias 2012). The miserable prognosis can be explained by the fact that acute metabolic acidosis is known to have several deleterious effects, a decrease in cardiac contractility and cardiac output, hypotension due to peripheral vasodilation, and decreased binding of insulin and catecholamines, for instance (Oliva 1970). In Western countries, most cases of acidosis with metabolic rather than respiratory origin are caused by lactic acidosis or ketoacidosis (Kraut and Madias 2012). Nonetheless, in clinical situations that make the administration of large volumes of saline fluids necessary, a metabolic acidosis might occur due to dilutional effects. Dilutional acidosis by definition develops, if the buffer base bicarbonate is diluted due to administration of volume replacement solutions that are devoid of bicarbonate, whereas the

buffer acid carbon dioxide is not (Waters *et al.* 1999). In the clinical setting outlined above, colloidal volume replacement solutions are often used due to their oncotic properties in contrast to pure crystalloid solutions. Concerning their composition, colloidal solutions differ as regards the contained colloid, electrolytes and possibly metabolizable anions.

Recently, we have established a model of severe stepwise normovolemic hemodilution to a final hematocrit of 10 ± 1 % in rats (Teloh *et al.* 2013). For hemodilution, we employed three different approved volume replacement solutions (one per experimental group), which all have been commonly used in clinical routine (Gelafundin, Voluven, Volulyte). Starting at a value of -0.6 mEq/l, current base excess fell to a value of -3.5 mEq/l at the end of the experiment in all groups, indeed indicating the existence of an acute metabolic acidosis, but to a much lesser extent than expected. Despite the severe degree of hemodilution, we did not detect any drop in plasma pH in none of the three experimental groups (Gelafundin, Voluven, Volulyte), instead staying at a rather constant level. To find an explanation for this unexpected finding and since the urinary pH decreased in all groups from an initial value slightly above neutrality to approximately 6.3 in the observation phase, we decided to measure phosphate and ammonium content in residual urine probes of this experiment and compared these values quantitatively with the amount of bicarbonate removed during stepwise severe normovolemic hemodilution.

Methods

We took residual urine probes from our previous experiments (Teloh *et al.* 2013). In those experiments, stepwise normovolemic hemodilution in male Wistar rats (strain WIS/WU, body weight 410-470 g, estimated blood volume of 25-29 ml according to the equation of Lee and Blaufox 1985) until a final hematocrit of 10 ± 1 % using one of the colloidal volume replacement solutions Voluven, Volulyte or Gelafundin was performed. For blood withdrawal and volume replacement, one catheter was placed within the right femoral artery and the right femoral vein each. Per step of dilution, 3 ml of blood were exchanged, followed by a pause interval of 15 min. For urine collection, a catheter was inserted into the bladder and the urine collected hereby pooled for the respective phase of the experiment (initial, phase of dilution and observation phase).

Measurements

For ammonium quantification, capillary electrophoresis was used. For this purpose, a fused silica capillary was employed with an effective length of 50 cm, an I.D. of 75 µm and an O.D. of 375 µm. Samples of initial urine were diluted with ultrapure water 1:50, whereas samples of the phase of dilution as well as observation phase were diluted 1:100. Analysis was performed using a cation analysis kit (ABSciex, Fullerton, USA). Per analysis, the volume was applied using pressure injection. The subsequent separation proceeded using a voltage of 30 kV and normal polarity of the capillary. Indirect detection was performed employing a photo diode array at a wavelength of 200 nm.

Quantification of phosphate in urine samples was realized with the help of a colorimetric test at a wavelength of 340 nm (Daly and Ertingshausen 1972). Here, inorganic phosphate reacts with ammonium molybdate to form an unreduced phosphomolybdate complex. The obtained results had already been corrected for background absorption by individual blank measurements that were automatically subtracted from the sample's absorbance by the analyzer.

Calculations

Bicarbonate having been removed during every single step of normovolemic hemodilution was calculated with the help of the Henderson-Hasselbalch equation on the basis of measured pH and carbon dioxide partial pressure at the respective point of time (Teloh *et al.* 2013). It was assumed, that during the pause interval with a length of 15 min after every step of dilution, a new equilibrium had adjusted.

The absolute amount of free protons in urine, which is responsible for its acidification, was obtained from the pH of the respective sample (Teloh *et al.* 2013). Absolute amounts of ammonium as well as phosphate were calculated taking into account the volumes of the respective urine samples.

Statistical analysis

Experiments were performed with six animals per group. The data are expressed as mean values $\pm$ standard error of the mean (SEM). Comparisons within a single group were performed using repeated measurement two-way independent analysis of variance (ANOVA) followed by a Dunnett *post hoc* analysis. Comparisons among multiple groups were performed

during two-way independent ANOVA followed by a Tukey *post hoc* analysis. A *P* value <0.05 was considered significant.

Results

The absolute amount of ammonium in initial urine varied between 0.012±0.003 mmol (Volulyte group) and 0.021±0.005 mmol (Gelafundin group) (Fig. 1). During the phase of dilution, the absolute amount of ammonium increased to values of 0.194±0.022 mmol, 0.296±0.024 mmol for the Volulyte

and Voluven group, but to 0.455±0.051 mmol for the Gelafundin group. In the observation phase, the values amounted to 0.201±0.030 mmol, 0.160±0.044 mmol and 0.425±0.026 mmol, respectively.

The absolute phosphate amount in initial urine was 0.0004±0.0002 mmol (Voluven group) and 0.0010±0.0004 mmol (Gelafundin group) (Fig. 2). The urinary amount increased to 0.038±0.002 mmol, 0.015±0.007 mmol and 0.032±0.012 mmol (Voluven, Volulyte, Gelafundin) in the phase of dilution and was 0.012±0.003 mmol, 0.022±0.014 mmol and 0.011±0.005 mmol, respectively, in the observation phase.
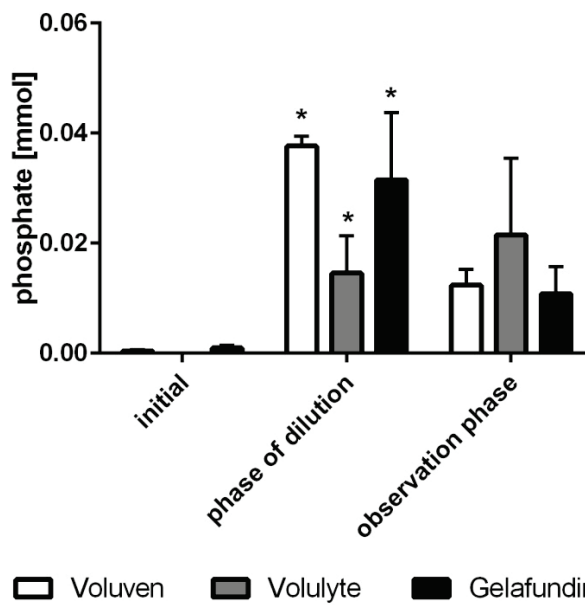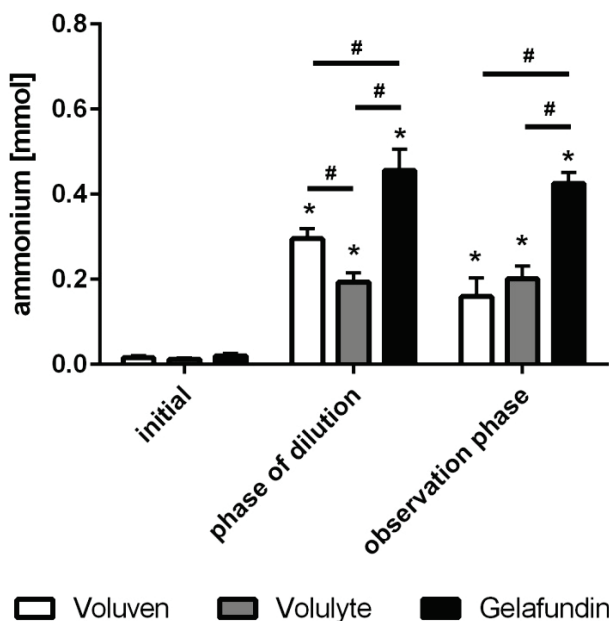
Fig. 1. Ammonia excretion during and subsequent to severe normovolemic hemodilution with Voluven, Volulyte and Gelafundin. After insertion of the urinary catheter, the initial urine was collected. Collection was continued during the phase of hemodilution as well as during the observation phase. Experimental groups differ from each other in the colloidal volume replacement solution employed for normovolemic hemodilution. * p<0.05 compared with the initial value. # p<0.05. Sample size for every group: n=3 at initial due to the small urine volume; n=6 at phase of dilution; n=4 at observation phase due to 2 missing samples.

Fig. 2. Phosphate excretion during and subsequent to severe normovolemic hemodilution with Voluven, Volulyte and Gelafundin. After insertion of the urinary catheter, the initial urine was collected. Collection was continued during the phase of hemodilution as well as during the observation phase. Experimental groups differ from each other in the colloidal volume replacement solution employed for normovolemic hemodilution. * p<0.05 compared with the initial value. Sample size for every group: n=3 at initial due to the small urine volume; n=6 at phase of dilution; n=4 at observation phase due to 2 missing samples.

Table 1. Calculation of excess protons generated during the phase of severe stepwise normovolemic hemodilution with Voluven, Volulyte and Gelafundin whereby the exchanged volume of the last of nine steps of dilution was kept variable to reliably reach the final hematocrit of 10 %.

Volume replacement solution used for hemodilution	Current HCO_3^- [mmol]	Exchanged volume during dilution [ml]	Excess H^+ [mmol]
Voluven	23	26	0.60 ± 0.02
Volulyte	26	26	0.68 ± 0.02
Gelafundin	23	25	0.58 ± 0.04

Calculations were based on the values of six individual animals per group at three different points of time. Therefore, calculations using mean values above may differ.

Discussion

During stepwise normovolemic hemodilution, bicarbonate is removed as a result of blood withdrawal and replacement of blood by non-bicarbonate-containing fluids. In the present experiments, the amount of bicarbonate removed that way approximated 0.6 mmol (mean of the three experimental groups, Table 1). Since every molecule bicarbonate removed left behind one proton, the deficit of bicarbonate equaled the surplus of protons and thus, about 0.6 mmol protons had been generated during the phase of hemodilution.

Generally, three different renal mechanisms exist to excrete or neutralize excess protons (Adam 1981, Rector 1973). First, excretion of free protons into urine. However, since the pH of urine is restricted between 4.5 and 7.5, only small amounts of protons can be excreted this way (Lim 2007, Rector 1973, Rose and Post 2001). In the present experiments, during hemodilution and the subsequent observation time, urinary pH decreased from approximately 7.3 to 6.3 (Teloh *et al.* 2013), indicating an additionally excreted amount of free protons of about $5 \cdot 10^{-6}$ mmol. Hence, the excretion of free protons is quantitatively negligible as compared to the amount of 0.6 mmol protons formed during normovolemic hemodilution.

Second, removal of excess protons with the help of the phosphate buffer system. Attributable to the acidification of urine, HPO_4^{2-} being present in the urine is further protonated to yield $H_2PO_4^-$. However, this buffer system is barely gradable due to limited available phosphate (Lim 2007, Pitts 1973, Rose and Post 2001). In our experiments, compared to the initial value, during and following hemodilution, the absolute amount of phosphate excreted is increased (Fig. 2), but these values are still around the reference of daily phosphate excretion in rats that amounts to approximately 0.35 mmol, i.e. 0.04 mmol within 3 h (Waynforth and Flecknell 1992). In total, excretion of protons *via* further phosphate protonation and increase in phosphate excretion may amount to approximately 0.03 mmol. Thus, as compared to a value in the order of 0.6 mmol protons formed, this mechanism also does not constitute an adequate machinery to excrete the surplus of protons being generated during hemodilution.

Third, enhanced urinary ammonium excretion. Ammonium release from glutamine and hereinafter glutamate takes place in the kidney and is mediated by two enzymes, namely glutaminase I and glutamate dehydrogenase (Damian and Pitts 1970, Fahien and Strmecki 1969, Goldstein and Schooler 1967). In acidotic states, urinary ammonium excretion rises resulting from an interplay between renal glutamine degradation with a concomitant fall in glutamine concentration and increased ammonium excretion due to urinary acidification. In the liver, re-synthesis of glutamine occurs for disposal of waste nitrogen at the expense of ureagenesis. Since nitrogen excretion *via* ammonium does not utilize any bicarbonate, but nitrogen excretion *via* urea does, the saved bicarbonate can neutralize excess protons. Compared with the initial value, the absolute amount of ammonium in urine increased in all groups during experimental time (Fig. 1). According to the literature (Oliver and Bourke 1975), and also reference values of other in-house animals (unpublished data), animals are supposed to excrete approximately 0.42 ± 0.01 mmol ammonium within 24 h under physiological conditions. For the duration of the phase of dilution (3 h) and the subsequent observation phase (2.5 h), this amounts to 0.05 mmol and 0.04 mmol, respectively. Therefore, the amount of ammonium excreted in the respective experimental phases (0.32 mmol and 0.26 mmol, respectively, on average) is distinctly elevated as opposed to the aforementioned physiological baseline conditions. The sum of excreted ammonium during the phase of dilution and observation phase in each group was close to or even above 0.6 mmol. These values clearly show that the vast majority of protons produced during normovolemic hemodilution was compensated by urinary ammonium excretion. However, animals having received Gelafundin as volume replacement excreted in sum (phase of dilution and observation phase) approximately twice as much ammonium as animals of the Volulyte group, being the group that did excrete fewest of all (Gelafundin 0.88 mmol, Voluven 0.46 mmol and Volulyte 0.40 mmol) suggesting a possible correlation of ammonium excretion with the colloid's chemical structure. Gelafundin comprises succinylated gelatin whose terminal carboxyl groups are deprotonated at physiological pH, hence, carrying a negative charge. Due to the fact that succinylated gelatin is rapidly excreted *via* the urine (Teloh *et al.* 2013), it could have favored the excretion of cations like ammonium for electrochemical reasons. In contrast, hydroxyethyl starch, being the colloidal component in Voluven and Volulyte, is uncharged at physiological pH. Based on the increased ammonium excretion in the Gelafundin group, one might even

speculate that infused succinylated gelatin may lead to a disproportional ammonium excretion favoring alkalization. In addition to the colloid, the carrier solution's composition appears to be also of importance as regards the ability to excrete ammonium. Volulyte is the only solution of the three employed here whose carrier solution is plasma-adapted and therefore contains a metabolic anion in terms of acetate. Based on the theory of metabolizable anions (Zander 2009), metabolic acidosis should be antagonized by producing bicarbonate during their metabolization. Taking into account absolute values of ammonium excretion (Fig. 1), our data of the Volulyte group are in line with this theory. In sum (phase of dilution plus observation phase), urine contained only 0.40 mmol ammonium (in contrast to 0.46 mmol and 0.88 mmol of the Voluven and Gelafundin group, respectively) but base excess values were simultaneously closest to neutrality of the three groups (Teloh *et al.* 2013), indicating, that the remaining surplus of protons is neutralized with the aid of bicarbonate originating from acetate metabolism.

In the past, a lot of experimental animal studies were conducted examining ammonium excretion as a response to acute metabolic acidosis (Bishop *et al.* 2010, Goldstein and Schooler 1967, Lee *et al.* 2009, Leonard and Orloff 1955, Oliver and Bourke 1975, Parry and Brosnan 1978, Veverbrants *et al.* 1977), showing,

that urinary ammonia excretion is the primary component of the increase in net acid excretion (Bishop *et al.* 2010, Hamm and Simon 1987, Weiner and Verlander 2013). All of these studies more or less uniformly showed that metabolic acidosis enhanced urinary ammonium excretion within a few hours after acidosis induction. However, metabolic acidosis was always induced by administration of either hydrochloric acid or ammonium chloride. Therefore, these models were highly artificial compared to patients developing acute metabolic acidosis. In contrast, in the present study, normovolemic hemodilution was the employed model to establish an acidosis of dilutional origin which regularly develops in the clinical setting, especially in patients undergoing cardiac surgery with the need of an extracorporeal circulation (Teloh *et al.* 2017). Thus, we here confirmed that urinary ammonium excretion is also the primary component of net acid excretion in the context of normovolemic hemodilution and thus suffices to mitigate metabolic acidosis in terms of a compensatory mechanism. Additionally, differences in the need for urinary ammonium excretion became obvious due to the presence or absence of metabolizable anions in the colloidal volume replacement solution.

Conflict of Interest
There is no conflict of interest.

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