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Renal tubular effects of prolonged therapy with the GLP-1 receptor agonist lixisenatide in patients with type 2 diabetes mellitus

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Abstract

Glucagon-like peptide-1 (GLP-1) receptor agonists (RA's) are well-established glucose-lowering drugs for type 2 diabetes mellitus (T2DM)-management. Acute GLP-1RA-administration increases urinary excretion of sodium and other electrolytes. The renal tubular effects of prolonged GLP-1RA-treatment are, however, largely unknown. In this secondary analysis of a randomised trial, we determined the renal tubular effects of 8-week treatment with lixisenatide 20 μg, a short-acting (prandial) GLP-1RA, versus titrated once-daily insulin glulisine in 35 overweight T2DM-patients on stable insulin glargine background therapy (age 62±7years, HbA1c 8.0±0.9%, estimated GFR >60mL/min/1.73 m^2). After a standardised breakfast, lixisenatide increased absolute and fractional excretions of sodium, chloride, and potassium and increased urinary pH. In contrast, lixisenatide reduced absolute and fractional excretions of magnesium, calcium, and phosphate. At Week-8, patients treated with lixisenatide had significantly more phosphorylated sodium-hydrogen exchanger isoform 3 (NHE3) in urinary extracellular vesicles than those on insulin glulisine treatment, which suggested decreased NHE3 activity in the proximal tubule. A rise in postprandial blood pressure with lixisenatide partly explained the changes in the urinary excretion of sodium, potassium, magnesium and phosphate, and urinary pH. In conclusion, lixisenatide affects postprandial urinary excretion of several electrolytes and increases urinary pH compared to insulin glulisine in T2DM patients after 8 weeks of treatment. This is most likely explained by a drug-induced rise in blood pressure, or direct inhibitory effects on NHE3 in the proximal tubule.

Introduction

Among the several dozen peptides that are secreted from the gut after meal ingestion, glucagon-like peptide (GLP)-1 has been the most widely studied, because of its therapeutic potential in glucose metabolism. GLP-1 is a parent compound mediating the actions of two drug-classes that are now widely used for glucose-lowering treatment of type 2 diabetes mellitus (T2DM), ie. GLP-1 receptor agonists (RA's) and dipeptidyl-peptidase (DPP)-4 inhibitors.[1] Moreover, GLP-1 –in concerted action with other gut-derived hormones and neuronal signals– also regulates water and electrolyte balance in the postprandial state through rapid feed-forward effects on the kidney.[1] A such, it is expected that GLP-1RA's also influence tubular handling of electrolytes. Evidence from both animal and human studies indicate that acute administration of native GLP-1 or a GLP-1RA augments urinary excretion (UE) of sodium.[2-8] Moreover, acute subcutaneous injection or infusion with GLP-1(RA) stimulates UE of chloride, potassium, and calcium in healthy males and T2DM patients.[2,5,8] In contrast to actions in the acute setting, clinical effects of prolonged GLP-1RA-therapy on renal tubular functions and plasma electrolytes in patients with T2DM are largely unknown.[9] Moreover, uncertainties remain regarding the mechanisms by which GLP-1RA's mediate UE of electrolytes, most notably sodium.[1,8,10] Studies in rodents suggest that the GLP-1R is expressed in proximal tubular epithelial cells, and that direct GLP-1R-stimulation inhibits the activity of the sodium-hydrogen exchanger isoform 3 (NHE3) in the proximal tubule.[11,12] However, another study that used a well-validated monoclonal antibody against GLP-1R, was unable to locate the receptors in the proximal tubule of primates.[10]

Lixisenatide, a short-acting (prandial) GLP-1RA, reduces postprandial glucose excursions by decreasing gastric emptying rate and suppressing glucagon secretion.[1] In contrast, prandial insulin decreases postprandial glucose by stimulating peripheral glucose uptake and by inhibiting hepatic glucose production.[13] Recently, we demonstrated that 8-week treatment of lixisenatide versus titrated once-daily insulin glulisine (iGlu) does not affect postprandial renal haemodynamics but leads to a sustained increase in postprandial urinary sodium excretion and urinary pH in overweight insulin glargine treated T2DM-patients.[14] In this secondary analysis, we aimed to detail the magnitude and mechanisms by which lixisenatide affects renal handling of electrolytes in T2DM. Furthermore, in a subgroup of patients, we explored effects of lixisenatide on NHE3 in urinary extracellular vesicles (uEVs). We hypothesised that lixisenatide increases urinary sodium excretion by inhibiting tubular NHE3-activity and also leads to changes in urinary excretion of other electrolytes through secondary (sodium-dependent) effects on various transporters.

Methods

Trial design

This was a secondary analysis of a phase-4, single-centre, randomised, open-label, comparator-controlled, parallel-group, intervention trial. The trial design and methodology have been previously described.[14] The trial was originally designed to determine postprandial effects of lixisenatide versus once-daily iGlu on gold-standard measured renal haemodynamics in T2DM

patients.[14] All patients underwent a 4-week run-in period, during which glucose-lowering treatment was not changed, followed by an 8-week randomised intervention period. The study protocol, protocol amendments and any other protocol-specific documents were approved by the ethics review board of the VU University Medical Center (Amsterdam, The Netherlands). The study complied with the Declaration of Helsinki and Good Clinical Practice guidelines and was registered at ClinicalTrials.gov (ID: NCT02276196).

Study population

Eligible patients were Caucasian, men or post-menopausal women, aged 35-75 years, with T2DM and a body mass index (BMI) >25 kg/m^2. Patients received a stable dose of basal insulin glargine, with or without a stable (ie. >3 months) dose of metformin. Blood pressure was under control (i.e. <140/90 mmHg) and hypertension and/or albuminuria was treated with at least a stable dose of a renin-angiotensin-system (RAS)-inhibitor. Exclusion criteria included estimated glomerular filtration rate (eGFR) <60 ml/min/1.73 m^2, urinary retention (bladder ultrasonography at screening-visit was performed to objectively assess bladder emptying), active glomerulonephritis, current urinary tract infection, or use of diuretics that could not be stopped 3 months prior to and during the intervention period.

Study protocol and measurements

Patients were randomised (1:1) to lixisenatide or iGlu. Lixisenatide (Sanofi Aventis, Paris, France) was initiated at a dose of 10 µg once-daily for 2 weeks, injected subcutaneously ~30 minutes before breakfast, followed by a dose-increase to 20 µg once-daily for the remainder of the study. iGlu (Sanofi Aventis, Frankfurt am Main, Germany) treatment was started with 2 units/day once-daily, injected <15 minutes before breakfast, followed by self-adjustment of daily-dose according to a prespecified treat-to-target algorithm (i.e. 2-hour postprandial self-monitored blood glucose target ≥ 5.0 and ≤ 8.0 mmol/L).[14]

Patients were instructed to control their intake of protein (1.5–2.0 g/kg/day) and sodium chloride (9–12 g/day; ~150-200 mmol sodium) 2 days prior to the testing day, to abstain from alcohol and vigorous physical activity for ≥ 24 hours, and not to use nicotine or caffeine for ≥ 12 hours. Patients arrived in the fasting state, after consuming 500 mL of tap water to stimulate diuresis, and were requested to delay all morning medications except for metformin and thyroid hormone replacement therapy. A venous cannula was inserted in an antecubital vein of the dominant arm for infusion of the renal tracer substance, and in the non-dominant arm for venous blood sampling. Before the renal tests, blood samples were taken to determine plasma electrolytes, urea, angiotensin-II, aldosterone and metabolic parameters.[14] In addition, a spot urine specimen was collected to measure electrolytes, urea, osmolality, creatinine, pH and glucose.

Then, the renal protocol commenced by infusing inulin (Inutest®, Fresenius Kabi Austria GmbH, Graz, Austria) (Figure 1). After 35 minutes, patients consumed a standardised mixed breakfast, which consisted of bread with cheese and jam, and milk (416 kcal; 14 g fat, 48 g carbohydrates and 22 g protein), together with 1.5 g of acetaminophen in suspension form

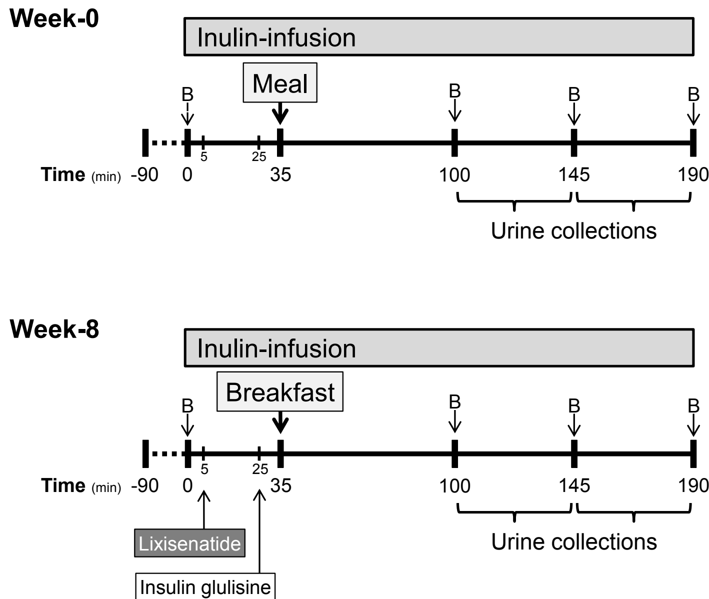

Figure 1. Study Design. Abbreviations: B, blood sampling for plasma electrolytes and urea.

to measure gastric emptying rate.[14] After the fasting measurements at Week-8, patients administered lixisenatide or iGlu 30 and 10 minutes before breakfast, respectively.

From minute-100, urine was collected by spontaneous voiding for two periods of 45 minutes, in which electrolytes, urea, osmolality, creatinine, pH, glucose and inulin were measured. Blood samples were taken before and after each urine collection period to measure electrolytes, urea, creatinine and inulin, while hematocrit, angiotensin-II and aldosterone were determined at the midpoint of the two urine collection periods. In addition, we measured meal-induced changes in blood glucose, serum insulin and acetaminophen, and plasma glucagon.[14] Plasma and urine electrolytes and urea, and urinary glucose and osmolality were analysed at our in-house Department of Clinical Chemistry by conventional methods, while other laboratory analyses have been described.[14] The amount of oral water intake to stimulate diuresis during the testing day and the total sodium load administered through flushing of the intravenous lines has been described.[14] Body water percentage was measured before and during the renal tests, directly after bladder emptying, using single-frequency bioelectrical impedance (BF-906, Maltron International Ltd, Essex, UK). Mean arterial pressure (MAP) was measured by an automated oscillometric device at baseline and minute-120.[14]

Isolation and analysis of urinary extracellular vesicles

From the first 20 patients that completed the study, from whom sufficient urine for uEV analysis was stored, we selected 5 patients randomised to lixisenatide and 5 randomised to iGlu who had the highest response in postprandial urinary sodium excretion from baseline to Week-8 for further exploratory analyses.[14] uEVs of fasting and post-breakfast urine samples at Week-8

were isolated using differential ultracentrifugation. First, urine was centrifuged at 17,000 x g for 20 minutes to remove larger vesicles and debris. This step was repeated on the pellet with dithiothreitol to disrupt Tamm-Horsfall networks to enhance yield.[15] Then, the samples were ultracentrifuged at 200,000 x g for 120 minutes. The pellets were solubilized in Laemmli buffer and heated for 10 minutes at 60°C. Immunoblotting was performed using 4-20% precast polyacrylamide gels (Bio-rad, USA). We used antibodies against total NHE3 (StressMarq), serine 552 phosphorylated NHE3 (pS552, Santa Cruz, referred to in this article as pNHE3) and CD9 (Santa Cruz). The amount of sample loaded was normalised by the urinary creatinine concentration. Technical replicates were not performed because of the limited amount of protein sample.

Statistics

Urinary flow and glomerular filtration rate (GFR; which was calculated from inulin clearances, based on timed urine sampling) were corrected for body surface area.[14] Postprandial fractional excretion was calculated using inulin as reference substance, while creatinine was used as reference substance to calculate fasting fractional excretion as well as the difference in postprandial and fasting fractional excretion (i.e. meal-induced effects). Postprandial fractional excretion, absolute UE and plasma levels of electrolytes and urea were averaged over the two urine collection periods. The time-averaged mean during both renal clearance periods was calculated for blood glucose, serum insulin and plasma glucagon. The net area under the curve (AUC) was calculated for acetaminophen.[14] Statistical analyses were performed in the per protocol population using SPSS Statistics for Windows, V22.0 (IBM Corp, Armonk, NY). Within-group comparisons were analysed using paired T-tests (for normally distributed data) or Wilcoxon signed rank tests (for not normally distributed data). The Mann-Whitney test was used to assess differences between lixisenatide and iGlu in postprandial and meal-induced urinary glucose excretion. Multivariable linear regression models were used to examine effects of lixisenatide compared to iGlu, in which corresponding baseline-values were added as covariate to correct for possible between-group differences at baseline. We also explored whether changes in postprandial or meal-induced fractional excretions of electrolytes and urinary pH were statistically explained by differences in predefined variables that we considered of influence (i.e. blood pressure,[8,14,16] blood glucose, serum insulin, plasma glucagon,[14] angiotensin-II and aldosterone), by adding the Week-0 to Week-8 difference of the explanatory variable as covariate to the model. A variable was considered of influence when the effect size (exemplified by the regression coefficient) was reduced by the arbitrary cut-off point of >15%. A T-test was used to analyse western blot data of uEVs of the subgroup. Statistical significance was considered at a two-sided α-level of < 0.05. Data are presented as median [IQR] or baseline-corrected mean treatment-difference ± SEM, or otherwise as stated.

Results

Of the 40 patients randomised, 35 were included in the final analyses (N=17 randomised to iGlu, N=18 randomised to lixisenatide).[14] Demographic and clinical characteristics of the two treatment groups were well balanced (Table 1).[14]

Metabolic, hormonal and haemodynamic effects

Treatment-induced changes in metabolic, hormonal, renal and systemic haemodynamic parameters have been described previously.[14] HbA_{1c} reduced from baseline to Week-8 by 0.8±0.1% with lixisenatide (p<0.001) and by 0.6±0.1% with iGlu (p=0.001); there was no between-group difference (p=0.897). Change from baseline to Week-8 in fasting glucose did not differ between- or within treatment-groups. Time-averaged postprandial glucose decreased in both groups (p<0.001), with a greater effect of lixisenatide compared to iGlu (-1.8±0.5 mmol/L, p=0.002). Lixisenatide compared to iGlu lowered time-averaged postprandial insulin (-71±36 pmol/L, p=0.057), glucagon (-8.0±2.6 pmol/L, p=0.005), and acetaminophen AUC (-748±127 mg/L/3.1hr, p<0.001); lixisenatide also reduced these measurements within-group (all p≤0.001). Lixisenatide increased meal-induced aldosterone within-group (from -18.4 [-37.2 to -8.6] to -4.3 [-18.5 to 14.3], p=0.015), while postprandial or meal-induced aldosterone or angiotensin-II did not change compared to iGlu.[14] From baseline to Week-8, there was no within- or between-

Table 1. Baseline characteristics

Variables	Insulin glulisine (N=18)	Lixisenatide (N=17)
Age, years	61 ± 7	62 ± 7
Male, *n* (%)	11 (61)	12 (71)
Bodyweight, kg	95.8 ± 17.7	99.2 ± 15.2
Body mass index, kg/m^2	31.5 ± 4.1	31.4 ± 4.1
Diabetes duration, years	14 ± 8	11 ± 5
HbA_{1c}, %	7.8 ± 0.8	8.3 ± 0.8
HbA_{1c}, mmol/mol	62 ± 9	67 ± 9
Fasting plasma glucose, mmol/l	7.3 ± 2.1	6.7 ± 1.8
eGFR (CKD-EPI 2009), ml/min/1.73m^2	82 ± 12	89 ± 11
UACR, mg/mmol*	1.53 [1.24-3.17]	0.94 [0.42-2.96]
Systolic blood pressure, mmHg	138.1 ± 17.7	129.7 ± 12.9
Diastolic blood pressure, mmHg	76.8 ± 9.9	75.0 ± 8.9
Mean arterial pressure, mmHg	99.8 ± 14.0	94.7 ± 9.6
RAS inhibitor use, *n* (%)	11 (61)	11 (65)
· ACE inhibitor use, *n* (%)	6 (33)	8 (47)
· ARB use, *n* (%)	5 (28)	3 (18)

Data are mean ± SD, median [IQR] or N(%). Using appropriate tests, there were no significant between-group differences. *UACR was <3 mg/mmol in 27 patients. UACR was ≥3 mg/mmol in 8 patients (5 randomised to insulin glulisine and 3 to lixisenatide). Abbreviations: ACE, angiotensin-converting enzyme; ARB, angiotensin-II receptor blocker; CKD-EPI, chronic kidney disease epidemiology collaboration as described[1]; eGFR, estimated glomerular filtration rate; HbA_{1c}, glycated haemoglobin; RAS, renin-angiotensin-system; UARC, urinary albumin/creatinine ratio.

group difference in GFR following the breakfast, while postprandial MAP increased compared to iGlu (9±2 mmHg, p=0.001) and within-group (p=0.002).[14]

Meal-induced effects on plasma electrolytes and their excretion

At baseline, mean fasting plasma electrolyte and urea concentrations were in the normal range (Figure 2 and Table 2). At baseline in all patients, plasma calcium (p<0.001) and phosphate (p=0.038) increased following meal-ingestion, while plasma sodium, chloride and urea decreased (all p<0.001) (Figure 2). Moreover, increases were observed in meal-induced fractional excretions of magnesium, phosphate, urea, and calcium (all p<0.001). A trend towards an increase in meal-induced fractional sodium excretion was observed (p=0.09) (Figure 2). Also, the meal increased UE of glucose (p=0.002). In contrast, decreases were observed in meal-induced fractional excretion of chloride and urinary pH (both p<0.001), and a tendency towards decreased meal-induced fractional potassium excretion (p=0.052).

Treatment-induced effects on plasma electrolytes and their excretion

In the fasting state, there were generally no between- or within changes in plasma electrolyte or urea concentrations from baseline to Week-8; only iGlu increased fasting plasma magnesium within-group (Table 2). Postprandially, lixisenatide increased plasma sodium (+1.8±0.7 mmol/L, p=0.011) and calcium (+0.05±0.02 mmol/L, p=0.027) compared to iGlu. There were no between-group differences in plasma chloride, potassium, magnesium, phosphate or urea concentrations postprandially; only lixisenatide increased plasma chloride and iGlu increased plasma magnesium (within-group) (Table 2).

From baseline to Week-8, there were no changes in fasting fractional excretion of electrolytes or urea, UE of glucose or urine osmolality (p>0.05; data not shown). From baseline to Week-8, lixisenatide compared to iGlu increased fractional excretions of sodium, chloride and potassium, and urinary pH after the meal (Figure 3A). Lixisenatide also increased absolute UE of chloride and potassium (Figure 3B). In contrast, lixisenatide compared to iGlu reduced fractional excretions of magnesium, calcium and phosphate postprandially, while fractional excretions of calcium (p=0.08) and phosphate (p=0.052) tended to decrease after the meal (Figure 3A). Lixisenatide also decreased absolute UE of magnesium (Figure 3B).

Lixisenatide increases pNHE3 in urinary extracellular vesicles

At Week-8 in a subgroup of patients, pNHE3 in uEVs in the fasting state was significantly higher in patients using lixisenatide compared with iGlu (p=0.02); total NHE3 demonstrated a similar trend but did not reach statistical significance (Figure 4). Lixisenatide did not change post-meal expression of pNHE3 or total NHE3 in uEVs (Figure 4).

Determinants of treatment-induced renal tubular effects

Changes in MAP partly explained changes in meal-induced fractional excretions of sodium (reduction in the regression coefficient of 16%), potassium (52%), magnesium (28%) and

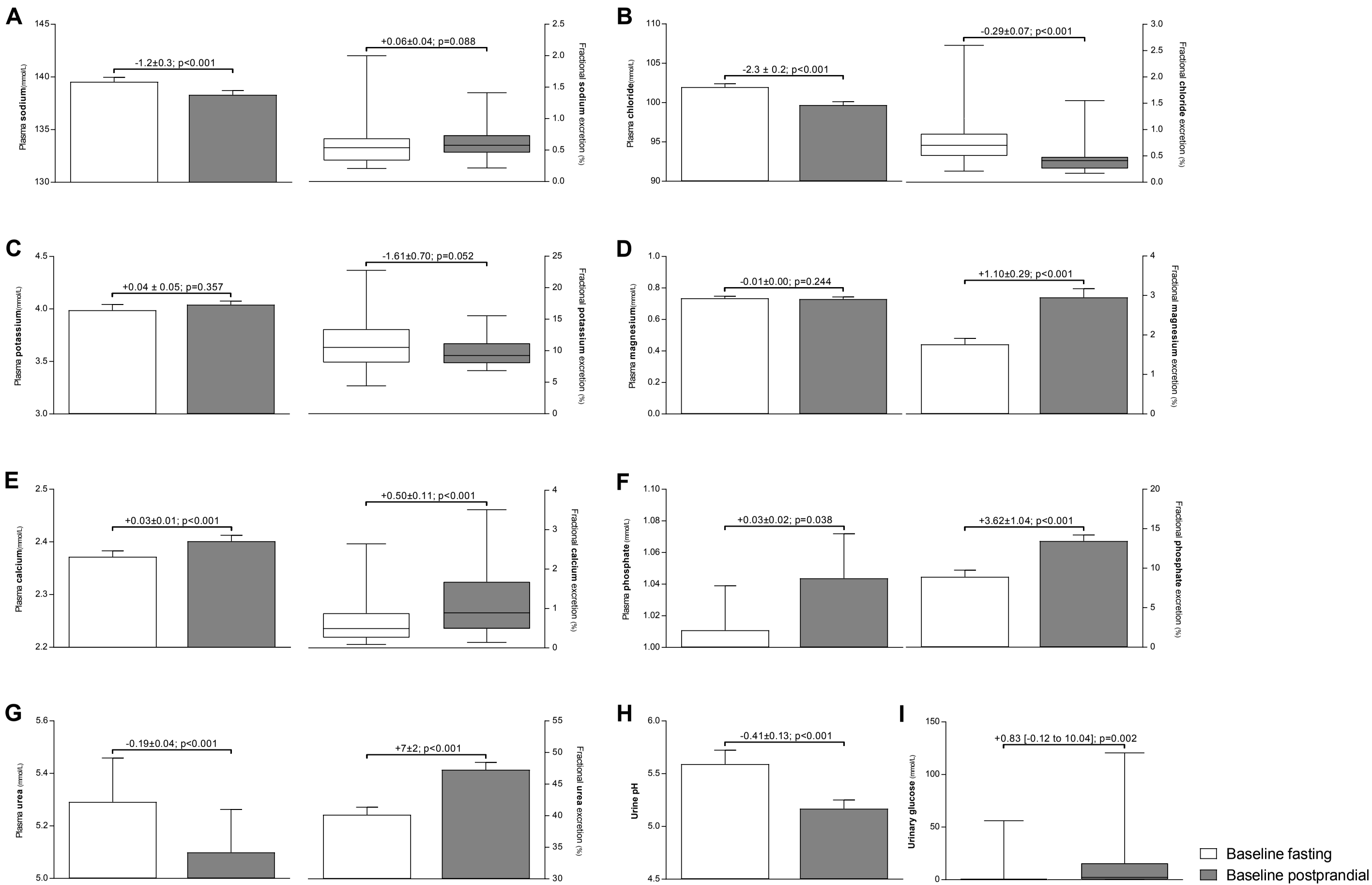

Figure 2. Postprandial plasma and tubular responses at baseline in all participants. Data are mean ± SEM or median [IQR and range]. Paired t-tests or Wilcoxon signed rank tests were used for within-group comparisons.

Table 2. Changes from baseline to Week-8 in plasma electrolytes and urea

Variables	Insulin glulisine (N=18)		
	Baseline	**Week-8**	**Within-group p-value**
Sodium			
Fasting, mmol/L	140.0 [138.0-140.3]	139.5 [137.8-143.0]	0.964
Postprandial, mmol/L	139.2 [137.1-140.2]	139.7 137.7 141.8	0.097
Chloride			
Fasting, mmol/L	101.5 [100.8-103.3]	102.5 [100.0-104.0]	0.682
Postprandial, mmol/L	100.0 ± 0.7	100.5 ± 0.6	0.326
Potassium			
Fasting, mmol/L	3.80 [3.70-4.05]	4.00 [3.80-4.20]	0.107
Postprandial, mmol/L	3.98 [3.93-4.08]	3.87 [3.75-4.13]	0.088
Magnesium			
Fasting, mmol/L	0.72 ± 0.02	0.75 ± 0.02	**0.032**
Postprandial, mmol/L	0.72 ± 0.02	0.75 ± 0.02	**0.015**
Calcium			
Fasting, mmol/L	2.37 ± 0.02	2.37 ± 0.01	0.813
Postprandial, mmol/L	2.41 [2.35-2.46]	2.40 [2.33-2.45]	0.744
Phosphate			
Fasting, mmol/L	1.00 ± 0.04	1.07 0.04	0.062
Postprandial, mmol/L	1.00 ± 0.04	1.04 0.04	0.182
Urea			
Fasting, mmol/L	5.23 ± 0.23	5.57 ± 0.27	0.154
Postprandial, mmol/L	4.73 [4.43-5.84]	5.38 [4.53-6.03]	0.163

Data are mean ± SEM or median [IQR]. Multiple linear regression was used to examine baseline-corrected lixisenatide-induced effects compared to insulin glulisine.

Table 3. Influence of postprandial blood pressure and glucose, insulin, glucagon and acetaminophen concentration on changes in tubular functions

Variables	Uncorrected		MAP-corrected		Time-averaged glucose-corrected	
Meal-induced FENa, %	0.25 ± 0.08	**0.003**	0.21 ± 0.10	**0.042**	0.21 ± 0.08	**0.019**
Meal-induced FECl, %	0.41 ± 0.13	**0.003**	–	–	–	–
Postprandial FEK, %	2.79 ± 1.29	**0.038**	–	–	–	–
Meal-induced FEK, %	4.31 ± 1.74	**0.019**	2.08 ± 2.08	0.326	–	–
Postprandial FEMg, %	-0.96 ± 0.37	**0.014**	-0.69 ± 0.44	0.133	–	–
Postprandial FECa, %	-0.39 ± 0.19	**0.049**	–	–	–	–
Postprandial FEPhos, %	-4.32 ± 2.07	**0.044**	-1.41 ± 2.30	0.544	-3.10 ± 2.22	0.172
Postprandial urinary pH	0.45 ± 0.17	**0.013**	0.25 ± 0.20	0.214	–	–
Meal-induced urinary pH	0.73 ± 0.21	**0.001**	0.49 ± 0.24	0.053	–	–

Data are mean ± SEM. – indicates that regression coefficient did not change >15%. Postprandial FE was calculated using inulin as reference substance. Meal-induced FE was calculated as the difference in postprandial and fasting FE using creatinine as reference substance. Abbreviations: ACT, acetaminophen; AUC, area under the curve; FECa, fractional calcium excretion; FECl,

Lixisenatide (N=17)			Difference lixisenatide-insulin glulisine	p-value
Baseline	Week-8	Within-group p-value		
140.0 [137.5-142.0]	140.0 [138.0-141.5]	0.620	0.3 ± 0.6	0.691
138.3 [137.2-139.2]	140.3 [139.5-142.7]	**<0.001**	1.8 ± 0.7	**0.011**
103.0 [100.0-104.0]	102.0 [100.0-104.0]	0.765	-0.2 ± 0.6	0.801
99.4 ± 0.5	101.0 ± 0.5	**0.001**	0.9 ± 0.6	0.145
4.05 [3.90-4.20]	4.00 [3.80-4.20]	0.102	-0.10 ± 0.07	0.170
4.17 [3.90-4.25]	4.00 [3.88-4.23]	0.380	0.07 ± 0.06	0.268
0.76 ± 0.02	0.74 ± 0.01	0.263	-0.03 ± 0.01	0.074
0.74 ± 0.02	0.76 ± 0.01	0.137	0.00 ± 0.02	0.900
2.37 ± 0.02	2.36 ± 0.02	0.523	-0.01 ± 0.02	0.757
2.37 [2.34-2.45]	2.43 [2.40-2.48]	**0.007**	0.05 ± 0.02	**0.027**
1.02 0.04	1.00 0.05	0.435	-0.09 ± 0.05	0.062
1.09 0.04	1.07 0.05	0.682	-0.04 ± 0.04	0.341
5.36 ± 0.25	5.28 ± 0.29	0.763	-0.38 ± 0.33	0.255
5.30 [4.83-5.87]	4.83 [4.33-5.55]	0.155	-0.57 ± 0.29	0.059

Paired t-tests or Wilcoxon signed rank tests were used for within-group comparisons. Significant differences indicated in bold font.

Time-averaged insulin-corrected		Time-averaged glucagon-corrected		ACT AUC-corrected	
–	–	–	–	–	–
–	–	–	–	–	–
–	–	–	–	1.88 ± 1.68	0.273
–	–	–	–	–	–
–	–	–	–	–	–
-0.26 ± 0.18	0.165	–	–	-0.21 ± 0.22	0.340
–	–	–	–	-2.57 ± 2.41	0.294
0.37 ± 0.17	**0.040**	–	–	0.30 ± 0.22	0.188
–	–	–	–	–	–

fractional chloride excretion; FEK, fractional potassium excretion; FEMg, fractional magnesium excretion; FENa, fractional sodium excretion; FEPhos, phosphate; MAP, mean arterial pressure.

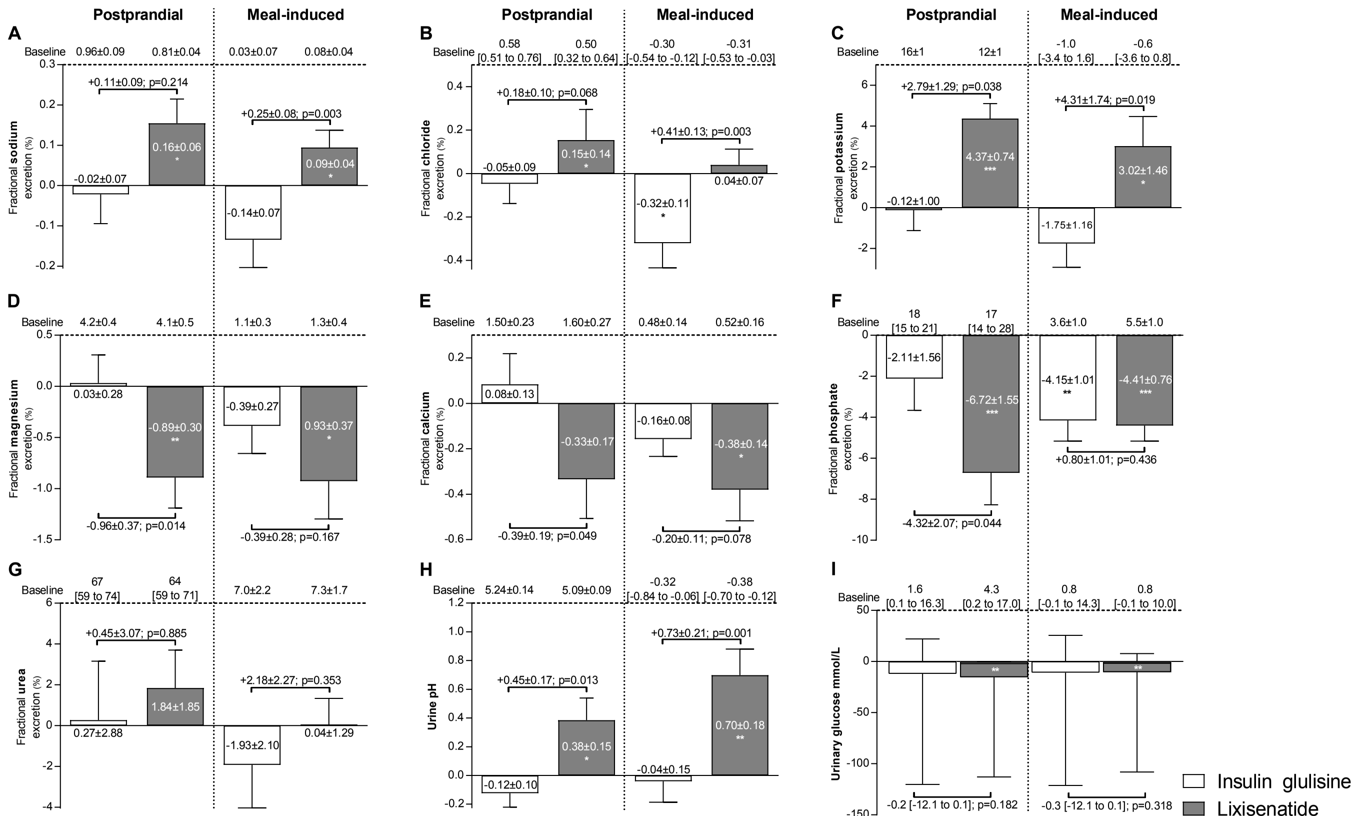

Figure 3A. Changes from baseline to Week-8 in postprandial effects and meal-induced effects (difference in postprandial and fasting) on fractional electrolyte and urea excretions, urinary pH and urinary glucose. Data are mean ± SEM or median [IQR]. Postprandial fractional excretion was calculated using inulin as reference substance. Meal-induced fractional excretion was calculated as the difference in postprandial and fasting fractional excretion using creatinine as reference substance. Multiple linear regression was used to examine baseline-corrected lixisenatide-induced effects compared to insulin glulisine, except for urinary-glucose for which the Mann-Whitney test was used. Paired t-tests or Wilcoxon signed rank tests were used for within-group comparisons (* p<0.05, ** p<0.01, *** p<0.001).

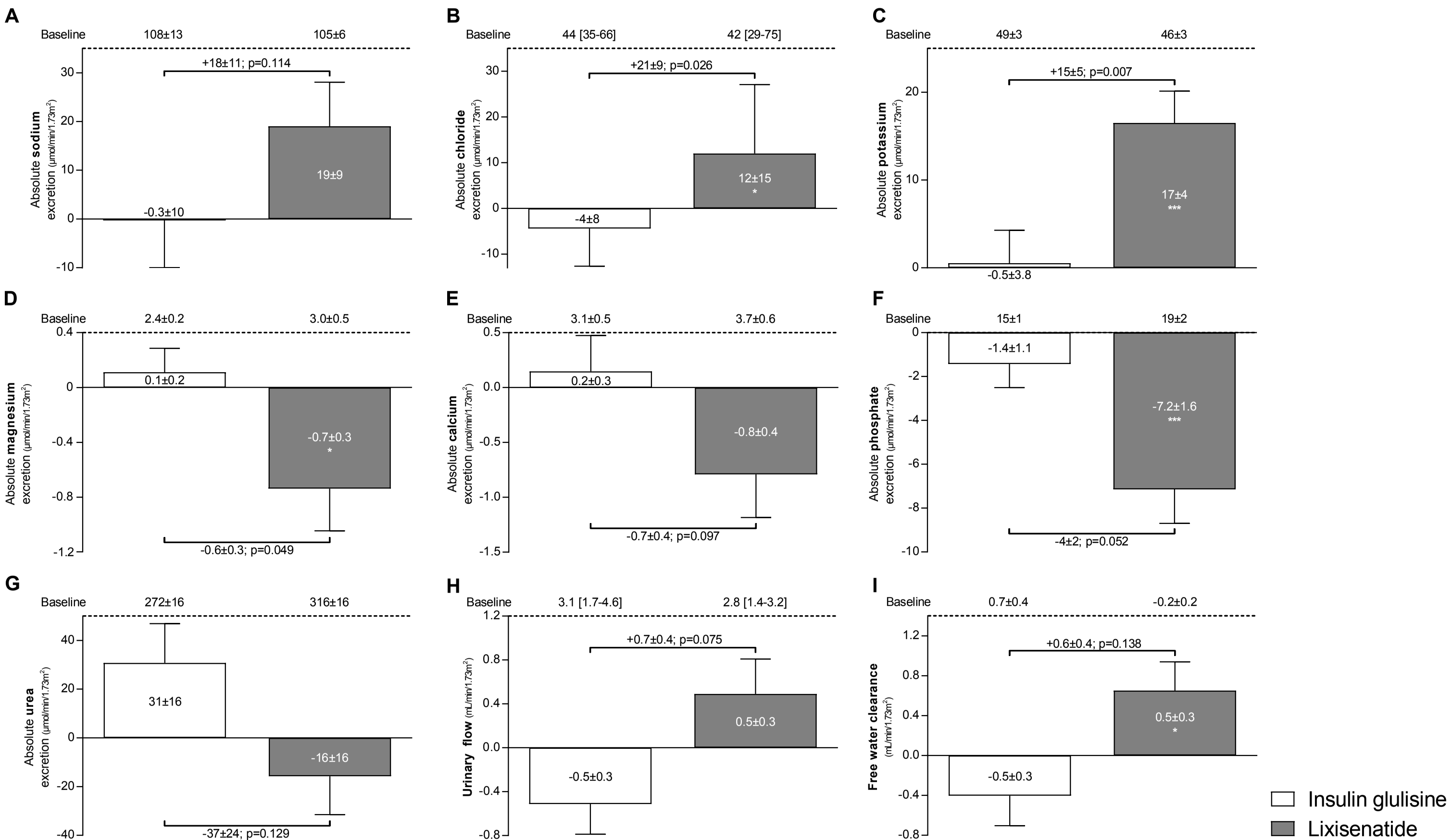

Figure 3B. Changes in absolute electrolyte and urea excretions, urinary flow and free water clearance. Data are mean ± SEM or median [IQR]. Multiple linear regression was used to examine baseline-corrected lixisenatide-induced effects compared to insulin glulisine, except for urinary-glucose for which the Mann-Whitney test was used. Paired t-tests or Wilcoxon signed rank tests were used for within-group comparisons (* p<0.05, ** p<0.01, *** p<0.001).

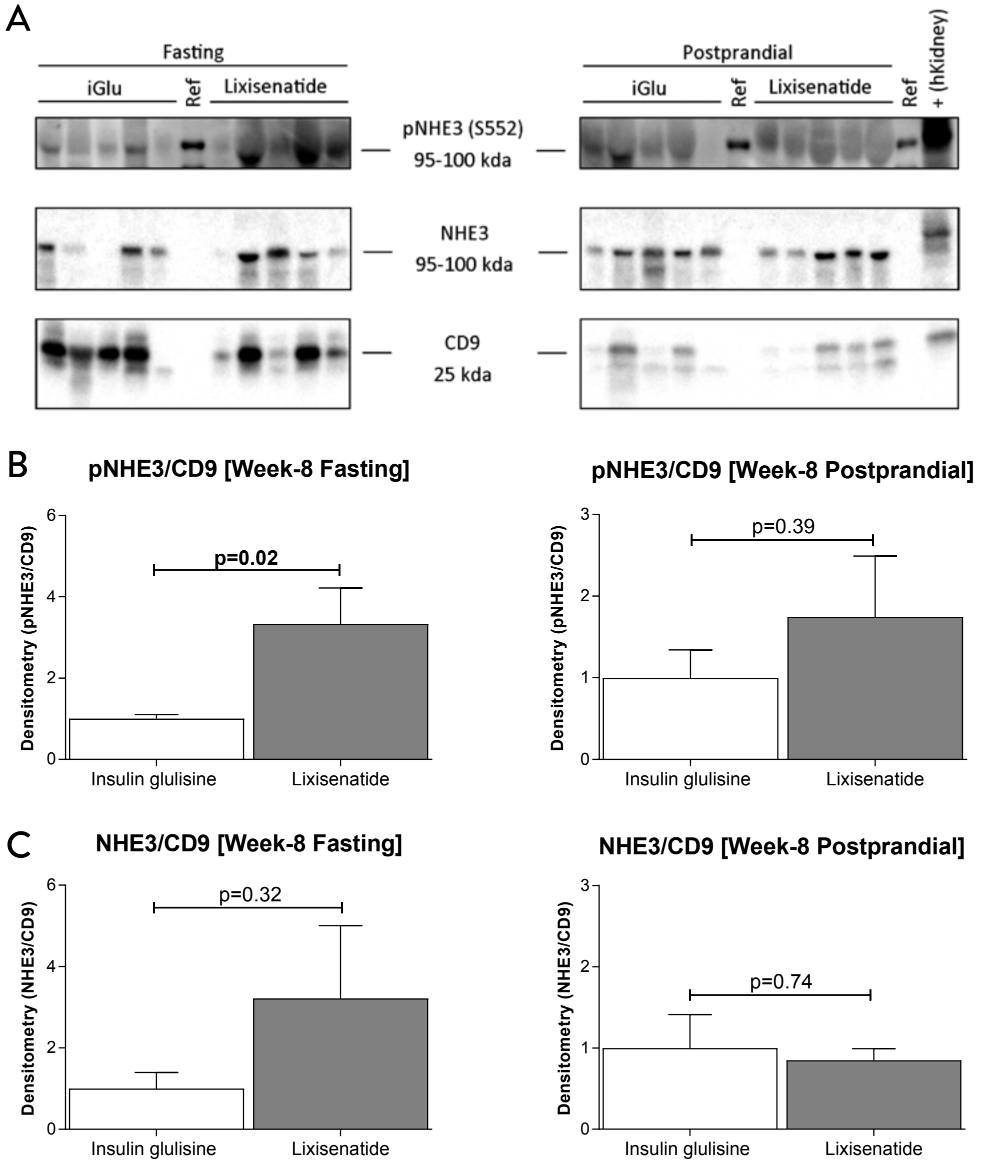

Figure 4. Fasting and postprandial pNHE3 and NHE3 abundance in urinary extracellular vesicles at Week-8. (**A**) Immunoblot of isolated urinary extracellular vesicles (uEV) content of fasting (left panel) and postprandial (right panel) patients, showing abundance of phosphorylated sodium-hydrogen exchanger 3 (pNHE3), sodium-hydrogen exchanger 3 (NHE3) and uEV-marker CD9. Sample loading was normalised to urinary creatinine. *Ref* is the reference by prestained protein ladder; *hKidney* (human kidney) was used as positive control. (**B**-**C**) Densitometry of pNHE3 (**B**) and NHE3 bands (**C**) [mean ± SEM], corrected for CD9 to normalise for loading and isolation variation. 1 fasting iGlu sample was excluded because CD9 was not quantifiable resulting in an outlier ($P<0.01$). A T-test was used to calculate P-values.

phosphate (67%), in addition to postprandial and meal-induced urinary pH (44% and 33%, respectively) (Table 3). Differences in blood glucose influenced the effect on meal-induced fractional excretions of sodium (17%) and phosphate (17%), while changes in serum insulin partly explained differences in postprandial fractional calcium excretion (33%) and urinary pH (17%). Changes in aldosterone partly influenced changes in meal-induced fractional potassium excretion (20%). Plasma glucagon or angiotensin-II did not explain changes in fractional excretion of electrolytes or urinary pH. We specifically explored the influence of urinary flow on postprandial and meal-induced fractional potassium excretion. Here, we observed that the regression coefficient of postprandial fractional potassium excretion decreased by 20%, while meal-induced fractional potassium excretion did not change.

Discussion

This is the first study that assessed the integrated effects of prolonged GLP-1RA-treatment on renal tubular handling and plasma concentrations of electrolytes in overweight T2DM patients. After 8 weeks, lixisenatide compared to iGlu did not affect fasting fractional UE of electrolytes or urea, but increased postprandial absolute and fractional UE of sodium, chloride, and potassium and of urinary pH (in conjunction with increased pNHE3 in uEVs in fasting patients), and reduced absolute and fractional UE of magnesium, calcium and phosphate (Table 4). Moreover, lixisenatide did not affect fasting plasma electrolytes or urea levels, but modestly increased plasma sodium and calcium concentrations following the breakfast.

The effects of lixisenatide on the tubular handling of electrolytes in our prolonged intervention trial is in line with several clinical studies that investigated effects of *acute* GLP-1(RA) administration.[1] In individuals with and without T2DM, urinary sodium excretion and urinary pH increased after native GLP-1-infusion,[2,5] exenatide-infusion,[4,8] and a single subcutaneous

Table 4. Summary of the main tubular effects of lixisenatide compared to insulin glulisine

	Effect of lixisenatide vs. insulin glulisine	
Variables	**Fasting**	**Postprandial**
UE of sodium	↔	↑
UE of chloride	↔	↑
UE of potassium	↔	↑
UE of magnesium	↔	↓
UE of calcium	↔	↓
UE of phosphate	↔	↓
UE of urea	↔	↔
Urine pH	↔	↑
pNHE3 in uEVs	↑	↔
NHE3 in uEVs	↔	↔

↑, increase; ↓, decrease; ↔, no difference; (p)NHE3, (phosphorylated) sodium-hydrogen exchanger isoform 3; UE, urinary excretion; uEV, urinary extracellular vesicles

injection of liraglutide.[6] In addition, GLP-1-infusion increased urinary chloride excretion in healthy individuals,[2,5] while exenatide-infusion raised urinary potassium excretion in T2DM patients.[8] In contrast to our findings, urinary calcium excretion increased after GLP-1 infusion in non-diabetic subjects.[2,5] In a prolonged intervention study, we showed that liraglutide did not alter fasting UE of sodium, potassium, urea and of urinary pH and urinary flow in T2DM patients after 12-weeks, while UE of sodium, potassium and urea were also unaffected after 2 weeks.[16] The differential response between lixisenatide and liraglutide may reflect differences in pharmacokinetics. Lixisenatide is a short-acting/prandial GLP-1RA producing short-lived receptor-activation, while long-acting GLP-1RA's such as liraglutide continuously activate the GLP-1R.[17] Due to their continuous receptor stimulation, long-acting GLP-1RA's lead to receptor tachyphylaxis, thereby losing their ability to decrease gastric emptying rate,[1,17] to inhibit hedonic feeding areas in the brain[1,17] and to induce natriuresis at the tubular levels (as previously suggested[14,16]). Current data supports that lixisenatide does not result in receptor tachyphylaxis and/or compensatory mechanisms at the tubular level, although head-to-head studies are needed to confirm this. Alternatively, the limited renal filtration of liraglutide-metabolites may lead to a reduced effect on the putative GLP-1R in renal tubule, thereby explaining differences between the drugs.[16]

Mechanisms by which lixisenatide affects tubular transport are incompletely understood. Increases in urinary sodium excretion and urinary pH suggest inhibitory actions on NHE3-activity in the proximal tubule.[14] Accordingly, studies in rodents and humans demonstrate that acute GLP-1R-stimulation increases UE of lithium (a marker of proximal sodium reabsorption),[1] while in rodents, NHE3-activity is inhibited by phosphorylation of the transporter at protein kinase A consensus sites Ser552 and Ser605.[11,12]

In a subgroup of the current study, we also observed an increase in NHE3 phosphorylation at serine 552 in the lixisenatide- compared to the iGlu treated patients at Week-8. Surprisingly, increased pNHE3 was observed in the fasting state, while only a trend was observed upon administration of the study drug, which could suggest that the effect of lixisenatide on pNHE3 is relatively long lasting and was thus not detected in acute-on-chronic administration. This may also explain why NHE3 is phosphorylated already in the fasting state at Week-8, almost 24 hours and 8 half-lives after the last injection. The absence of difference in fasting urinary sodium excretion may be explained by a new steady state in which urinary sodium excretion reflects dietary intake.

Changes in tubular-transport of other electrolytes upon lixisenatide therapy may also be explained by NHE3-inhibition. As such, the increase in urinary chloride excretion could result from the coupling of Na^+/H^+ exchange with $Cl^-/base^-$ and Cl^-/anion exchange.[18] Moreover, inhibition of NHE3 may induce kaliuresis by increasing sodium delivery to the distal nephron,[19,20] and by inducing flow-mediated kaliuresis.[20,21] Finally, reduced UE of magnesium, calcium and phosphate could be explained by increased sodium-dependent transport of magnesium and calcium in the thick ascending limb (paracellular transport) or distal convolute (transcellular transport), and of phosphate in the loop of Henle and the distal convolute through unknown reabsorptive pathways.[22] However, a recent investigation in primate kidneys, that used the most

extensively validated antibody to date, could not detect GLP-1R's in the proximal tubule,[10] suggesting that lixisenatide may indirectly decrease NHE3-activity. Indeed, our statistical analyses suggest that other factors may be involved. First, the increase in urinary sodium excretion and urinary pH was partly explained by an increase in postprandial MAP, which may lead to redistribution and subsequent de-activation of NHE3 (*i.e.* pressure-natriuresis).[14,23] Changes in MAP also partly explained changes in UE of potassium, magnesium and phosphate. Second, reductions in blood glucose partly explained effects on urinary sodium excretion, which could indicate reduced sodium-coupled glucose-uptake by sodium-dependent glucose cotransporters (SGLT's) in the proximal tubule.[14] In line, urinary glucose excretion decreased within-group with lixisenatide. Third, we explored the role of lixisenatide-induced reductions in insulin[24] and glucagon[25] concentrations, which may also inhibit NHE3-activity in the proximal tubule and thick ascending limb of Henle, respectively. However, our analyses did not indicate that these hormones contributed to a natriuretic and urine alkalising effect. Finally, angiotensin-II did not explain any of the tubular effects, while increased aldosterone is suggested to contribute to increased ENaC-activity.[14] Lixisenatide could also influence tubular transporters beyond NHE3. As magnesium and calcium are regulated in the proximal tubule, loop of Henle and distal tubule,[21] and phosphate reabsorption primarily occurs in the proximal tubule (~85%),[21] a reduction in UE of all these electrolytes is generally explained by increased proximal tubular reabsorption. However, since this conflicts with NHE3-inhibition, involvement of tubular transporters in the thick ascending limb[19,26] or distal convolute tubule[20] should be considered.

Our study has several limitations. First, although we aimed to investigate real-life effects by measuring lixisenatide-induced actions postprandially, the drug may potentially have reduced effects upon a system that is already activated by a meal, while the potential inhibitory effect of lixisenatide on NHE3 may have been indirectly blunted by a concurrent drug-induced decrease in gastric emptying rate.[12] Moreover, several factors that are known to influence phosphate and calcium homeostasis, such as parathyroid hormone and fibroblast growth factor 23, were not measured. However, since fasting plasma phosphate and calcium concentrations did not change, we do not expect that these factors were affected. Furthermore, although lixisenatide most likely affects electrolyte excretion only acutely after drug administration, we did not perform measurements over a 24-hour period to confirm this. Also, we did not use 24-hour urine to assess compliance of the dietary instructions prior to the testing days. Finally, we are the first to study NHE3 and pNHE3 in human uEVs. Although this currently is the only non-invasive method to study transporter changes in human kidney, animal studies should be performed to assess whether abundance of NHE3 in uEVs corresponds to its abundance in the proximal tubule, as is the case for NCC and AQP2.[27]

In conclusion, following a meal, lixisenatide increases UE of sodium, chloride, and potassium and of urinary pH, and reduces UE of magnesium, calcium and phosphate in overweight T2DM patients after prolonged therapy. This is most likely explained by a BP-mediated and/or direct inhibitory effects on the NHE3 in the proximal tubule. However, prolonged treatment with a short-acting GLP-1RA probably only affects diurnal rhythms in tubular electrolyte handling, leaving overall electrolyte balance unchanged.

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