Mechanisms mediating the diuretic and natriuretic actions of the incretin hormone glucagon-like peptide-1

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Crajoinas RO, Oricchio FT, Pessoa TD, Pacheco BP, Lessa LM, Malnic G, Girardi AC. Mechanisms mediating the diuretic and natriuretic actions of the incretin hormone glucagon-like peptide-1. Am J Physiol Renal Physiol 301: F355–F363, 2011. First published May 18, 2011; doi:10.1152/ajprenal.00729.2010.—Glucagon-like peptide-1 (GLP-1) is a gut incretin hormone considered a promising therapeutic agent for type 2 diabetes because it stimulates beta cell proliferation and insulin secretion in a glucose-dependent manner. Cumulative evidence supports a role for GLP-1 in modulating renal function; however, the mechanisms by which GLP-1 induces diuresis and natriuresis have not been completely established. This study aimed to define the cellular and molecular mechanisms mediating the renal effects of GLP-1. GLP-1 (1 μg·kg−1·min−1) was intravenously administered in rats for the period of 60 min. GLP-1-infused rats displayed increased urine flow, fractional excretion of sodium, potassium, and bicarbonate compared with those rats that received vehicle (1% BSA/saline). GLP-1-induced diuresis and natriuresis were also accompanied by increases in renal plasma flow and glomerular filtration rate. Real-time RT-PCR in microdissected rat nephron segments revealed that GLP-1 receptor-mRNA expression was restricted to proximal tubule and glomerulus and proximal convoluted tubule. In rat renal proximal tubule, GLP-1 significantly reduced Na+/H+ exchanger isoform 3 (NHE3)-mediated bicarbonate reabsorption via a protein kinase A (PKA)-dependent mechanism. Reduced proximal tubular bicarbonate flux rate was associated with a significant increase of NHE3 phosphorylation and by downregulation of NHE3 activity in the renal proximal tubule. Moreover, our findings support the view that GLP-1-based therapeutic strategies are known as “based on incretins” (7). Cumulative evidence supports a role for GLP-1 in modulation of renal function and blood pressure control (8, 20, 21, 36, 40). The effect of chronic administration of GLP-1 was explored in a genetic model of experimental hypertension, the Dahl salt-sensitive rat, fed with a high-sodium chloride diet for 2 wk (40). In this strain, chronic administration of GLP-1 attenuated significantly the development of hypertension, reduced cardiac and renal injury, and improved endothelial function. Those rats presented an increase in urinary flow and sodium excretion, indicating that GLP-1 has natriuretic and diuretic properties that contribute to its anti-hypertensive effect (40). The incretin mimetic exendin-4 has been shown to exert an anti-hypertensive effect in two different mice models of salt-sensitive hypertension by improving the renal ability of these animals to handle a salt load (21). Furthermore, recent studies from our laboratory showed that 8-day treatment with the incretin enhancer sitagliptin attenuates blood pressure rising in young prehypertensive spontaneously hypertensive rats, at least in part by diminishing proximal tubular sodium reabsorption (33). Collectively, these studies suggest that the incretin-based anti-hyperglycemic agents may confer anti-hypertensive and renoprotective effects as well. The mechanisms by which GLP-1 induces diuresis and natriuresis have not been completely established. Schlatter and colleagues (36) recently detected the GLP-1 receptor in primary porcine proximal tubular cells, where it decreases sodium reabsorption. Furthermore, in vitro studies performed in our laboratory showed that the GLP-1R agonist exendin-4 decreases Na+/H+ exchanger isoform 3 (NHE3)-mediated sodium-dependent pH recovery in LLC-PK1 cells (8). However, it is still unknown whether GLP-1 affects proximal tubular function in vivo and whether other cell types within the kidney are responsive to this incretin hormone. Therefore, the aim of this study was to define the cellular and molecular mechanisms mediating the renal effects of GLP-1.
MATERIALS AND METHODS

Reagents and antibodies. All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise noted. GLP-1 and exendin-4 were purchased from Bachem (Philadelphia, PA). P32/98 (Ile-thiazolidine) (35), a specific competitive inhibitor of dipetidyl peptidase IV, was purchased from Enzo Life Sciences (Plymouth Meeting, PA). S3226 was kindly donated by Dr. Jurger Punten from Sanofi-Aventis Deutschland GmbH (Frankfurt, Germany). A monoclonal antibody (mAb) raised to the renal brush-border NHE3, clone 2B9 (5), was kindly provided by Drs. D. Biemesderfer and P. Aronson (Yale University, New Haven, CT). Phosphospecific NHE3 mAbs to NHE3 (phospho-serines 552 and 605) anti-PS552 (14D5), anti-PS605 (10A8) (24), and a goat polyclonal antibody to villin (C-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal antibody to GLP-1-IR was purchased from Abcam (Cambridge, MA). A mAb to actin (JLA20) was purchased from Calbiochem (San Diego, CA). Horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit, and rabbit anti-goat secondary antibodies were purchased from Life Technologies (Carlsbad, CA).

Animal protocols. Animal procedures and protocols were followed in accordance with the ethical principles in animal research of the Brazilian College of Animal Experimentation and were approved by the institutional animal care and use committee. Experiments were performed using male Wistar rats (220–260 g) housed under standardized conditions (constant temperature of 22°C, 12:12-h light-dark cycle, and relative humidity of 60%) at the University of São Paulo Medical School animal facility. On the day of study, rats were anesthetized with ketamine-xylazine-acepromazine (64.9, 3.20, and 0.78 mg/kg sc, respectively) and placed on a heated surgical table to maintain body temperature. After tracheotomy, polyethylene catheters were inserted into the jugular vein and the urinary bladder for drug infusion and urine collection, respectively. GLP-1 (1.0 μg·kg⁻¹·min⁻¹) or vehicle (1% BSA/saline) was intravenously infused at a rate of 3 μl/min for the period of 1 h. For the experiments using incretin mimetics or incretin enhancers, the compounds were dissolved in PBS and adjusted to neutral pH. An intraperitoneal injection (2.0 μl/kg) containing 50 μg/kg of the DPP4 inhibitor DPP4/98, 5 μg/kg exendin-4, or vehicle was then administered to experimental rats. Right after intraperitoneal administration of the drugs, the animals were prepared for bladder urine collection as mentioned above. Arterial blood was collected at the time of death. Kidneys were then removed for RNA extraction and cortical microsomal or microvilli membrane vesicle preparations.

Blood and urine analyses. pH, bicarbonate concentration, and PCO₂ were measured on a Radiometer ABL5 blood-gas analyzer (Radiometer, Copenhagen, Denmark). Sodium, potassium, and lithium concentrations were determined by flame photometry (Micronal B262, São Paulo, SP, Brazil). Urinary creatinine concentration was determined by using the kinetic alkaline picrate (Jaffe’s) method and plasma creatinine was measured on a Beckman Coulter Synchron CX7 Analyzer (Beckman Coulter, Fullerton, CA). Creatinine clearance was used to estimate glomerular filtration rate (GFR), paraaminobiphenyluric acid (PAH) clearance to estimate renal plasma flow (RPF) (1), and lithium clearance as a measure of end-proximal tubule delivery.

Urinary cAMP excretion. cAMP concentration in urine containing 1 mM phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine was determined by enzyme immunoassay (cAMP Direct Biotrak EIA, GE Healthcare) according to the manufacturer’s instructions. cAMP excretion was calculated by multiplying the urinary volume by the urine cAMP concentration.

Microdissection of nephron segments. Anesthetized animals were subjected to laparotomy after a midline abdominal incision and the aorta was cannulated with polyethylene tubing below the left kidney. The left kidney was perfused initially with 20 ml ice-cold dissection solution containing 135 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 3 mM sodium acetate, 1.2 mM Na₂SO₄, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 5.5 mM glucose, 5 mM HEPES (pH 7.4); and, right after, with 10 ml of the same solution containing 100 mg collagenase B and 1 mg/ml bovine serum albumin. The kidney was removed and decapsulated and thin coronal sections were cut. The sections were transferred into tubes containing 10 ml of the same collagenase solution and incubated with 95% O₂-5% CO₂ bubbling for 40 min at 37°C. Then, these sections were transferred to Petri dishes on ice filled with dissection solution. Nephron segments were isolated according to the methodology previously described by Moriyama et al. (30). The microdissection was done under stereomicroscopic observation and the segments were identified and isolated by using anatomical and morphological criteria (25). Mapping of GLP-1-IR transcripts was performed on the following nephron segments: glomerulus, proximal convoluted tubules (PCTs), proximal straight tubules (PSTs), thick ascending limb (TAL), cortical collecting duct (CCD), and medullary collecting duct (MCD).

RNA extraction, real-time reverse transcription PCR, and RT-PCR. RNA was isolated from the the microdissection samples using TRizol reagent (Life Technologies, Gaithersburg, MD) as per the manufacturer’s instructions. First-strand cDNA synthesis was performed with Super-Script III Reverse Transcriptase following the manufacturer’s guidelines. Fifty nanograms cDNA were used for real-time RT-PCR reaction (SYBR Green PCR Master Mix-PX Applied Biosystems) in an ABI Prism 7700 Sequence Detection System (Applied Biosystems). All samples were assayed in triplicate. The comparative threshold cycle method was used for data analyses. RT-PCR was carried out using Taq polymerase under the following conditions: initial denaturation for 5 min at 95°C followed by 40 cycles of denaturation for 15 s at 95°C, annealing for 1 min at 60°C, extension for 1 min 72°C, and final extension for 10 min at 72°C. The PCR products were analyzed by electrophoresis on agarose gel. The gene control 28S ribosomal RNA was used to normalize the results. The following oligonucleotide primers were used: GLP-1IR, 5′-CCGCTCTTGGGCAGCGGATGA-3′ (forward) and 5′-AGCCGTCCTCAGCTTTCGCGA-3′ (reverse), villin, 5′-CTGCAAGCAGCTTGCACAACT-3′ (forward) and 5′-CAGCGCCCTTTTTTCGGATCAC-3′ (reverse), and 28S, 5′-TCTACGACCCCCAGAACAGG-3′ (forward) and 5′-GATTCGCGGATGTTG-3′ (reverse).

Preparation of kidney membranes. Brush-border microvilli membrane vesicles (MMV) were isolated from renal corticis of saline- or drug-treated rats as described previously (2). Renal cortical microsomal membranes were isolated from rats euthanized by intraperitoneal injection of pentobarbital sodium. Kidneys were excised, minced with opposing razor blades, and homogenized in a Potter-Elvehjem-style tissue grinder for 25 strokes in ice-cold PBS (10 mM phosphate, 140 mM NaCl, pH 7.2) containing 1 μM pepstatin, 1 μM leupeptin, and 230 μM PMSF. Aliquots of this homogenate were saved and kept at −80°C until analysis. The homogenate was then centrifuged at 2,000 g for 15 min at 4°C. The supernatant was removed and subjected to a further 1 h of centrifugation at 100,000 g at 4°C to pellet the microsomal fraction. The supernatant was discarded and the microsomes were resuspended in fresh PBS containing protease inhibitors and stored at −80°C.

Radioactive sodium uptake. For 32P uptake assays, MMVs were washed and equilibrated for 2 h at room temperature in 254 mM mannitol, 35 mM KOH, 68 mM HEPES, 50 mM Mes, pH 6.0. MMVs were then centrifuged and resuspended in the same solution at a final protein concentration of 10 μg/μl. Subsequently, the uptake of 22Na was assayed by mixing 10 μl of the MMV suspension with 90 μl of a “hot solution” containing 0.1 μCi of 22Na, 300 mM mannitol, 42 mM KOH, 80 mM HEPES, pH 7.5. The influx of radioactive sodium was terminated after 10 s by three rapid washes of 3 ml ice-cold solution containing 100 mM KCl, 42 mM KOH, 80 mM HEPES, pH 7.5. MMVs were collected on 0.65-μm Millipore filters. After an additional wash with 9.0 ml of “stop solution,” the Millipore filters were transferred to vials and the radioactivity was measured using a liquid scintillation counter.
than two groups were compared, statistical significance was determined by ANOVA followed by Tukey’s post hoc test. P values <0.05 were considered significant.

RESULTS

Dose-dependent effect of GLP-1 on cAMP urinary excretion. The GLP-1R is a G protein-coupled receptor whose activation stimulates the formation of intracellular cAMP (14). Urinary cAMP is considered to be a reflection of hormonal action in renal tissue that is due to increased production and secretion by the kidneys. To test whether GLP-1 infusion may activate rat renal GLP-1R and to establish at which dose this effect would be more pronounced, cAMP concentration was measured in the urine of rats treated or not with different doses of this hormone incretin. The dose-dependent profile for mean cAMP urinary excretion of rats treated for 1 h with GLP-1 is summarized in Fig. 1. Continuous infusion of 0.5 μg·kg⁻¹·min⁻¹ GLP-1 induced an increase in the urinary excretion of cAMP compared with controls (68 ± 9 vs. 8 ± 3 pmol/min, P = 0.003; Fig. 1). As seen in Fig. 1, maximum urinary cAMP excretion (121 ± 9 pmol/min) was obtained with GLP infusion at doses of ≥1.0 μg·kg⁻¹·min⁻¹. In view of the fact that higher doses of GLP-1 (5 μg·kg⁻¹·min⁻¹) did not significantly enhance the levels of cAMP in urine (127 ± 11 vs. 121 ± 9 pmol/min, P = 0.44), the dose of 1.0 μg·kg⁻¹·min⁻¹ was employed in our experiments.

Effect of GLP-1 infusion on renal function. Consistent with previous reports (19, 20, 29), we found that continuous intravenous infusion of 1.0 μg·kg⁻¹·min⁻¹ GLP-1 for the period of 1 h caused significant increase of urine flow and sodium excretion compared with rats that were infused with saline/BSA (control; Table 1). The GLP-1 natriuretic and diuretic effects were accompanied by an enhancement of glomerular filtration rate and of renal plasma flow, as estimated by creatine and PAH clearance, respectively. The incretin also significantly increased the lithium clearance and the fractional potassium excretion. Analysis of acid-base parameters showed that rats acutely treated with GLP-1 displayed increased bicarbonate reabsorption, r is the tubule radius, and (HCO₃⁻) is the half-time of the exponential reduction of the injected HCO₃⁻ concentration to its stationary level.

Stationary in vivo microperfusion. Experiments were carried out essentially as described previously including anesthesia and surgical preparation for in vivo micropuncture (10). Proximal tubules were punctured by means of a double-barreled micropipette, one barrel being used to inject FDC-green-colored Ringer perfusion solution, and the other to inject Sudan-black-colored castor oil. To measure luminal pH, proximal tubules were impaled by a double-barreled asymmetric microelectrode, the larger barrel containing H⁺-ion-sensitive ion-exchange resin silanized with hexamethyldisilazane (Sigma Fluka, Buchs, Switzerland) and the smaller barrel containing the reference solution (1 M KCl) colored by FDC-green. The voltage between the microelectrode barrels, representing luminal H⁺ activity, was continuously recorded by means of a microcomputer equipped with an AD converter (Lynx, São Paulo, Brazil). Luminal bicarbonate was calculated from luminal pH and arterial blood PCO₂ and the rate of tubular acidification was expressed as the half-time of the exponential reduction of the injected HCO₃⁻ concentration to its stationary level (t₁/₂). Net HCO₃⁻ reabsorption (JₜHCO₃⁻) per cm² of tubule epithelium was calculated by using the following equation: JₜHCO₃⁻ = In²(t/t₂) [(HCO₃⁻)₀ - (HCO₃⁻)ₜ]×t/s, where t₁/₂ is the half-time of bicarbonate absorption, r is the tubule radius, and (HCO₃⁻)₀ and (HCO₃⁻)ₜ are the concentrations of the injected HCO₃⁻ and HCO₃⁻ at the stationary level, respectively.

SDS-PAGE and immunoblotting. Protein samples were solubilized in Laemmli sample buffer and separated by SDS-PAGE using 7.5% polyacrylamide gels (26). For immunoblotting, proteins were transferred to PVDF (Millipore Immobilon-P, Millipore, Bedford, MA) at 350 mA for 8–10 h at 4°C with a TE 62 transfer electrophoresis unit (GE HealthCare). Sheets of PVDF containing transferred proteins were incubated first in Blotto (5% nonfat dry milk and 0.1% Tween 20 in PBS, pH 7.4) for 1 h to block nonspecific binding of antibody, followed by overnight incubation in primary antibody. Primary antibodies, diluted in Blotto, were used at dilutions ranging from 1:200 to 1:50,000. The sheets were then washed in Blotto and incubated for 1 h with an appropriate horseradish peroxidase-conjugated secondary antibody diluted 1:2,000 in Blotto. After being washed 5× in Blotto and 2× PBS (pH 7.4), an enhanced chemiluminescence detection system (GE HealthCare) with Kodak Biomax imaging film (Kodak) was used for visualization of the bound antibodies. The visualized bands were digitized using the ImageScanner III (GE HealthCare) and quantified using the Scion Image Software (Scion, Frederick, MD). In some experiments, PVDF blots were reprobed with additional primary antibodies after stripping away the first antibody. This was accomplished by incubating the PVDF sheets in Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL).

Statistical analyses. All data are presented as means ± SE. Comparisons between two groups were made by unpaired t-test. If more

| Table 1. Effect of GLP-1 infusion on rat renal function and systemic acid-base parameters |
|----------------|----------------|
|                | CTRL (n = 9)   | GLP-1 (n = 10)  |
| Body wt, g     | 245 ± 4        | 241 ± 6        |
| UO, μl·min⁻¹·kg⁻¹ | 32.7 ± 1.2    | 79.3 ± 3.7†   |
| GFR, ml·min⁻¹·kg⁻¹ | 3.7 ± 0.5    | 5.0 ± 0.6*    |
| RPF, ml·min⁻¹·kg⁻¹ | 11.1 ± 0.9  | 14.2 ± 1.1*   |
| FE Na⁺, %      | 0.42 ± 0.03   | 1.23 ± 0.21†  |
| FE K⁺, %       | 25 ± 4        | 44 ± 3†       |
| C₅₁, μl·min⁻¹·kg⁻¹ | 1.37 ± 0.32  | 2.99 ± 0.54†  |
| FE HCO₃⁻, %    | 0.22 ± 0.01   | 0.79 ± 0.05†  |
| Urine pH       | 6.00 ± 0.07   | 6.52 ± 0.10*  |
| Plasma HCO₃⁻, mM | 23.5 ± 1.1    | 20.7 ± 1.3    |
| Plasma Pco₂, mM | 45 ± 4        | 46 ± 6        |
| Plasma pH      | 7.35 ± 0.01   | 7.31 ± 0.04   |

Values are means ± SE. *P < 0.05 and †P < 0.01 vs. control (CTRL), assessed by unpaired t-test. Urinary output (UO) was measured gravimetrically. Creatinine clearance was used to estimate glomerular filtration rate (GFR) and PAH clearance to estimate renal plasma flow (RPF). Fractional excretion (FE) of an ion (i) was calculated as Ci/Co where Ci is the ion clearance and Co is creatinine clearance. Endogenous lithium clearance (CiL) was calculated as V × Uᵢ/Uᵢ where V is urine output, Uᵢ is urinary lithium concentration, and Pᵢ is plasma lithium concentration. GLP-1, glucagon-like peptide-1.
bonate excretion and acidified their urine to a lesser extent than controls. However, as seen in Table 1, this bicarbonaturia did not significantly decrease blood pH and bicarbonate concentration and therefore differences in systemic acid-base status were not statistically significant between control and GLP-1-treated rats. Altogether, these data suggest that the diuretic and natriuretic responses induced by GLP-1 infusion are mediated by changes in both renal hemodynamics and tubular function.

Distribution of the GLP-1 receptor along the rat nephron. To shed light on the mechanisms by which GLP-1 alters renal function, we first located the potential sites of action of the incretin by examining the distribution of the GLP-1R-mRNA along the nephron. The nephron segments tested were glomerulus, PCT, PST, TAL, CCD, and MCD. Real-time RT-PCR (Fig. 2A) and conventional RT-PCR (Fig. 2B) in these microdissected rat nephron segments revealed GLP-1R-mRNA expression to be confined to both glomerulus and PCT. As seen in Fig. 2, A and B, expression of GLP-1R transcript was normalized by 28S-RNA. Given the possibility of contamination of isolated glomerulus preparation with PCT constituents, amplification of the proximal tubular marker villin was used to account for the purity of the samples. As can be seen in Fig. 2C, the glomeruli showed no expression of rat villin-mRNA, excluding the possibility of proximal tubular contaminants in the isolated glomeruli preparation.

Next, immunoblotting analyses were performed to evaluate the pattern of distribution of the GLP-1R within the renal cortex (Fig. 2D). As well as the microvillar core protein villin, the GLP-1R was enriched in the microvillus membrane fraction (MMV), compared with homogenate and renal cortical microsomes. As expected, the basolateral Na\(^+\)/K\(^+\)-ATPase was predominantly enriched in the microsomal fraction and no significant amount of the pump could be detected at the MMV. It therefore suggests that along the renal cortex, the GLP-1R is mainly confined to the brush-border microvilli of the proximal tubule.
The distribution pattern of the GLP-1R combined with the fact that GLP-1 infusion increased urinary NaHCO_3 excretion suggests that the diuretic and natriuretic effects of this incretin are at least in part mediated by inhibition of NHE3-mediated Na^+/H^+ exchange in renal proximal tubule.

Effect of GLP-1 on NHE3-mediated Na^+/H^+ exchange in renal proximal tubule. The effect of GLP-1 on NHE3 activity was evaluated ex vivo, by pH-dependent ^22Na uptake into MMV, and in vivo, by stationary microperfusion and continuous measurement of luminal pH (Fig. 3). As seen in Fig. 3A, NHE3-mediated Na^+/H^+ exchange in isolated MMV was not significantly different between controls and GLP-1-treated animals. At first glance, these results seemed somehow unexpected. However, Kocinsky and colleagues (23) recently postulated that early inhibition of NHE3 activity by hormones such as PTH, which increase cAMP intracellular formation, may rely on factors that may not be present or functional in isolated MMVs. We therefore conducted stationary in vivo microperfusion experiments in individual PCTs of rats that were exposed or not to 20 nM GLP-1 (Fig. 3B). As indicated in Fig. 3B, 20 nM GLP-1 in the luminal fluid produced a great reduction in rate of bicarbonate flux (J_{HCO_3}) compared with control animals. No significant differences were observed in the rate of bicarbonate reabsorption that was insensitive to the specific inhibitor of NHE3 activity, S3226 (37). These results lead to the conclusion that GLP-1 significantly decreases NHE3-mediated bicarbonate reabsorption in the intact renal proximal tubule.

Role of cAMP-dependent PKA in mediating the inhibitory effect of GLP-1 on NHE3 transport activity. Given that GLP-1 infusion significantly enhanced urinary cAMP excretion (see Fig. 1), we next sought to examine whether the inhibitory effect of GLP-1 on NHE3-mediated bicarbonate reabsorption in proximal tubule was dependent on activation of the cAMP/ PKA signaling pathway. By means of stationary in vivo microperefusion, we were able to verify that the PKA inhibitor H89 (10 μM) fully prevented the inhibitory effect of GLP-1 on bicarbonate reabsorption in the renal proximal tubule (Fig. 4). When H89 was added alone to the luminal fluid, no influence was noticed in the baseline rate of bicarbonate reabsorption (Fig. 4).

Kocinsky and colleagues (24) recently developed phospho-specific mAbs that only recognize NHE when the serine 552 is phosphorylated (anti-PS552-NHE3) or when the serine 605 is phosphorylated (anti-PS605-NHE3). The generation of these mAb allowed demonstrating that those two consensus sites for PKA, present at the NHE3 COOH-terminal region, are physiologically regulated both in vitro (24) and in vivo (23, 24). We therefore test the hypothesis that activation of the GLP-1R in the proximal tubule that led to increases of cAMP accumulation and, subsequently, to PKA activation enhanced the levels of phosphorylated NHE3. As illustrated in Fig. 5, continuous infusion of GLP-1 into the systemic circulation increased the levels of phosphorylated NHE3 relative to total NHE3.
(pNHE3/total NHE3) at the proximal tubule brush-border microvilli at both PKA consensus sites. It is important to emphasize that NHE3 expression in MMV of GLP-1-treated rats was unchanged.

Taken together, these results suggest that modulation of NHE3 transport activity by the incretin hormone GLP-1 is mediated via PKA and is associated with increased levels of NHE3 phosphorylation at the PKA consensus sites 552 and 605.

Effect of incretin mimetics and enhancers on renal function. Taking into account that the clinical use of GLP-1 is limited, we also evaluated the renal effects of the incretin mimetic exendin-4 and of the incretin enhancer P32/98. The dose-dependent effects of intraperitoneal injections of exendin-4 or DPPIV inhibitor P32/98 on urinary cAMP excretion are shown in Fig. 6. Exendin-4 dose dependently elevated urinary cAMP excretion in rats and maximal cAMP accumulation was found at doses ≥5.0 μg/kg (Fig. 6A). The effects of single doses of the DPPIV inhibitor P32/98 on mean cAMP urine excretion were much less pronounced than the ones produced by exendin-4 (Fig. 5B). Higher cAMP urine excretion was reached at the dose of P32/98 (Fig. 6B). Based on these results, the doses of 5.0 μg/kg exendin-4 and 50 mg/kg P32/98 were employed in our study.

Table 2 summarizes the effect of the incretin mimetic exendin-4 and of the incretin-enhanced P32/98 on rat renal function and systemic acid-base parameters. The intraperitoneal administration of the GLP-1R agonist exendin-4 (5 μg/kg) or the DPPIV inhibitor P32/98 (50 mg/kg) caused a significant increase of urine flow, sodium and potassium excretion, and lithium clearance compared with controls (Table 2). Additionally, both exendin-4-treated rats and rats treated with P32/98 exhibited increased GFR and RPF compared with controls. Overall, the effects of the incretin mimetic exendin-4 on rat renal function were much more pronounced than the ones induced by the incretin enhancer P32/98. Additionally, exendin-4-treated rats exhibited increased GFR and RPF, whereas in rats treated with P32/98 these parameters did not reach statistical difference compared with controls. Both exendin-4 and P32/98 increased bicarbonate excretion. Consequently, urine pH was higher in rats treated with these drugs than in controls. However, as seen in Table 2, these changes in renal bicarbonate handling did not significantly affect the systemic...
Table 2. Effect of intraperitoneal injection of the incretin mimetic Ex-4 and the incretin enhancer P38/98 on rat renal function and systemic acid-base parameters

<table>
<thead>
<tr>
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<th>CTRL (n = 9)</th>
<th>Ex-4 (n = 6)</th>
<th>P32/98 (n = 6)</th>
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<tr>
<td><strong>Body wt, g</strong></td>
<td>240 ± 4</td>
<td>238 ± 8</td>
<td>239 ± 8</td>
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<td><strong>UO, µl-min⁻¹-kg⁻¹</strong></td>
<td>28.3 ± 0.7</td>
<td>62.5 ± 2.9†</td>
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<td><strong>GFR, ml-min⁻¹-kg⁻¹</strong></td>
<td>3.6 ± 0.2</td>
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<td><strong>RPF, ml-min⁻¹-kg⁻¹</strong></td>
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<td><strong>FE Na⁺, %</strong></td>
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<td><strong>FE K⁺, %</strong></td>
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<td><strong>Cl⁻, mmol-min⁻¹-kg⁻¹</strong></td>
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<td>2.61 ± 0.37†</td>
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<td><strong>FE HCO₃⁻, %</strong></td>
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<td><strong>Urine pH</strong></td>
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<td><strong>Plasma HCO₃⁻, mM</strong></td>
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<tr>
<td><strong>Plasma pH</strong></td>
<td>7.33 ± 0.01</td>
<td>7.29 ± 0.03</td>
<td>7.31 ± 0.02</td>
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Values are means ± SE. *P < 0.05 and †P < 0.01 vs. CTRL. Assessed by ANOVA followed by Tukey’s post hoc test. Defined terms as in Table 1 legend.

acid-base status of these animals. Collectively, these data suggest that, similarly to GLP-1, the incretin mimetic exendin-4 and the incretin enhancer P32/98 acutely inhibit NaHCO₃ reabsorption in rat renal proximal tubule.

Effect of exendin-4 and P32/98 on NHE3 transport activity and levels of phosphorylation. Microperfusion experiments were undertaken to test the hypothesis that the incretin-based agents exendin-4 (50 nM) and P32/98 (10 µM) affect in vivo NHE3 transport activity. As illustrated in Fig. 7, exendin-4 significantly inhibited the rate of JHCO₃⁻ in luminally perfused proximal tubules compared with controls. Interestingly, local inhibition of DPPIV by the P32/98 compound did not significantly affect proximal tubular bicarbonate reabsorption (Fig. 7).

We next evaluated whether systemic administration of these incretin-based agents would alter in vivo NHE3 phosphorylation status. As seen in Fig. 8, MMVs isolated from rats treated with exendin-4 (5 µg/kg), the DPPIV inhibitor P32/98 (50 mg/kg), or vehicle were analyzed by immunoblotting using the phosphospecific antibodies anti-PS552-NHE3 and anti-PS605-NHE3. These analyses showed that both exendin-4 and P32/98 increased the ratio of phosphorylated NHE3/total NHE3 at the PKA consensus sites 552 and 605. Similar to the results obtained by renal function evaluation, the effects of exendin-4 on NHE3 phosphorylation levels were more prominent than the ones caused by P32/98 (Fig. 8).

In concert, these results suggest that both exendin-4 and P32/98 are capable of regulating NHE3 when systemically administered. When these compounds are added to luminal fluid of the proximal tubule, only the GLP-1R agonist exendin-4 is capable of inhibiting NHE3 transport activity. These results strengthen our previous hypothesis that DPPIV inhibitors modulate NHE3 by increasing the half-life of a circulating endogenous peptide that tonically inhibits the activity of the transporter.

DISCUSSION

Several lines of evidence demonstrate that continuous administration of GLP-1 induces diuresis and natriuresis in both humans (19, 20) and experimental animal models (29, 40); however, the molecular and cellular mechanisms mediating the
renal actions of this incretin hormone have not been established. In the present work, we demonstrate that systemic infusion of GLP-1 increases GFR and RPF and decreases proximal tubular sodium, bicarbonate, and water reabsorption in part by inhibiting NHE3-mediated Na\(^{+}\)/H\(^{+}\) exchange. Inhibition of NHE3 seems to be due to increased intracellular cAMP accumulation, PKA activation, and phosphorylation of the exchanger’s COOH-terminal region at the PKA consensus sites, serines 552 and 605.

The GLP-1R is a G-coupled receptor whose activation stimulates adenylyl cyclase via Gsα leading to an increase in cyclic adenosine monophosphate and PKA activation. Our study shows that the effect of GLP-1 to reduce proximal tubular bicarbonate reabsorption is accompanied by a significant increase of NHE3 phosphorylation at serines 552 and 605 in MMV. Although acute NHE3 inhibition by PKA requires phosphorylation of the exchanger at serines 552 and 605 (24), phosphorylation per se does not seem to be sufficient to inhibit NHE3 activity (23). One possibility by which PKA phosphorylation may subsequently result in NHE3 inhibition is by altering NHE3 redistribution along the microdomains of the kidney brush border. This appears not to be the case, since the increase of NHE3 phosphorylation levels induced by GLP-1 is not accompanied by a decrease of NHE3 expression at the microvillar microdomain of the brush border, suggesting that the mechanism by which GLP-1 inhibits NHE3 activity does not involve subcellular trafficking of the exchanger. An alternative explanation is that phosphorylation modulates association of NHE3 with regulatory proteins. A number of proteins have been identified to interact with NHE3 and play a role in regulation of the transporter (11). Whether any of these regulatory proteins preferentially associate with NHE3 when it is phosphorylated contributing to PKA-mediated inhibition of transport function has yet to be experimentally demonstrated.

Our primary interest in studying the mechanisms by which GLP-1 induces diuresis and natriuresis in rats arose from our previous studies showing that NHE3 and DPPIV physically (15) and functionally (16, 17) interact in the renal proximal tubule. The fact that systemic administration of DPPIV inhibitors reduces the activity of NHE3 raised the hypothesis that DPPIV may degrade a peptide that inhibits the activity of the transporter (16, 33). The findings presented here strongly support this hypothesis, although they still do not validate it. Future studies are needed to evaluate whether the GLP-1/GLP-1R complex mediates the physical and/or the functional interaction between NHE3 and DPPIV in the proximal tubule.

Consistent with previous findings (29), we found that GLP-1 infusion alters renal handling not only of sodium but also of potassium. The primary event in potassium urinary excretion is tubular secretion by the cells of the distal tubule and collecting duct (28). The finding that the GLP-1R is only expressed in the glomerulus and in the PCT suggests that the increased potassium excretion in response to GLP-1 might be due to an indirect effect that stimulates potassium secretion such as a rise in the flow of tubular fluid and increased sodium delivery to the distal nephron.

The diuretic and natriuretic effects of GLP-1 are also mediated by changes of renal hemodynamics. The fact that this peptide increases both GFR and RPF suggests that GLP-1 might exert a direct effect on the renal vasculature, most likely by decreasing the resistance of the precapillary capillaries. The vasoactive actions of GLP-1 have been demonstrated in a variety of vascular beds including conduit and resistance vessels and the vasorelaxant effects induced by this gut peptide seem to occur by both endothelial-dependent (4, 32) and endothelial-independent mechanisms (18, 31). Moreover, ongoing studies from our laboratory showed that GLP-1 induces a dose-dependent relaxant effect on isolated rat renal artery rings that appears to be mediated, at least in part, via stimulation of cAMP (Couto GK, Pacheco BP, Rossoni LV, Girardi AC, unpublished data).

The coexistence of hypertension with type II diabetes is a prevalent condition that substantially increases the risk of cardiovascular morbidity and mortality (3, 4, 13, 38). A recent review pointed out that the chronic use of some of the available antidiabetic drugs may, in fact, promote negative cardiovascular outcomes in subjects, despite improvement in hyperglycemia. Therefore, the authors emphasized the importance of the antidiabetic therapy to be targeted not only at glycemic control, but also at reduction of the risk of cardiovascular complications and mortality (3). In this regard, numerous compelling studies, including ours, established that the incretin agents promote cardioprotective, vasoprotective, and renoprotective effects as well (4, 6, 21, 27, 31, 33, 40). Herein, we demonstrate that the beneficial effects of the incretin agents on renal function are mediated by an increase of GFR and RPF and by a decrease of proximal tubular NHE3 function. Our findings further support the view that the GLP-1-based therapeutics may also have a potential clinical usefulness in hypertension and other disorders of sodium retention.

In summary, the results of the present study suggest that binding of GLP-1 to its receptor GLP-1R activates the cAMP/PKA signaling pathway, leading, in turn, to phosphorylation of the PKA consensus sites at the NHE3 COOH-terminal region. Subsequently, inhibition of NHE3-mediated Na\(^{+}\)/H\(^{+}\) exchange in proximal tubule decreases sodium, bicarbonate, and water reabsorption. The same signaling cascade might be triggered to affect the vascular renal resistance leading to an increase in renal blood flow and GFR.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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