Intrarenal Ghrelin Infusion Stimulates Distal Nephron-Dependent Sodium Reabsorption in Normal Rats


Abstract—Ghrelin is a 28-amino acid peptide hormone that exerts powerful orexigenic effects. Ghrelin receptor expression has been reported in the kidney, but the role of ghrelin in the kidney is unknown. The present studies confirmed ghrelin receptor mRNA expression in the kidneys of normal Sprague Dawley rats (n=6) using reverse transcription polymerase chain reaction (PCR) and sequencing of the 588-bp PCR product. To test intrarenal ghrelin action, uninephrectomized rats received 3 cumulative 1-hour renal interstitial (RI) infusions of 5% dextrose in water (vehicle, n=21), ghrelin (n=10), ghrelin plus specific ghrelin receptor antagonist [D-Lys-3]-GHRP-6 (n=24), or [D-Lys-3]-GHRP-6 alone (n=32). Mean arterial pressure (MAP), urine sodium excretion rate (UNaV), glomerular filtration rate (GFR), fractional excretion of sodium (FE\textsubscript{Na}), and fractional excretion of lithium (FE\textsubscript{Li}) were calculated for each period. RI ghrelin infusion significantly decreased UNaV to 86±4.9% (P<0.05), 74±6.5% (P<0.01), and 62±10% (P<0.01) of baseline during periods 1 to 3, respectively. Ghrelin also significantly decreased FE\textsubscript{Na} to 68±11% (P<0.05), 57±8.6% (P<0.01), and 59±12% (P<0.01) of baseline, without changing GFR or FE\textsubscript{Li}. Identical ghrelin infusions in the presence of [D-Lys-3]-GHRP-6 failed to permit reductions in UNaV or FE\textsubscript{Na}. Following [D-Lys-3]-GHRP-6 infusion alone, UNaV increased from 0.06±0.01 to 0.18±0.03 µmol/min (P<0.0001). Concomitant increases in FE\textsubscript{Na} were also observed (0.23±0.03% to 0.51±0.06% [P<0.01]), without changes in MAP, GFR, or FE\textsubscript{Li}. Together, these data introduce a novel intrarenal ghrelin-ghrelin receptor system, which, on activation, significantly increases Na\textsuperscript{+} reabsorption at the level of the distal nephron. (Hypertension. 2011;57[part 2]:633-639.)

Key Words: sodium ■ kidney ■ tubular transport ■ ghrelin ■ obesity ■ hypertension

Ghrelin is a 28-amino acid peptide hormone characterized by a novel postranslational acylation that ensures bioactivity\textsuperscript{1} (Figure 1). It is secreted into the circulation by the stomach and small intestine but may also be synthesized by the pancreas, adrenal glands, and other tissues.\textsuperscript{2} Ghrelin is the most potent known endogenous orexigenic peptide, with a greater abundance in distal compared to proximal epithelia.\textsuperscript{3} Hypothalamic ghrelin receptors mediate these orexigenic effects, but recent studies have also confirmed widespread distribution of ghrelin receptors in tissues relevant to cardiovascular control.\textsuperscript{4–9}

Ghrelin receptors have been identified in the myocardium,\textsuperscript{6} brain stem,\textsuperscript{10} blood vessels,\textsuperscript{4,11} and spinal cord.\textsuperscript{12} Intravenous bolus administration of ghrelin significantly reduces mean arterial pressure (MAP) in rodents\textsuperscript{11} and healthy volunteers,\textsuperscript{6} while direct intrathecal stimulation of ghrelin receptors on sympathetic preganglionic neurons increases MAP.\textsuperscript{12} The hypotensive effect of peripherally administered ghrelin is thought to occur directly, since vasorelaxation has been demonstrated in isolated blood vessels.\textsuperscript{5,14} Also consistent with a peripheral site of action is the observation that intravenously administered ghrelin reduces MAP without affecting renal sympathetic nerve activity (RSA) in conscious rabbits.\textsuperscript{5} However, microinjection of ghrelin into the nucleus solitarius tract (NTS) reduces MAP and suppresses RSA, suggesting that part of the ghrelin-induced reduction in MAP may be due to central sympathoinhibitory effects as well.\textsuperscript{5,15}

Ghrelin and ghrelin receptor expression have also been identified in the kidneys of humans and rodents. Preproghrelin mRNA is expressed in glomeruli, cultured mesangial cells, and podocytes.\textsuperscript{16} Immunoelectron microscopy images localize ghrelin to tubular rather than glomerular cells,\textsuperscript{17} with a greater abundance in distal compared to proximal epithelia.\textsuperscript{17,18} Studies using reverse-phase high-performance liquid chromatography (coupled with radioimmunoassay) demonstrate 10 times greater ghrelin immunoreactivity in kidneys compared to plasma,\textsuperscript{16} suggesting that ghrelin is produced locally in the kidney. Ghrelin receptor mRNA has also been confirmed in the kidney using intron-spanning primers,\textsuperscript{16} although other studies have reported conflicting results regarding the presence of ghrelin receptors in the kidney.\textsuperscript{19,20}

The central purpose of the current investigations is to define the functional role of ghrelin in normal kidneys. MAP responses are often determined by renal regulation of sodium
(Na\(^+\)) homeostasis, and the present studies demonstrate that renal interstitial (RI) acyl ghrelin infusion increases distal nephron-dependent Na\(^+\) reabsorption. Furthermore, intrarenal blockade of ghrelin receptors induces a significant increase in urine sodium excretion rate (UNaV), suggesting a tonic effect of endogenous ghrelin to regulate Na\(^+\) reabsorption in normal rodents.

**Methods**

**Animal Preparation**

The experimental protocols were approved by the Animal Care and Use Committee at the University of Virginia and were conducted on 12-week-old female Sprague Dawley (SD) rats (Harlan; n=87) that were housed in a vivarium under controlled conditions (temperature 21±1°C; humidity 60±10%; light 8:00 AM to 8:00 PM). Rats were placed under short-term anesthesia with ketamine and xylazine via an intraperitoneal injection, and a retro-peritoneal flank incision was made to remove the right kidney using a sterile technique. Following a 72-hour recovery, the rats were placed under general anesthesia with pentobarbital (50 mg/mL) given 5 mg/100 g of body weight via an intraperitoneal injection. A tracheostomy was performed to assist respiration, and the right internal jugular vein was cannulated with PE-10 tubing to provide intravenous access through which vehicle (5% dextrose in water [D5W]) with and without inulin and lithium (Li\(^+\)) chloride was infused at 20 \(\mu\)L/min. The hydration state was identical for all protocols included in these studies. Direct cannulation of the right carotid artery with PE-50 tubing provided arterial access for monitoring MAP. Following a midline laparotomy, a microcatheter (PE-10) was inserted into the ureter of the remaining (left) kidney to collect urine for the quantification of UNaV. Experiments were initiated at the same time each day to prevent any effect of diurnal variation in blood pressure (BP).

**BP Measurements**

MAP was measured by the direct intracarotid method with the use of a digital BP analyzer (Micromed), recorded every 5 minutes, and averaged for all periods of study.

**Renal Cortical Interstitial Infusion**

An open-bore microinfusion catheter (PE-10) was inserted under the renal capsule into the cortex of the remaining (left) kidney for the RI infusion of either D\(_5\)W or a pharmacological agent at 2.5 \(\mu\)L/min with a syringe pump (Harvard; model 55-222) as reported previously. When >1 agent was simultaneously infused into the kidney, the coinfusion was performed via the PE-10 catheter.

**Figure 1.** Schematic representation of acyl ghrelin formation.

**Figure 2.** Direct RI infusion of acyl ghrelin significantly reduces UNaV in normal rats. Coinfusion of the ghrelin receptor antagonist [D-Lys-3]-GHRP-6 abolishes the effect. A, UNaV in response to RI vehicle infusion with D\(_5\)W (\(\square\), n=6), cumulative RI acyl ghrelin infusion (0.3 to 3 \(\mu\)g/min) (\(\square\), n=10), and the coinfusion of acyl ghrelin (0.3 to 3 \(\mu\)g/min) plus [D-Lys-3]-GHRP-6 (6 \(\mu\)g/min) (\(\square\), n=14). B, MAP responses to the corresponding infusions in A. Results are reported as percentage of corresponding baseline value. Data represent mean±1 SE; *P<0.05, **P<0.01 from corresponding baseline value.
kidney, separate interstitial catheters were used. Vetbond tissue adhesive (3M Animal Care Products) was used to secure the catheter and prevent interstitial pressure loss.

**Pharmacological Agents**

Rat acyl ghrelin (0.3 and 3 μg/min) and [D-Lys-3]-GHRP-6 (4, 6, and 8 μg/min), a specific and potent antagonist of ghrelin receptors (IC₅₀=0.9 μmol/L), were purchased from Tocris and infused directly into the RI space. The dose of acyl ghrelin was based on the tenets that the normal rat kidney receives ~20% to 25% of the cardiac output and that the volume of RI fluid in a normal rat kidney is ~350 μL. Thus, acyl ghrelin was calculated at one fourth of the dose given systemically to decrease MAP in normal subjects, corrected for the volume of distribution in the rat cortical interstitium. The concentration of [D-Lys-3]-GHRP-6 was based on the IC₅₀ of the compound, ensuring adequate blockade of intrarenal ghrelin receptors.

**Measurement of Glomerular Filtration Rate, Fractional Excretion of Sodium, and Fractional Excretion of Lithium**

Urinary and plasma Na⁺ and Li⁺ concentrations were measured using a flame photometer (Instrumentation Laboratory 943). Glomerular filtration rate (GFR) was measured by inulin clearance using a method described previously. Tubular Na⁺ reabsorption was determined by calculating the fractional excretion of sodium (FEₙa), and renal proximal tubule Na⁺ reabsorption was estimated using the fractional excretion of lithium (FEₗi) (maximum error of 4%), as published previously.

**Effects of RI Ghrelin, Coinfusion of Ghrelin and MAP**

12-week–old SD rats were studied on normal Na⁺ intake (0.28% Na⁺). Seventy-two hours after undergoing uninephrectomy, the remaining kidney was infused for 1 hour with 5% dextrose in water (D₅W). This period was designated as the control period. Following the 1-hour D₅W infusion, the kidney was infused directly into the RI space for 3 consecutive 1-hour experimental periods with one of the following: (1) continued D₅W at 2.5 μL/min, n=21; (2) acyl ghrelin alone (0.3 μg/min during the first period, followed by 3 μg/min during the last 2 periods), n=10; (3) acyl ghrelin (0.3 and 3 μg/min) plus [D-Lys-3]-GHRP-6 (6 μg/min), n=24; or (4) [D-Lys-3]-GHRP-6 (4, 6, or 8 μg/min) alone, n=32. Inulin and Li⁺ chloride in D₅W were infused throughout the study via the internal jugular catheter. UNaV, GFR, FEₙa, FEₗi, and MAPs were calculated and/or recorded for each period.

**Determination of Renal Ghrelin Receptor mRNA**

Reverse transcription-polymerase chain reaction (RT-PCR) was used to determine the presence of ghrelin receptor mRNA in normal kidneys from 12-week–old female SD rats (n=6). Under anesthesia, the kidneys were harvested, and total RNA was extracted using TRIZol reagent (Invitrogen). RT was performed using 2 μg of total RNA in a 20 μL volume according to the manufacturer’s instructions (RT2 First Strand cDNA Synthesis kit; SuperArray). PCR was performed in 50-μL volumes using 2 μL of cDNA obtained in the RT reactions and 25 μL of the 2× GoTaq polymerase mix (Promega). The following primers were used: rat ghrelin receptor sense 5’-CGTGAAGCTGGTCATCCTTGT-3’ and antisense 5’-GAACCTCTACCTCTGAAGTGTT-3’ (both at 2.5 μmol/L; Eurofins mwg operon), yielding a 588-bp product. Following an initial activation cycle at 94°C for 4 minutes, the reaction consisted of 40 cycles with denaturation at 94°C (30 seconds), annealing at 60°C (30 seconds), and extension at 72°C (60 seconds), followed by a final extension at 72°C (5 minutes). The PCR samples were separated on a 1.2% agarose gel along with a low–molecular-weight DNA ladder (New England Biolabs). The gel was stained with SYBR-Gold (Invitrogen) and scanned on a Typhoon 9400 scanner (Molecular Dynamics). To determine the identity of the 588-bp PCR product, it was sequenced using the following protocol. The PCR samples were

**Figure 3.** Direct RI infusion of acyl ghrelin significantly decreases FEₙa without affecting the GFR or FEₗi. The reduction in FEₙa induced by ghrelin is not observed in the presence of ghrelin receptor blockade. A, GFR in response to RI vehicle infusion with D₅W (n=15), cumulative RI acyl ghrelin infusion (0.3 to 3 μg/min) (n=10), and the coinfusion of acyl ghrelin (0.3 to 3 μg/min) plus [D-Lys-3]-GHRP-6 (6 μg/min) (n=10). B, FEₙa in response to the corresponding infusions in A. C, FEₗi in response to the corresponding infusions in A. Results are reported as percentage of corresponding baseline value. Data represent mean±1 SE; *P<0.05, **P<0.01, ***P<0.001 from corresponding baseline value.
separated on a 1.2% agarose gel and stained with ethidium bromide. The band at 588 bp was excised from the gel, and the DNA was extracted with a QIAquick Gel Extraction kit (Qiagen) and sequenced by direct sequencing with BigDye Terminator v3.1 labeling chemistry with AmpliTaq polymerase; FS (Applied Biosciences). The sequence obtained shared 99.3% identity with the known rat ghrelin receptor sequence.

Statistical Analysis
Statistically significant differences among D5W, ghrelin receptor blocker [D-Lys-3]-GHRP-6, acyl ghrelin, and [D-Lys-3]-GHRP-6 plus acyl ghrelin effects were estimated by ANOVA, including a repeated-measures term, by using the general linear models procedure of SAS (version 9.1; SAS Institute Inc). Multiple comparisons of individual pairs of effect means were conducted by the use of a least-square means pooled variance. Data are expressed as mean±1 SE. Statistical significance was defined at P<0.05.

Results

Effects of RI Acyl Ghrelin Alone and RI Coinfusion of [D-Lys-3]-GHRP-6 Plus Acyl Ghrelin on UNaV and MAP in Normal SD Rats
As demonstrated in Figure 2A, RI acyl ghrelin infusion (n=10) significantly decreased UNaV to 86±4.9% (P<0.05), 74±6.5% (P<0.01), and 62±10% (P<0.01) of baseline during experimental periods 1 to 3, respectively. RI coinfusion of acyl ghrelin plus [D-Lys-3]-GHRP-6 (n=14) abolished the decrease in UNaV seen with acyl ghrelin alone. RI D5W infusion (n=6) had no effect on UNaV. MAP responses remained unchanged following all RI infusions (Figure 2B).

Renal Function Studies in Response to RI Acyl Ghrelin Alone and RI [D-Lys-3]-GHRP-6 Plus Acyl Ghrelin Infusion in Normal SD Rats
As demonstrated in Figure 3B, RI acyl ghrelin infusion (n=10) significantly decreased FENa to 68±11% (P<0.05), 57±8.6% (P<0.001), and to 59±12% (P<0.01) of baseline during experimental periods 1 to 3 respectively. RI coinfusion of acyl ghrelin plus [D-Lys-3]-GHRP-6 (n=10) abolished the decrease in FENa seen with acyl ghrelin alone. RI D5W infusion (n=15) had no effect on FENa. Concomitant reductions in GFR or FE(In) were not observed during RI acyl ghrelin, acyl ghrelin plus [D-Lys-3]-GHRP-6, or D5W infusions (Figure 3A and 3C).

Effects of RI [D-Lys-3]-GHRP-6 Infusion on UNaV, MAP, GFR, FENa, and FE(In) in Normal SD Rats
As demonstrated in Figure 4A, RI ghrelin receptor blockade significantly increased UNaV from a baseline of 0.06±0.01 µmol/min to 0.10±0.02 µmol/min (P<0.05), 0.15±0.02 µmol/min (P<0.001), and 0.18±0.03 µmol/min (P<0.0001), respectively, following 4, 6, and 8 µg/min of RI [D-Lys-3]-GHRP-6 infusions. As seen in Figure 5B, FENa significantly increased from a baseline value of 0.23±0.03% to 0.41±0.06% (P<0.01), 0.51±0.06% (P<0.001), and 0.39±0.06% (P<0.01) following 4, 6, and 8 µg/min of RI [D-Lys-3]-GHRP-6 infusion, respectively. The significant increases in UNaV and FENa following RI [D-Lys-3]-GHRP-6 infusion occurred in the absence of changes in MAP (Figure 4B), GFR, or FE(In) (Figure 5A and 5C).

Ghrelin Receptor Gene Expression in the Rat Kidney
As shown in Figure 6, ghrelin receptor mRNA was present in rat kidneys (n=6). In negative control experiments, where all the procedures were similar except that reverse transcriptase was omitted, no PCR product was obtained. Following sequencing, the 588-bp band amplified using RT-PCR was found to share 99.3% homology with the known rat ghrelin receptor sequence.

Discussion
To the best of our knowledge, this is the first study to define a direct functional role for ghrelin and its receptor in normal kidneys. Specific intrarenal ghrelin infusion significantly reduced UNaV in normal SD rats. The reduction in Na+ excretion rate was not accompanied by similar reductions in GFR, suggesting a direct tubular, rather than renal hemodynamic, mechanism of action. To localize the specific tubular site, events distal to the renal proximal tubule were identified by changes in FENa that occurred independently of changes in FE(In). In this light, RI ghrelin infusion significantly decreased FENa without altering FE(In), suggesting distal nephron-dependent Na+ reabsorption. Since ghrelin-induced Na+ reabsorption was not observed in the presence of ghrelin receptor blockade, the effect was mediated through intrarenal ghrelin receptors. Furthermore, RI antagonism of the ghrelin receptor alone significantly increased UNaV and FENa, without affecting FE(In). Thus, the ghrelin antagonist results strongly suggest a tonic effect of endogenous ghrelin to regulate tubular Na+ reabsorption at the level of the distal nephron.
Previous studies have reported conflicting results regarding the presence of ghrelin receptors in the kidney. Antibodies directed toward the ghrelin receptor lack specificity, resulting in heavy reliance on detection of ghrelin receptor message expression. The first study to report ghrelin receptor mRNA in the kidney utilized sense and antisense primers that were originally synthesized based on the known rat ghrelin receptor sequence.16 However, confirmation sequencing of the resultant 511-bp product was never reported in this study.16 Moreover, other investigators failed to demonstrate ghrelin receptor expression in the kidney utilizing alternate primer sequences.20 However, data from binding studies demonstrated strong binding and displacement of ghrelin and ghrelin mimetics in renal tissue.19 Because of these controversial results, the present study utilized intron-spanning primers directed against the known rat ghrelin receptor sequence, followed by direct sequencing of the 588-bp product. The product was found to share 99.3% homology with the known rat ghrelin receptor sequence, clearly demonstrating ghrelin receptor mRNA in renal tissues.

In the past, studies investigating ghrelin and the kidney have focused on changes in circulating levels of ghrelin, or salutary effects of systemic ghrelin administration in subjects with chronic kidney disease (CKD). Several investigators have observed that total ghrelin levels are increased in patients with CKD,28–31 and that desacylated ghrelin accounts for ≈90% of this increase.32 A pathogenic role has been suggested for desacyl ghrelin in the anorexia of hemodialysis patients,33 while acyl ghrelin administration (12 μg/kg) for 1 week has been shown to increase daily energy intake in malnourished dialysis patients.34 Similarly, in a rat model of CKD, treatment with acyl ghrelin increased food intake and lean body mass and decreased circulating inflammatory cytokines.35 None of these studies, however, administered ghrelin directly into the kidney to determine local effects on renal parameters such as GFR or Na⁺ excretion. The only reported effects of acyl ghrelin infusion on UNaV are in subjects with congestive heart failure, wherein 60 minutes of intravenous acyl ghrelin infusion resulted in a trend toward a reduction in UNaV.36 If the duration of this infusion had been extended, a statistically significant change in UNaV may have been observed.

The RI route and dose of acyl ghrelin used in the present studies did not alter MAP responses in normal rats. As
mentioned previously, the route of administration of acyl ghrelin often determines its effect on MAP, with intrathecal administration increasing MAP\textsuperscript{12} and intravenous\textsuperscript{4,14} and NTS administration\textsuperscript{5,15} reducing MAP. The lack of associated changes in MAP during RI infusions suggests that the infused compounds were confined to the kidney during the experimental periods. The doses of acyl ghrelin infused into the RI space were pharmacological and not physiological, based on reported circulating and intrarenal acyl ghrelin levels that are in the femtomoles per milliliter range.\textsuperscript{16}

To investigate the role of endogenous ghrelin in the kidney, the ghrelin receptor antagonist [D-Lys-3]-GHRP-6 was infused alone. The resultant increase in natriuresis was striking, and the magnitude of the effect was not dissimilar to the increase in UNaV observed under identical experimental conditions during renal angiotensin type-1 receptor (AT\textsubscript{1}R) blockade with candesartan.\textsuperscript{21} Candesartan-induced natriuresis is primarily attributed to inhibition of proximal tubular Na\textsuperscript{+} reabsorption,\textsuperscript{21,37} while ghrelin receptor antagonism results in distal nephron-dependent natriuresis. This raises the possibility of additive or even synergistic effects between AT\textsubscript{1}R blockers and ghrelin receptor antagonists.

Given the significance of excess Na\textsuperscript{+} retention in the pathogenesis of many disorders, characterization of the specific distal-nephron site(s) (cortical collecting duct and/or distal tubule), as well as the source of ghrelin (local versus circulating) responsible for mediating the effects on Na\textsuperscript{+} reabsorption, remains an important topic of future investigations.

**Perspectives**

Ghrelin is a powerful orexigenic hormone that is being targeted for the treatment of obesity. The present studies demonstrate that it also has significant sodium-retaining properties at the level of the distal nephron. Since obesity-related disorders such as hypertension and nephropathy are often characterized by excess sodium retention, identification of the renal effects of this hormone may be important in linking obesity with such complications.

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**Disclosures**

None.

**References**


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