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ORIGINAL RESEARCH

Role of Renal Sympathetic Nerves in GLP-1 (Glucagon-Like Peptide-1) Receptor Agonist Exendin-4-Mediated Diuresis and Natriuresis in Diet-Induced Obese Rats

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BACKGROUND: The gut-derived hormone GLP-1 (glucagon-like peptide-1) exerts beneficial effects against established risk factors for chronic kidney disease. GLP-1 influences renal function by stimulating diuresis and natriuresis and thus lowering arterial blood pressure. The role of the sympathetic nervous system has been implicated as an important link between obesity with elevated arterial pressure and chronic kidney disease. The primary aim of this study was to determine the contribution of renal sympathetic nerves on intrapelvic GLP-1-mediated diuresis and natriuresis in high-fat diet (HFD)-induced obese rats.

METHODS AND RESULTS: Obesity was induced in rats by HFD for 12 weeks, followed by either surgical bilateral renal denervation or chronic subcutaneous endopeptidase neprilysin inhibition by sacubitril for a week. Diuretic and natriuretic responses to intrapelvic administration of the GLP-1R (GLP-1 receptor) agonist exendin-4 were monitored in anesthetized control and HFD rats. Renal GLP-1R expression and neprilysin expression and activity were measured. The effects of norepinephrine on the expression of GLP-1R and neprilysin in kidney epithelial LLC-PK1 cells were also examined. We found that diuretic and natriuretic responses to exendin-4 were significantly reduced in the HFD obese rats compared with the control rats (cumulative urine flow at 40 minutes, 387±32 versus 650±65 μL/gkw; cumulative sodium excretion at 40 minutes, 42±5 versus 75±10 μEq/gkw, $P<0.05$). These responses in the HFD rats were restored after ablation of renal nerves (cumulative urine flow at 40 minutes, 625±62 versus 387±32 μL/gkw; cumulative sodium excretion at 40 minutes, 70±9 versus 42±5 μEq/gkw, $P<0.05$). Renal denervation induced significant reductions in arterial pressure and heart rate responses to intrapelvic GLP-1 in the HFD rats. Renal denervation also significantly increased the GLP-1R expression and reduced neprilysin expression and activity in renal tissues from the HFD rats. Chronic subcutaneous neprilysin inhibition by sacubitril increased GLP-1–induced diuretic and natriuretic effects in the HFD rats. Finally, exposure of the renal epithelial cells to norepinephrine in vitro led to downregulation of GLP-1R expression but upregulation of neprilysin expression and activity.

CONCLUSIONS: These results suggest that renal sympathetic nerve activation contributes to the blunted diuretic and natriuretic effects of GLP-1 in HFD obese rats. This study provides significant novel insight into the potential renal nerve–neprilysin–GLP-1 pathway involved in renal dysfunction during obesity that leads to hypertension.

Key Words: glucagon-like peptide-1 ■ neprilysin ■ obesity ■ renal nerve ■ sodium retention

Increasing evidence shows that obesity is associated with elevated arterial blood pressure, which is a major driver of various cardiovascular and kidney diseases.[1,2] In obesity, abnormal renal function and excessive tubular sodium reabsorption are thought to initiate hypertension. Elevated arterial pressure (AP) further causes renal injury and worsens the development of drug-resistance hypertension. The mechanisms of obesity-associated chronic kidney disease (CKD) are complex and multifactorial such as oxidative stress,

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CLINICAL PERSPECTIVE

What Is New?

- We found that renal sympathetic nerve activation led to (1) blunted diuretic and natriuretic responses to intrapelvic GLP-1 (glucagon-like peptide-1) injection in obese rats and (2) decreased renal GLP-1 receptor expression but increased expression and activity of the neutral endopeptidase neprilysin in obese rats.
- Chronic subcutaneous neprilysin inhibition increased GLP-1–induced diuretic and natriuretic effects in obese rats.
- Direct action of norepinephrine led to downregulation of GLP-1 receptor expression but upregulation of neprilysin expression and activity in kidney epithelial cells in vitro.

What Are the Clinical Implications?

- This finding provides potential therapeutics targeting the renal nerve–neprilysin–GLP-1 pathway to treat obesity-associated chronic kidney disease and hypertension.

Nonstandard Abbreviations and Acronyms

AP	arterial pressure
DPP-4	dipeptidyl-peptidase IV
GLP-1	glucagon-like peptide-1
GLP-1R	glucagon-like peptide-1 receptor
HFD	high-fat diet
HR	heart rate
RDN	renal denervation

inflammation, lipotoxicity, activated renin-angiotensin system and adipocyte-derived hormones are involved.[3–5] The role of the sympathetic nervous system has been also well documented in linking obesity with elevated blood pressure and CKD.[6,7] Particularly, increased renal sympathetic nerve activity in individuals with obesity is thought to contribute to the increases in renin secretion and sodium reabsorption and consequently lead to elevated AP. It has been reported that renal denervation (RDN) attenuates sodium retention and normalizes AP in obese animals, suggesting a critical role for renal nerves in the development of obesity-associated hypertension and CKD.[8,9] However, the underlying mechanisms related to the renal sympathetic nerve activation and renal dysfunction in obesity have not been fully elucidated.

GLP-1 (glucagon-like peptide-1) is an incretin hormone released by endocrine L-cells of distal gut. It has been shown that GLP-1 plays important role in the maintenance of glucose homeostasis via insulin stimulation and inhibition of glucagon secretion.[10,11] GLP-1 improves glucose sensitivity in pancreatic beta cells, stimulates beta-cell proliferation and reduces food intake. The actions of GLP-1 are mediated by its specific receptor, the GLP-1 receptor (GLP-1R), which is expressed in pancreatic beta cells, as well as in various other tissues. Recently, several GLP-1R agonists or dipeptidyl-peptidase IV (DPP-4) inhibitors have been introduced for management of hyperglycemia in type 2 diabetes.[12,13] The GLP-1R is also present in various sites within the kidneys, including the proximal tubular cells.[14] Recent studies using GLP-1R agonist show that GLP-1 exerts potential renoprotective effects in patients with diabetic nephropathy.[15,16] The protective actions on the kidneys of GLP-1R agonist in CKD may be through its diuretic and natriuretic effects and thus lowering AP, which are thought to be independent of its glucose-lowering effects.

GLP-1 has diuretic and natriuretic effects, likely involving the increasing in renal plasma flow and glomerular filtration rate, and inhibition of the sodium-hydrogen exchanger 3 in the proximal tubular cells.[17] In addition, some studies indicate a role for the modulation of the renin-angiotensin system and the neural pathway.[18,19] Our previous studies have indicated that the renal nerve contributes to the epithelial sodium channel–mediated sodium and water retention in the higher sympathetic activation states, such as myocardial infarct–induced chronic heart failure.[20] RDN alleviates renal sodium and water retention and improves renal function in rats with chronic heart failure. Recently, we also reported that the excessive activation of neural circuitry of afferent and efferent renal sympathetic nerves suppressed diuretic and natriuretic responses to GLP-1.[21] However, the direct effects and modulation of GLP-1 actions within the kidney by renal nerves in the obese condition have not been elucidated.

Some studies have revealed that GLP-1 can be degraded and inactivated by neprilysin, a widely expressed neutral endopeptidase, which is upregulated in metabolically altered states such as obesity and type 2 diabetes.[22,23] Reducing neprilysin could be beneficial for patients with diabetes by increasing active GLP-1 levels. It is reported that removing the influence of the renal nerves on renal function attenuates neprilysin activity in the heart failure condition.[24] Based on this, the present study was designed to investigate the contributions of renal nerves in the modulation of GLP-1-mediated diuresis and natriuresis in high-fat diet (HFD)-induced obesity. Further, the potential renal nerve–neprilysin–GLP-1 axis pathway involved in renal dysfunction during obesity in rats was also assessed.

METHODS

All supporting data are available within the article.

Animals

All the procedures on animals were approved by the Institutional Animal Care and Use Committee of the University of South Dakota. The experiments were in accordance with the American Physiological Society and the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

Male Sprague-Dawley rats weighing 130 to 150 g (age 6–7 weeks) were obtained from Envigo (Indianapolis, IN) and housed in a 12-hour light/dark cycle. Rats were fed with either HFD (TD.88137, 42% of calories are from fat; Envigo) or regular chow as the control group. Body weight and food consumption were monitored weekly. All the acute experiments were performed after 13 weeks of exposure to a high-fat or normal diet.

Renal Denervation

Twelve weeks after HFD or normal chow fed, a cohort of HFD and control rats underwent RDN under anesthesia (2%–3% isoflurane inhalation). Kidneys were exposed through a retroperitoneal flank incision. Complete RDN was achieved by cutting the visible renal nerves around the renal artery and vein bilaterally, followed by painting the exposed vessels with 70% ethanol. This method has been shown to ablate the afferent and efferent renal nerves.[20,25] Renal tissue norepinephrine content was measured to confirm the completeness of RDN. All the experiments were performed 1 week after RDN (13 weeks after induction of obesity).

Measurement of Renal Norepinephrine Content

Rats were euthanized at 19 to 20 weeks of age. The kidneys were removed, and renal tissue was homogenized using 1 mmol/L EDTA and 4 mmol/L sodium metabisulfite in 0.1 N HCl. Norepinephrine concentration in the supernatants was measured using a commercially available ELISA kit (LSBio, Seattle, WA), following the manufacturer's instructions. The analytical sensitivity for the norepinephrine concentration was 2.5 ng/mL. The norepinephrine content was expressed as nanograms per gram of kidney weight.

Measurements of Visceral Fat, Plasma Glucose, and GLP-1 Levels

After the rats were euthanized, the visceral fat was collected and immediately weighed. Plasma glucose level was monitored weekly from the tail blood sample and measured by Accu-chek. The blood samples used for GLP-1 measurement were supplemented with DPP-4 inhibitor (MilliporeSigma, Burlington, MA) and protease inhibitor cocktail (Promega, Madison, WI). Plasma GLP-1 level was measured by chemiluminescence ELISA kit (ALPCO, Salem, NH). The analytical sensitivity for the GLP-1 concentration was 1.5 pg/mL.

Implantation of Osmotic Minipump for Neprilysin Inhibitor Infusion

In separate groups of control and HFD rats, a ALZET osmotic minipump (model 2001; Durect Corporation, Cupertino, CA) was implanted subcutaneously under isoflurane anesthesia in the dorsal neck region for systemic infusion of either saline or neprilysin inhibitor, sacubitril (MilliporeSigma), at a rate of 1 μg/kg per minute. After 7 days of infusion (13 weeks after induction of obesity), rats were subjected to acute experiments.

Hemodynamic Measurement, Intrapelvic Injection, and Urine Collection

On the day of the acute experiment, rats were anesthetized with Inactin (100 mg/kg IP). Body temperature was controlled at 36 to 38°C by a heated stage. After tracheal intubation, the left femoral vein was cannulated with PE-50 tubing for administration of supplemental anesthesia and isotonic saline. The femoral artery was cannulated and connected to the MacLab (ADInstruments, Colorado Springs, CO) for computer-based recording of AP and heart rate (HR).

The left kidney was exposed through a retroperitoneal flank incision. The renal pelvis was cannulated with a 32-G double-lumen catheter (ReCathCo, Allison Park, PA) via the ureter for intrapelvic injection and urine collection, concomitantly. Surgery was completed within 30 minutes. An additional 30 minutes stabilization period was allowed before the start of the first urine collection. Urine was collected in preweighed tubes from left kidney, and urine volume was measured gravimetrically.

Measurements of Diuretic and Natriuretic Responses to Intrapelvic GLP-1 Injection

In 4 groups of rats (control, HFD, control+RDN, and HFD+RDN; n=6/group), 2 urine collections (10 minutes each) were obtained over 20 minutes to represent a baseline period. After baseline collection, 10 μL of isotonic saline (as volume control) was injected via the pelvic catheter, and urine was collected over 10 minutes. Then, a bolus dose of GLP-1 agonist exendin-4 (1 μg, Cayman Chemical, Ann Arbor, MI) in 10 μL isotonic saline was injected. After injection of exendin-4, urine was collected at 10-, 20-, 30-, and 40-minute time points. Urine volume was measured by subtracting

the weight of the tube before collecting urine from the weight of the tube containing urine. Sodium concentration of each urine sample was analyzed (Horiba Sodium Ion Meter, Kyoto, Japan). At the end of the experiments, rats were euthanized, and kidneys were harvested. Similar procedures were also performed in the set of rats with chronic subcutaneous neprilysin inhibitor sacubitril infusion (control+saline, HFD+saline, control+sacubitril, HFD+sacubitril; n=6/group).

Western Blot Analysis of GLP-1R and Neprilysin Expression in the Kidney

Renal tissue (≈100 mg) from cortex was homogenized in 400 µL of RIPA buffer containing 1% protease inhibitor cocktail. The total protein concentrations were measured with a bicinchoninic acid assay kit (Pierce, Rockford, IL); 4× loading buffer was added and 50 µg protein samples were loaded onto SDS-PAGE gel and subjected to electrophoresis and transferred to a polyvinylidene fluoride membrane. Then, the membrane was incubated with primary antibody (mouse anti-GLP-1R, 1:250, sc-390774; mouse anti-neprilysin, 1:500, sc-46656; mouse anti-β-actin, 1:1000, sc-8432; Santa Cruz Biotechnology, Santa Cruz, CA) overnight. After the incubation with secondary antibody conjugated with fluorescent dye (1:10 000, Thermo Fisher Scientific, Waltham, MA), the bands were detected using an Odyssey scanner (LI-COR Biosciences, Lincoln, NE). The intensity of the band was quantified using ImageJ software (National Institutes of Health, Rockville, MD). The protein expression was calculated as the ratio of the intensity of the protein to the intensity of β-actin.

Immunofluorescence Staining for GLP-1R and Neprilysin in the Kidney

Kidneys were dissected and fixed in HistoChoice MB tissue fixative (Amresco, Solon, OH) and dehydrated in 70% ethanol. Paraffin-embedded renal tissue sections from rats were processed according to standard histochemical methods. Antigen retrieval was performed by boiling slides in 10 mmol/L calcium citrate, followed by permeabilization, and then blocking. The primary antibody (mouse anti-GLP-1R, 1:100; mouse anti-neprilysin, 1:200, Santa Cruz Biotechnology) was incubated with the sections overnight, and the next day the sections were incubated with Alexa Fluor 594 donkey anti-mouse IgG (1:500, Jackson ImmunoResearch, West Grove, PA) for 2 hours. The sections were coverslipped with Vectashield mounting medium (Vector Laboratory, Burlingame, CA). The fluorescent images of the kidney were imaged using a Leica SP8 lightning confocal microscope (Leica, Germany). The intensity of fluorescence signaling was measured in 4 randomly chosen high-power (×400 magnification) fields per kidney within the cortex and pelvis using ImageJ software.

Renal Neprilysin Activity Assay

Neprilysin enzyme activity in renal tissue homogenates (100 µg protein) was measured with a fluorometric assay using a commercially available assay kit (MilliporeSigma). The neprilysin activity assay kit uses the ability of an active neprilysin to cleave a synthetic o-aminobenzoyl peptide substrate (Abz-based peptide) to release a free fluorophore. The released Abz was quantified using a fluorescence microplate reader (PerkinElmer Victor, Waltham, MA). The assay kit can detect as low as 20 µU/mg of neprilysin activity.

In Vitro Studies

Direct Effects of Norepinephrine on the Expression of GLP-1R and Neprilysin

Kidney epithelial LLC-PK1 cells (ATCC CL-101, Manassas, VA) were grown in Medium 199 (MilliporeSigma) with 3% fatal bovine serum. Cells were maintained at 37°C and 5% CO_2 until 70% to 80% confluent. Cells were then maintained in the medium without serum for differentiation purpose. Cells were treated with norepinephrine (MilliporeSigma) at a concentration range of 0.32 to 200 ng/mL or vehicle for 24 hours. Each treatment was repeated 4 times. Cultured cells were subject to protein extraction procedure and Western blot analysis for GLP-1R and neprilysin protein expression. Neprilysin activity in culture cells with norepinephrine treatment was also measured.

Statistical Analysis

All data are presented as means±SE. For comparison of values in >2 groups, statistical significance was determined by 1-way ANOVA followed by comparison for individual group differences with the Tukey's test or 2-way ANOVA for repeated measurements using Prism 7 (GraphPad Software, La Jolla, CA). For comparison of values between 2 groups, the Student unpaired *t* test was performed. $P<0.05$ was considered statistically significant.

RESULTS

General Characteristics of Control and HFD rats

Table summarizes the salient characteristics of the control and HFD groups used in the present study. After 13 weeks of HFD there was increased body weight, retroperitoneal fat pad weight, and epididymal fat pad weight. There were no significant differences in the weight of kidneys between groups. The level of

Table. General Characteristics of Control and HFD Rats With/Without RDN

	Control (n=8)	HFD (n=9)	Control+RDN (n=10)	HFD+RDN (n=10)
Body weight, g	404±24	483±21*	391±35	467±20
Kidney weight, g	1.18±0.08	1.27±0.11	1.04±0.05	1.20±0.09
Retroperitoneal fat pad, g	4.7±0.5	12.6±2.4*	4.3±0.4	13.4±2.1
Epydidymal fat pad, g	4.0±0.6	9.0±1.3*	3.8±0.5	9.6±0.6
Plasma glucose, mg/dL	130±21	183±22*	124±9	192±18
Plasma GLP-1, pg/mL	31.6±5.7	29.5±2.7	32.4±4.6	31.2±1.8
Kidney norepinephrine content, ng/g	189±36	401±42*	8±3†	15±5†
Baseline MAP, mm Hg	92±4	104±3*	90±2	100±5
Baseline HR, beat/min	351 + 21	396 + 20*	337±26	374±22

Values are mean±SE. HFD indicates high-fat diet; HR, heart rate; MAP, mean arterial pressure; and RDN, renal denervation.
*P<0.05 vs control group.
†P<0.05 vs without RDN group.

plasma glucose was significantly higher in the HFD rats compared with those in the control rats. The baseline of mean AP (MAP) and HR significantly increased in the HFD fed rats. The data confirmed that 13 weeks of HFD induced hyperlipidemia, hyperglycemia, and hypertension in the rats. There were no significant differences in the plasma GLP-1 levels between the control and HFD rats.

Kidney norepinephrine content was significantly greater in the HFD rats compared with the Controls. RDN reduced the kidney content of norepinephrine to very low levels in both control and HFD rats, which confirms the completeness of the RDN procedure. However, after 1 week RDN did not significantly change the visceral fat, plasma glucose, GLP-1 levels, baseline MAP, and HR in both control and HFD groups.

Effects of RDN on Diuretic and Natriuretic Responses to Pelvic Injections of GLP-1 Agonist

To assess the functional alteration of GLP-1 in the HFD rats, with and without RDN, the diuretic and natriuretic responses to the intrapelvic GLP-1R agonist

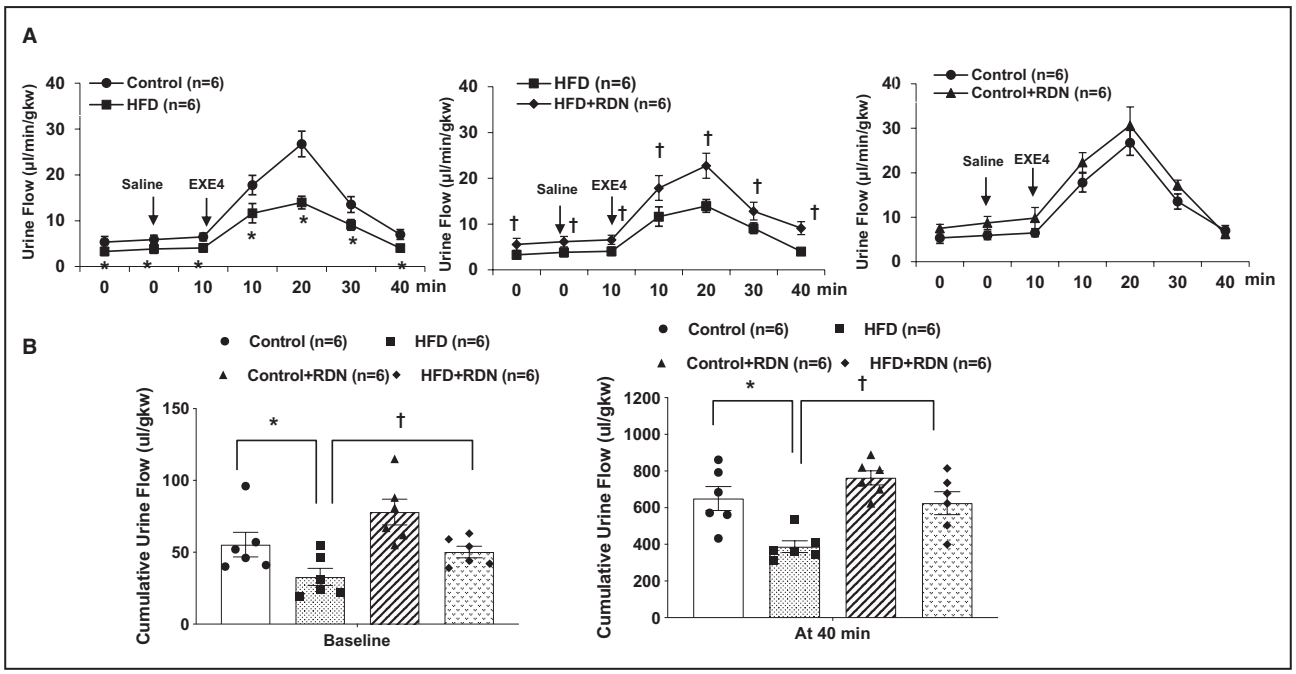

Figure 1. Effects of renal denervation (RDN) on diuretic response to pelvic injection of GLP-1 (glucagon-like peptide-1) agonist.
A, Urine flow in response to GLP-1 agonist exendin-4 (EXE4) injection in control and high-fat diet (HFD) rats with/without RDN. **B**, Cumulative urine flow at baseline and 40 minutes after exendin-4 injection in each group. *P<0.05 vs respective control group; †P<0.05 vs without RDN.

exendin-4 were monitored. The basal urine flow and sodium excretion before intrapelvic injections was significantly different between the control and HFD groups (cumulative urine flow at baseline 55.3±8.6 versus 32.8±5.9 μL/gkw per 10 minutes, n=6, *P*=0.0480; cumulative sodium excretion at baseline 6.1±1.3 versus 2.4±0.6 μEq/gkw per 10 minutes, n=6, *P*=0.0282) (Figures 1 and 2). As vehicle control, saline injection in the pelvic did not significantly increase the urine volume. Pelvic GLP-1 agonist injection produced diuresis and natriuresis in both groups of rats. However, both diuresis and natriuresis responses were significantly blunted in the HFD group compared with the corresponding control group after GLP-1 injection (cumulative urine flow at 40 minutes 387.3±32.1 versus 650.2±65.2 μL/gkw, n=6, *P*=0.0047; cumulative sodium excretion at 40 minutes 42.8±5.1 versus 75.0±10.3 μEq/gkw, n=6, *P*=0.0192) (Figures 1 and 2).

RDN significantly increased GLP-1-mediated urine flow and sodium excretion in the HFD group (cumulative urine flow at 40 minutes 625.3±62.3 versus 387.3±32.1 μL/gkw, n=6, *P*=0.0068; cumulative sodium excretion at 40 minutes 70.2±9.0 versus 42.8±5.1 μEq/gkw, n=6, *P*=0.0254) (Figures 1 and 2). RDN did not change the GLP-1–mediated urine flow and sodium excretion in the control rats. There were no significant differences between GLP-1–mediated urine flow and sodium excretion in the control+RDN and HFD+RDN groups.

Effects of RDN on AP and HR in Responses to Pelvic Injection of GLP-1 Agonist

To assess the hemodynamic responses to intrarenal GLP-1, AP, and HR responses to the GLP-1R agonist exendin-4 were monitored. The pelvic exendin-4 injection initially increased MAP and HR (first 1–2 minutes), followed by a rapid decrease, which lasted longer (≈30–40 minutes) in both the control and HFD groups. It was observed that the magnitude of the reduction in MAP and HR were significantly blunted in the HFD group compared with the control rats after GLP-1 agonist injection (ΔMAP –10.3±1.3 versus –27.5±1.8 mm Hg, n=6, *P*<0.0001; ΔHR –24.0±2.7 versus –60.8±5.9 bpm, n=6, *P*=0.0002) (Figure 3). These blunted hemodynamic responses in the HFD rats were abrogated by RDN (ΔMAP –20.8±2.5 versus –10.3±1.3 mm Hg, n=6, *P*=0.0045; ΔHR –40.0±5.2 versus –24.0±2.7 bpm, n=6, *P*=0.021) (Figure 3).

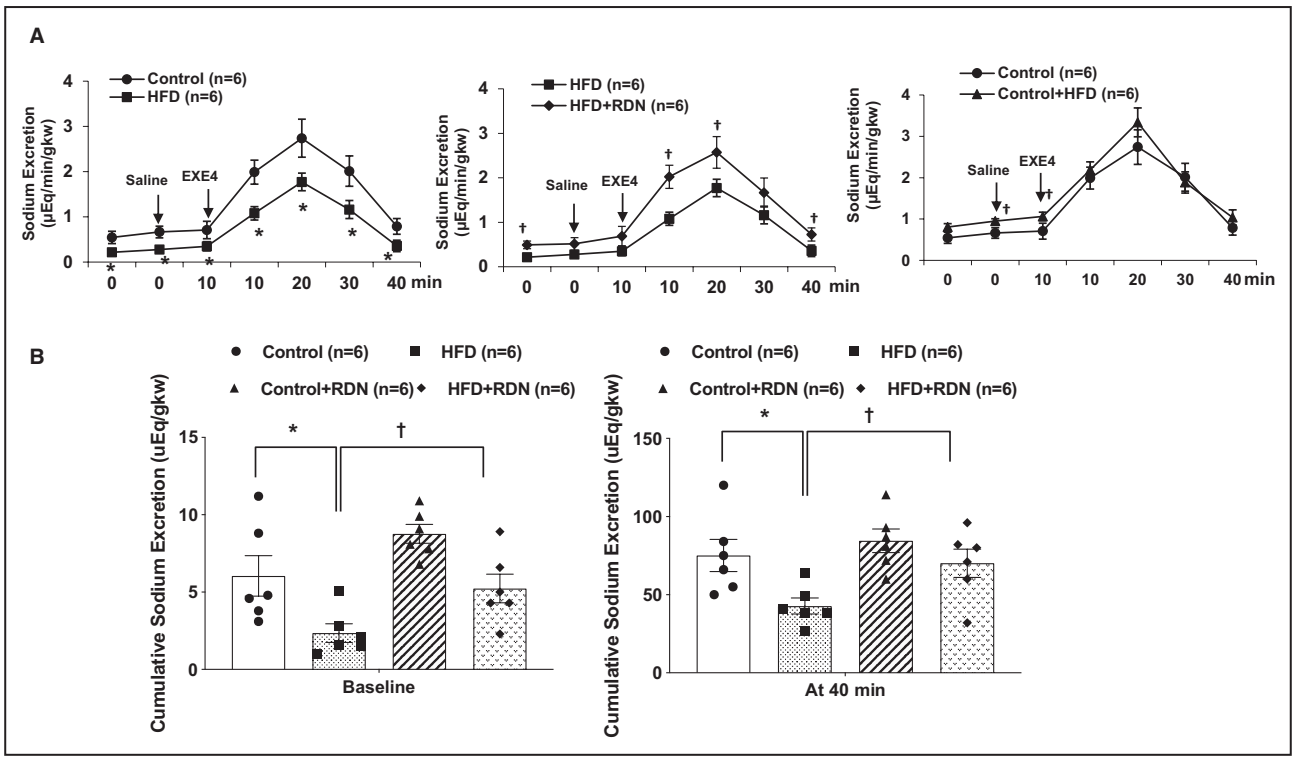

Figure 2. Effects of renal denervation (RDN) on natriuretic response to pelvic injection of GLP-1 (glucagon-like peptide-1) agonist.

A, Sodium excretion in response to pelvic GLP-1 agonist exendin-4 injection in control and high-fat diet (HFD) rats with/without RDN. **B**, Cumulative sodium excretion at baseline and 40 minutes after exendin-4 injection in each group. **P*<0.05 vs respective control group; †*P*<0.05 vs without RDN.

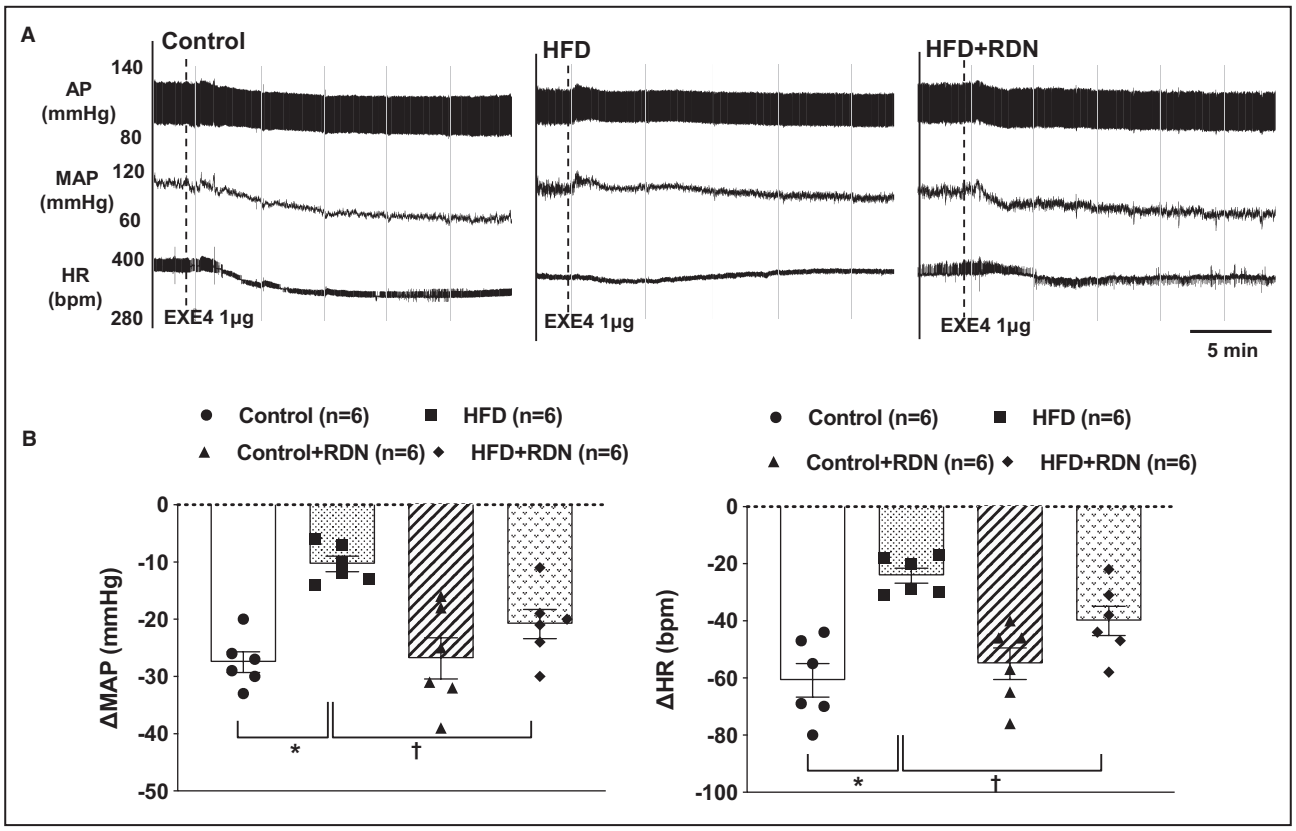

Figure 3. Effects of renal denervation (RDN) on arterial pressure (AP) and heart rate (HR) in responses to pelvic injection of GLP-1 (glucagon-like peptide-1) agonist.
A, AP, mean AP (MAP) and HR in response to pelvic GLP-1 agonist exendin-4 (EXE4) injection in control and high-fat diet (HFD) rats with/without RDN. **B**, Mean MAP and HR changes after exendin-4 injection in each group. **P*<0.05 vs respective control group; †*P*<0.05 vs without RDN.

Effect of RDN on GLP-1R Expression in the Kidney of HFD Rats

The immunofluorescent staining showed reduced fluorescent signal for GLP-1R within the pelvis and renal cortex of HFD rats (Figure 4A). Consistent with these observations the western blot analysis showed that HFD rats had significantly lower protein levels of GLP-1R in the renal tissue compared with the controls (ratio of intensity: 0.86±0.10 versus 1.40±0.09, n=4, *P*=0.0074). RDN significantly enhanced the renal GLP-1R expression of HFD rats (ratio of intensity: 1.30±0.14 versus 0.86±0.10, n=4, *P*=0.0411) (Figure 4B).

Effect of RDN on Neprilysin Expression and Activity in the Kidneys of HFD Rats

The immunofluorescent staining showed increased fluorescent signal for the neutral endopeptidase neprilysin within the renal cortex in the HFD rats (Figure 4A). Western blot analysis showed significantly higher levels of neprilysin protein in the kidneys of HFD rats compared with the controls (ratio of intensity, 0.73±0.07 versus 0.32±0.06, n=4, *P*=0.0046) (Figure 4B). Consistent with these observations, the enzyme activity of neprilysin also significantly increased in the HFD rats (1.337±0.062 versus 0.768±0.039 mU/min per mg, n=6, *P*<0.0001) (Figure 4C). RDN reduced neprilysin expression (ratio of intensity, 0.49±0.08 versus 0.73±0.07, n=4, *P*=0.0482) and activity (0.913±0.055 versus 1.337±0.062 mU/min per mg, n=6, *P*=0.0005) in the renal tissues of HFD rats to control levels (Figure 4B and 4C).

Effects of Neprilysin Inhibition on Diuretic and Natriuretic Responses to Pelvic Injection of GLP-1 Agonist

Chronic neprilysin inhibition by sacubitril significantly increased GLP-1–mediated urine flow and sodium excretion in rats with HFD compared with the HFD rats with chronic saline infusion (vehicle infusion controls) (cumulative urine flow at 40 minutes, 533.7±62.2 versus 357.5±30.4 μL/gkw, n=6, *P*=0.0290; cumulative sodium excretion at 40 minutes, 65.2±4.4 versus 43.3±4.2 μEq/gkw, n=6, *P*=0.0053) (Figure 5). Neprilysin inhibitor sacubitril did not significantly change the GLP-1–mediated urine flow and sodium

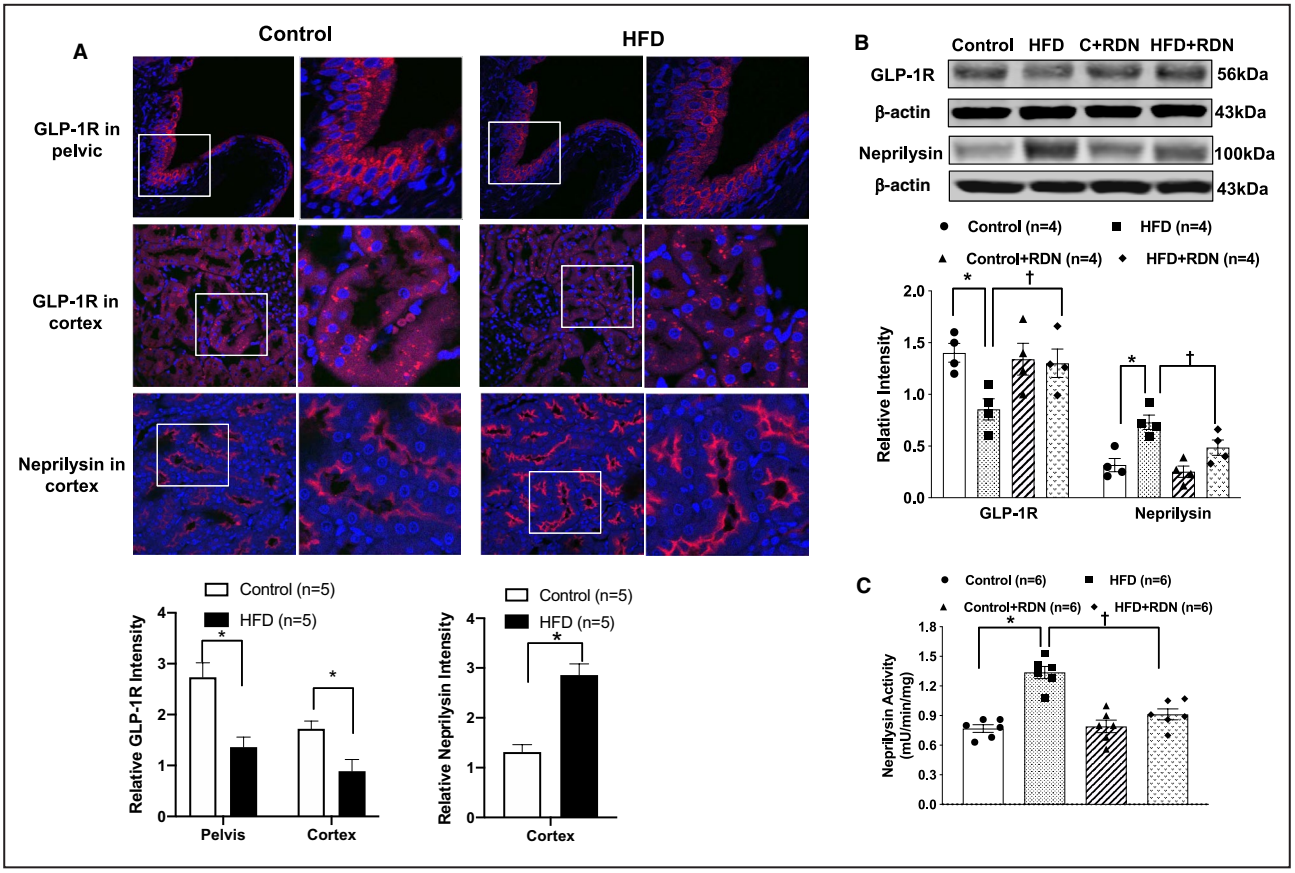

Figure 4. Effects of renal denervation (RDN) on GLP-1R (glucagon-like peptide-1 receptor) and neprilysin expression in the kidney of high-fat diet (HFD) rats.

A, Immunofluorescent microscopy of renal sections for GLP-1R (red staining) (top, pelvis; middle, cortex) and neprilysin (red staining, bottom panel) in the kidney from ontrol and HFD rats (magnification, ×400, right panel image showing the magnification image of small box). Blue: DAPI for nucleus; (**B**) Representative western blots and mean values of GLP-1R and neprilysin protein expression in the cortex of renal tissue from control, HFD, control+RDN, and HFD+RDN rats. **C**, Neprilysin activity in the renal tissue from control, HFD, control+RDN, and HFD+RDN rats. **P*<0.05 vs respective control group; †*P*<0.05 vs without RDN. C indicates control.

excretion in the control rats. There were no significant differences between GLP-1–mediated urine flow and sodium excretion in the control+sacubitril and HFD+sacubitril groups.

Direct Effects of Norepinephrine on the Expression of GLP-1R and Neprilysin (In Vitro)

Western blot analysis revealed that norepinephrine treatment for 24 hours significantly reduced GLP-1R protein expression in cultured kidney LLC-PK1 cells, in vitro (ratio reduced to 0.28±0.03 at norepinephrine dose 200 ng/mL, n=4, *P*<0.0001) (Figure 6A). Conversely, norepinephrine treatment increased the expression of neprilysin protein (ratio increased to 1.90±0.09 at norepinephrine dose 200 ng/mL, n=4, *P*<0.0001) (Figure 6A), as well as neprilysin activity (ratio increased to 3.16±0.36 at norepinephrine dose 200 ng/mL, n=4, *P*<0.0001) (Figure 6B) in the cultured kidney LLC-PK1 cells.

DISCUSSION

Previously, we have shown that increased efferent renal sympathetic nerve activity negates the diuresis and natriuresis produced by GLP-1.[26] The present study was designed to investigate the contributions of renal sympathetic nerve activation on intrapelvic GLP-1–mediated diuresis and natriuresis in the HFD-induced obese rats. The major findings in this study showed that renal nerve activation led to (1) blunted diuretic and natriuretic responses to intrapelvic GLP-1 injection in the HFD rats and (2) decreased GLP-1R expression but increased expression and activity of neutral endopeptidase neprilysin in the renal tissues from the HFD rats. As a corollary chronic subcutaneous neprilysin inhibition by sacubitril significantly increased GLP-1–induced diuretic and natriuretic effects in the HFD rats. Finally, direct action of norepinephrine led to downregulation of GLP-1R expression but upregulation of neprilysin expression and activity in kidney epithelial cells in vitro.

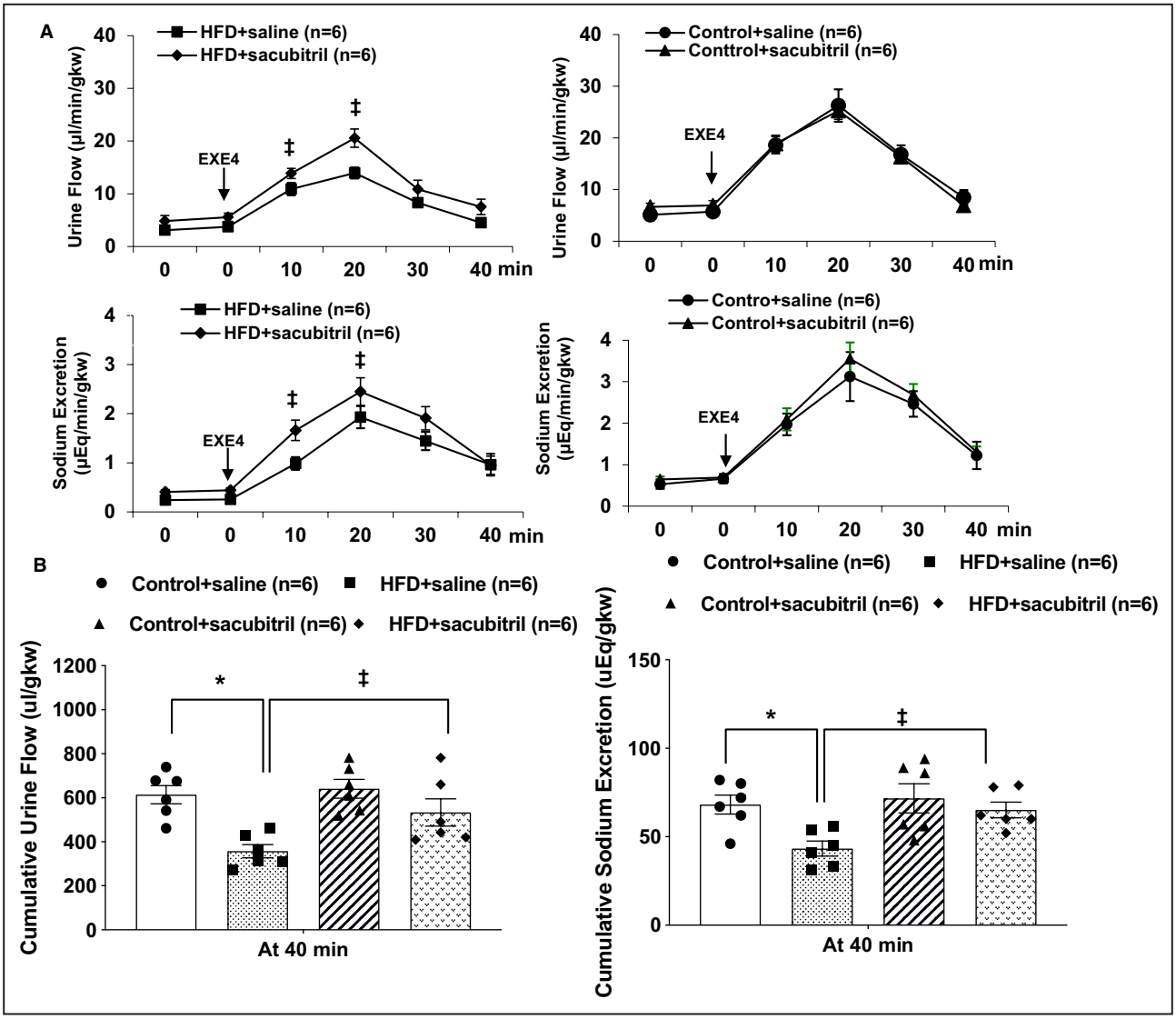

Figure 5. Effects of neprilysin inhibition on diuretic and natriuretic responses to pelvic injections of GLP-1 (glucagon-like peptide-1) agonist.

A, Urine flow and sodium excretion in response to pelvic GLP-1 agonist exendin-4 injection in control and high-fat diet (HFD) rats with/without subcutaneous neprilysin inhibitor sacubitril infusion. **B**, Cumulative urine flow at baseline and 40 minutes after exendin-4 injection in each group. **P*<0.05 vs respective control group; ‡*P*<0.05 vs without sacubitril.

The gut-generated hormone GLP-1 has been shown to have hemodynamic and renoprotective capacity besides its better characterized glucoregulatory actions.[15] GLP-1 can induce diuresis and natriuresis via inhibition of sodium-hydrogen exchanger 3 in the renal proximal tubule and increasing the renal plasma flow and glomerular filtration rate in healthy individuals.[27,28] However, there have been several studies addressing the changes in response to GLP-1 in disease conditions. Some of these studies suggest that GLP-1–induced renal diuresis and natriuresis are attenuated in spontaneously hypertensive rats compared with normotensive rats.[29,30] It is observed that obese db/db mice and diabetic mice have lower glomerular filtration rate changes in response to GLP-1/exendin-4 infusion. The renal vasodilator response to GLP-1R activation could be blunted in diabetes.[31] Obesity and diabetes may impair the beneficial effects of GLP-1 treatments, with the clear implication that the population who will most commonly be treated with GLP-1–related agents may not accrue the expected metabolic benefits.[31] In the current study, we observed that a direct intrapelvic injection of GLP-1R agonist exendin-4 increased urine volume and sodium excretion in both control and HFD obese rats. However, these responses were significantly blunted in the obese rats. This indicates that there is an altered GLP-1 mechanism related to the impaired kidney excretory function and sodium retention

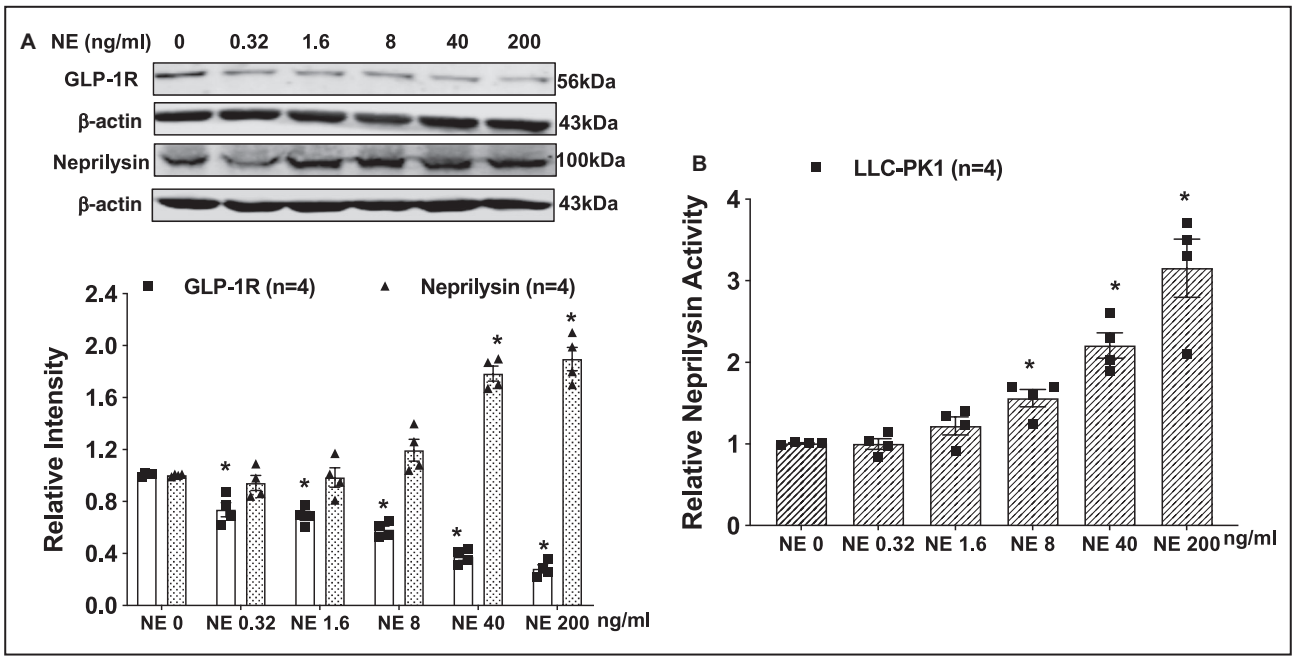

Figure 6. Direct effects of norepinephrine (NE) on the expression of GLP-1R (glucagon-like peptide-1 receptor) and neprilysin (in vitro).
A, Representative gel and mean protein expressions of GLP-1R and neprilysin after NE incubation in kidney epithelial LLC-PK1 cells. The cells were incubated with NE (0.32–200 ng/mL) for 24 hours. **B**, Neprilysin activity of kidney LLC-PK1 cells after incubation with NE for 24 hours. *P<0.05 vs control group without NE treatment.

in the obese condition. As a long-acting GLP-1 analogue, exendin-4 is more resistant to DPP-4 cleavage and has a significant effect on the kidney than GLP-1.[17,31]

We investigated the plasma GLP-1 levels and GLP-1R expression in the kidneys of HFD rats. We have found that GLP-1R was mainly expressed in the renal pelvis and cortex by immunohistochemistry. Although the plasma GLP-1 level was not significantly different after HFD feeding, the protein expression of GLP-1R within the kidney decreased in the HFD rats. This may be one explanation for the mechanism by which there would be blunted diuretic and natriuretic response to GLP-1 agonist, since there is reduced expression of GLP-1R within the kidneys of the obese rats. This altered mechanism may contribute to the development and progression of CKD in obesity. Exendin-4 induces diuresis and natriuresis via activation of the GLP-1R.[32] Although some studies show that DPP-4 inhibition protects the kidney against nephrotoxin through both GLP-1R–dependent and GLP-1R–independent mechanisms,[33] the activation of GLP-1/GLP-1R system is critical to excrete sodium load and suppress diabetic kidney injury.[34]

Multiple lines of evidence indicate that increased renal sympathetic nerve activity contributes to hypertension during obesity and obesity-associated CKD.[6,7] RDN has been demonstrated to markedly reduce sodium retention and hypertension in obese animals and obese patients with resistant hypertension.[8] Besides the direct effects on the kidney, various studies including ours have reported that GLP-1 regulates diuresis and natriuresis via neural mechanisms as well.[18,26] Previously, we have shown that GLP-1 was regulated in an inhibitory manner by neural circuitry of afferent and efferent renal nerves. Diuretic and natriuretic responses to intravenous infusion of GLP-1 were enhanced by total RDN through cutting both afferent and efferent renal nerves.[26] In the present study, the results showed that bilateral RDN significantly improved GLP-1–induced diuretic and natriuretic effects in obese rats, suggesting that the renal nerve can modulate GLP-1 function in the kidney. RDN also increased the expression of GLP-1R in obese rats. These results suggest that the higher tonic sympathetic activation in obese rats produces inhibitory effects on GLP-1/GLP-1R system in the obese condition. The dysregulation of GLP-1–related neural mechanisms may contribute to renal sodium and fluid retention observed in obesity.

In this study, we also observed the hemodynamic changes in response to the intrapelvic GLP-1 injection. We have seen that pelvic GLP-1 agonist injection transiently increased AP and HR in both control and HFD rats. This transient pressor effect of GLP-1 observed in the present study may be because of the GLP-1–mediated autonomic effects via the afferent renal sympathetic nerve to increase sympathetic nerve activity to the heart and arterioles. Previous other

studies have shown that systemic acute administration of GLP-1R agonist likely produces a temporal increase in AP and HR in rodents.[26,35,36] Pharmacologic studies support the role of central GLP-1R–mediated pressor responses in rodents via the central nervous system reflex pathway.[36,37]

In our study, after transient increasing in AP and HR, there was a rapid and substantial decrease of AP and HR in both control and HFD rats. This is consistent with some other reports showing that administration of GLP-1R agonists increases diuresis and natriuresis and consequently elicits a modest decrease in AP.[38,39] Systemic administrated GLP-1 may also bind cardiac GLP-1R to promote the secretion of atrial natriuretic peptide to elicit a decrease in systemic blood pressure.[40] It should be noted that most of the previous observations are based on the intravenous administration of GLP-1 or GLP-1R agonist. In contrast, our current results are based on the observation of the direct intrapelvic GLP-1 agonist injection to reveal the effects of GLP-1/GLP-1R system in the kidney on the hemodynamics and renal function. The blood pressure–lowering effects of GLP-1 observed in the present study are more likely attributed to the extended and increased urine volume. Moreover, we found that the blood pressure–lowering effects of GLP-1 in obese rats were significantly attenuated. However, after renal nerve ablation, the hemodynamic responses of intrarenal GLP-1 in the HFD rats recovered markedly. This might be because of the alleviation of sympathetic activation to enhance the blunted renal GLP-1/GLP-1R system function in the obese condition.

To explore the potential mechanism related to the role of sympathetic renal nerves on GLP-1/GLP-1R system function, we further investigated the interactions of renal nerve, endopeptidase neprilysin and GLP-1 related to the regulating of renal function. Some studies report that neprilysin activity is increased in plasma and metabolic tissues of mice with diet-induced obesity.[41,42] Neprilysin inhibition could be beneficial for patients with diabetes by increasing active GLP-1 levels by preventing the neprilysin-mediated proteolysis of GLP-1 and reducing DPP-4 activity.[42] Neprilysin can cleave active GLP-1 at 6 sites in the central and C-terminal regions, eliminating its ability to bind to the GLP-1R. In our study, we found higher levels of neprilysin expression and activity in the kidneys of obese rats. Chronic inhibition of neprilysin by sacubitril improved GLP-1-mediated diuretic and natriuretic activity in obese rats, suggesting that the pharmacologic neprilysin inhibition was associated with elevated GLP-1 activity in the kidney.

Obesity is accompanied by increased activation of both the sympathetic nervous system and the renin-angiotensin system to cause overactivity of neprilysin.[43] Another study has shown that RDN significantly inhibits renal neprilysin activity in spontaneously hypertensive rats and in rats with heart failure.[24] Further, chronic treatment with adrenergic β_1 receptor antagonist showed an inhibition of renal neprilysin activity in rats with heart failure.[24] Our results showed that RDN reduced renal neprilysin expression and activity in obese rats, suggesting the effects of tonic sympathetic activity on the neprilysin. To explore the novel observation of renal nerve–neprilysin–GLP-1 axis pathway, our in vitro study provided further evidence that norepinephrine directly increased neprilysin expression and activity and reduced GLP-1R expression in the kidney epithelial cells. Taken together, this evidence supports the hypothesis that renal sympathetic nerve activation may modulate GLP-1 activity via increasing neprilysin in the kidney. It may well be that RDN improves GLP-1 function via inhibition of neprilysin activity.

Our studies provide evidence that enhanced renal sympathetic nerve activation in the HFD obese rats contributes to the blunted diuretic and natriuretic effects of GLP-1. This may well be mediated by renal nerve–neprilysin–GLP-1 pathway to alter the effects of GLP-1 in the kidneys of obese rats (Figure 7). The combination treatment of GLP-1 agonist and neprilysin inhibition may provide additional therapeutic benefits for patients with obesity-associated CKD and hypertension. We also speculate that RDN may provide a novel targeted therapeutic technique, giving additional

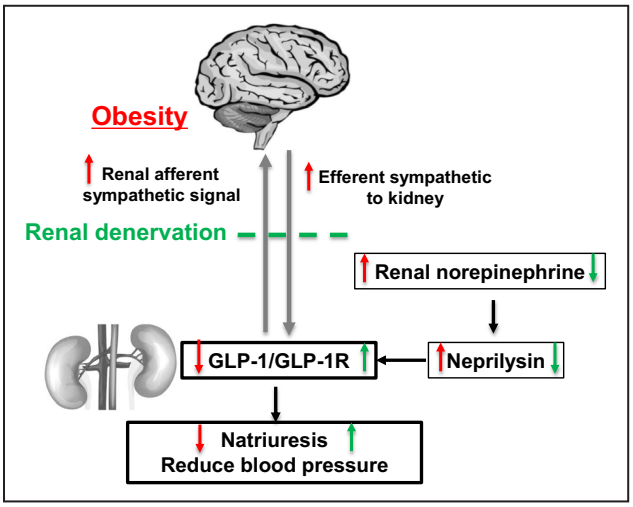

Figure 7. Schematic graph shows the proposed mechanism that increased renal sympathetic nerve activation contributes to the blunted renal diuretic and natriuretic effects of GLP-1 (glucagon-like peptide-1) in the high-fat diet (HFD) obese rats.

This may be via renal nerve–neprilysin–GLP-1 axis pathway to alter the effects of GLP-1 in the kidneys of obese rats. Red arrows indicate the changes attributable to obesity and green arrows indicate the changes elicited by renal denervation (RDN) in obesity.

insight into potential beneficial effects to treat obesity-associated CKD and hypertension.

ARTICLE INFORMATION

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Disclosures

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