NOTE *Pathology*

Change in intrarenal Ghrelin expression in immune complex-mediated glomerular disease in dogs

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(Received 5 July 2015/Accepted 24 July 2015/Published online in J-STAGE 8 August 2015)

ABSTRACT. Ghrelin is a peptide hormone that is mainly produced by the stomach. The kidney is a major source of local ghrelin, and maintaining body fluid balance is considered a critical role of renal ghrelin. However, there are no reports on renal ghrelin in small animal medicine. The present study investigated the intrarenal localization of and change in ghrelin expression in dogs with immune complex-mediated glomerulonephritis (ICGN). Ghrelin immunoreactivity (IR) was observed in the distal tubules of normal kidneys. Ghrelin IR was weak in ICGN kidneys, and the quantitative ghrelin IR score was significantly lower in ICGN kidneys than in normal kidneys. In cases of ICGN, plasma creatinine concentrations showed a positive correlation with the ghrelin IR score.

KEY WORDS: canine, ghrelin, glomerular disease, immune complex-mediated nephritis, immunohistochemistry

doi: 10.1292/jvms.15-0404; *J. Vet. Med. Sci.* 78(1): 109–112, 2016

Ghrelin, a peptide hormone, was first discovered in rat and human stomachs [8]. It plays several important roles including growth hormone release and appetite stimulation [9, 10, 12]. Various organs other than the stomach also synthesize local ghrelin [4, 5]; the kidney is a major source of local ghrelin. The intrarenal localization of ghrelin was first investigated in our study of rodent kidneys [18]. We found that it was primarily distributed in the distal tubules. A similar distribution has since been observed in the human kidney [2]. In diseased human kidneys, ghrelin immunoreactivity (IR) in renal tubules is decreased during proliferative glomerulopathy [3]. However, the intrarenal expression of ghrelin in normal and diseased canine kidneys has not been reported.

Glomerular disease is a common form of kidney disease in dogs and is divided into three major categories based on the pathological evaluation of renal biopsies: Immune complex-mediated glomerulonephritis (ICGN), non-ICGN and amyloidosis [1]. ICGN garners the greatest clinical interest as it has the highest prevalence among these categories [16]. The purpose of the present study was to clarify the intrarenal localization of ghrelin in normal canine kidneys and to evaluate how it changes in ICGN.

Normal kidney samples were obtained from clinically healthy male Beagles (n=6, 2–3 years old), who were euthanized after use in other surgical experiments. The stomach was chosen as a positive control tissue for ghrelin IR. Experiments were performed in accordance with the Guidelines for Animal Experimentation of Kagoshima University, Japan. Samples were fixed in 10% neutral buffered formalin and embedded in paraffin according to the routine procedure. ICGN samples (n=12) were obtained from renal biopsies of clinical cases in dogs. All cases were clinicopathologically diagnosed as protein-losing nephropathies. The owners provided informed consent for renal biopsies to definitively diagnose the kidney disease and for research use of the samples after diagnosis. Histopathological diagnosis of renal biopsies was based on light microscopy, transmission electron microscopy and immunohistochemical (immunoglobulin [Ig] G, IgA, IgM and complement C3) analyses.

Immunohistochemistry to detect ghrelin was performed using 3-μm paraffin sections. The procedure was as follows: (1) deparaffinization and rehydration; (2) antigen retrieval treatment by microwave heating in 10 mM citrate buffer (pH 6.0) with pre-warming for 5 min, heating for 10 min and cooling for 20 min; (3) incubation with 3% H_2O_2 for 30 min; (4) washing in 10 mM phosphate buffered saline (PBS, pH 7.4); (5) blocking with 0.25% casein (Sigma-Aldrich Corp., St. Louis, MO, U.S.A.) in PBS for 30 min; (6) incubation overnight at 4°C with goat anti-ghrelin antiserum (C-18) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) diluted 1:1,000 in blocking solution; (7) washing in PBS; (8) incubation for 30 min with biotinylated horse anti-goat IgG (H+L) (Vector Laboratories, Burlingame, CA, U.S.A.) diluted 1:200 in blocking solution; (9) washing in PBS; (10) incubation with peroxidase-conjugated streptavidin (KPL, Gaithersburg, MD, U.S.A.); (11) washing in PBS; (12) immunosignal detection using a 3,3′-diaminobenzidine (DAB) system (DAB-buffer tablet; Merck KGaA, Darmstadt, Germany); and (13) termination of the reaction with distilled water. Sections were counterstained with Mayer's

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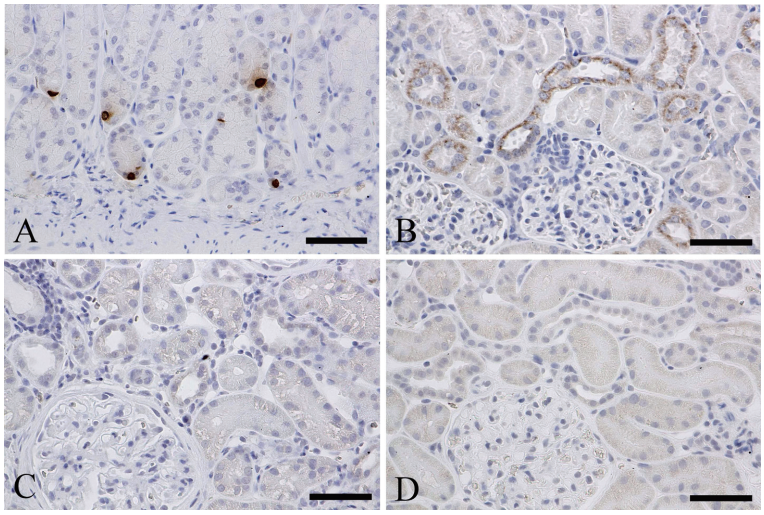

Fig. 1. Immunohistochemical detection of ghrelin immunoreactivity (IR). (A) Stomach from a normal dog. Cells with ghrelin IR are clearly observed in the gastric glands. (B) The kidney of a normal dog. Ghrelin IR is clearly detected in the distal tubules. (C and D) Kidneys from 2 cases of immune complex-mediated glomerulonephritis (ICGN). Ghrelin IR is weaker in the distal tubules than in the normal kidney. Bars indicate 50 μm.

hematoxylin. For the negative control sections, normal goat IgG (Santa Cruz Biotechnology) was used instead of the primary antibody. Specificity was tested using affinity pre-absorption of primary antibody with 10 μg/m*l* synthesized ghrelin peptide (Santa Cruz Biotechnology).

Ghrelin IR in tubules was evaluated using a point-counting method described in a previous report [11]. Digital images were captured at 200× magnification and prepared (approximately seven images/section). Then, 300 circles per image (approximately 2,100 circles/section) were created using Photoshop software (Adobe Systems, San Jose, CA, U.S.A.). The circles containing glomeruli and large vessels were considered exclusion points, and those containing ghrelin IR were considered positive points. The percentage of ghrelin positive points per evaluation points (total points − exclusion points) was calculated. To avoid interference from interstitial fibrosis, which decreases the tubular area, the percentage of ghrelin IR points was divided by the percentage of interstitial fibrosis, which was determined using the same point-counting method. The calculated value was used as the score for intrarenal ghrelin IR. The glomerulosclerosis score was also evaluated in a semi-quantitative manner as previously reported [14]. The glomerular diameter served as an index of glomerular hypertrophy and was measured using an ocular micrometer.

The difference between normal and ICGN kidneys in terms of the ghrelin IR score was evaluated using the Mann-Whitney U test. Relationships between the ghrelin IR score and extent of renal tissue damage (glomerulosclerosis, glomerular diameter and interstitial fibrosis) in ICGN samples were evaluated with the Spearman rank correlation coefficient. Relationships between the ghrelin IR score and clinicopathological data, such as the plasma concentrations

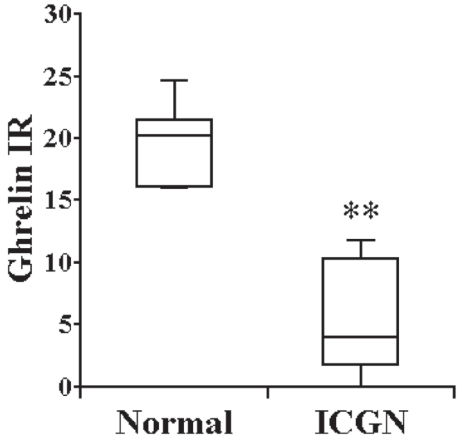

Fig. 2. Intrarenal ghrelin IR score in normal and ICGN kidneys. All data are represented as Box–Whisker plots. ** indicates significant difference compared to the normal kidney (P<0.01).

of urea, creatinine (pCre), phosphorus, total protein and albumin, urinary protein/creatinine ratio (UP/C), urine specific gravity (USG) and systolic blood pressure (SBP) measured using the Doppler method, were also evaluated with the Spearman rank correlation coefficient. All analyses were performed using the PASW software program for Windows (IBM SPSS Statistics, Armonk, NY, U.S.A.).

In the normal stomach, which works as a positive control, numerous cells with ghrelin IR were observed in the gastric glands (Fig. 1A). These cells were considered ghrelin-producing gastric endocrine cells, because the localization of immunopositive cells in the canine stomach was consistent

Table 1. Correlations between the ghrelin immunoreactivity score and data from clinicopathological and histomorphometrical analyses

Blood chemicals	Creatinine	Urea nitrogen	Total protein	Albumin	Phosphorus
	0.594* (n=12)	0.106 (n=12)	0.292 (n=11)	0.055 (n=10)	0.503 (n=8)
Blood pressure and Urinalysis	SBP (mmHg)	UP/C	USG		
	0.524 (n=8)	0.166 (n=12)	0.175 (n=11)		
Tissue damage	Glomerulosclerosis	Glomerular diameter	Interstitial fibrosis		
	0.063 (n=12)	0.472 (n=12)	0.289 (n=12)		

Values represent the Spearman rank correlation coefficient. * Statistical significances were defined as $P<0.05$. SBP, systolic blood pressure; UP/C, urinary protein/creatinine ratio; USG, urine specific gravity.

with patterns from previous reports in mice, humans and dogs [15]. The specificity of these immunosignals was confirmed by negative and pre-absorption tests. Therefore, we determined that the antibody used in the present study could successfully detect canine ghrelin with a high specificity in immunohistochemistry.

In kidneys from normal dogs, ghrelin IR was observed in the tubular epithelium. These positive tubules were identified as distal tubules (Fig. 1B), and the signal intensity was similar between the pars convoluta and pars recta. Weak signals were also observed in the proximal tubules. The specificity of ghrelin IR in the kidney was also confirmed by negative controls and pre-absorption controls.

In ICGN cases, ghrelin IR was observed in the distal tubules, but the signal intensity was weak compared to that in normal dogs (Fig. 1C and 1D). In quantitative analysis, the ghrelin IR score of the distal tubules was significantly less in ICGN cases than that in normal dogs (Fig. 2). Although none of the renal histopathological parameters showed significant correlations, the pCre level showed a significant positive correlation with the ghrelin IR score (Table 1).

The regulation of sodium reabsorption via the epithelial sodium channel is suspected to be a physiological role of intrarenal ghrelin in rat kidneys [6, 7]. Since the intrarenal localization of ghrelin in canine kidneys, as demonstrated in the present study, is similar to that in rat kidneys [18], intrarenal ghrelin in dogs may also regulate sodium reabsorption. In ICGN, downregulation of intrarenal ghrelin was suspected from quantitative analysis, and this might induce the dysfunction of ghrelin-dependent sodium reabsorption. Despite such downregulation, ghrelin IR positively correlated with pCre, which is the most reliable biochemical marker for evaluating renal function in veterinary medicine. This phenomenon might be correlated with the renoprotective effects of ghrelin. Such a potential of ghrelin has already been suggested in previous reports. In a model of ischemic-reperfusion-induced acute renal injury, renal damage and azotemia were attenuated by ghrelin treatment [13, 17]. In a model of endotoxemia-induced acute renal injury, a decrease in the glomerular filtration rate was prevented by ghrelin treatment [19]. These renoprotective effects of ghrelin were associated with changes in the serum or tissue concentration of certain molecules, such as interleukin-6, tumor necrosis factor-alpha, insulin-like growth factor-1 and neuronal nitric oxide synthase [13, 17].

In conclusion, the present study demonstrated that intrarenal ghrelin IR is distributed in the distal tubules in dogs. Although the ghrelin IR score was lower in the ICGN kidneys than in normal kidneys, this score was positively correlated with the pCre level in ICGN cases. These findings might be correlated with a renoprotective effect of ghrelin, and further investigation into this potential function in kidney disease is necessary.

ACKNOWLEDGMENT. This research was partially supported by the Japan Society for the Promotion of Science KAKENHI (Grant Number 25450446).

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