Kidney produces a novel acylated peptide, ghrelin

Kiyoshi Mori[a,*], Akihiro Yoshimoto[a], Kazuhiko Takaya[a], Kiminori Hosoda[a], Hiroyuki Ariyasu[a], Kensei Yahata[a], Masashi Mukoyama[a], Akira Sugawara[a], Hiroshi Hosoda[b], Masayasu Kojima[b], Kenji Kangawa[b], Kazuwa Nakao[a]

[a] *Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan*
[b] *Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka 565-8565, Japan*

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Abstract Ghrelin is a novel growth hormone-releasing peptide with a unique acylated structure. Here we reveal that prepro-ghrelin gene is expressed in the mouse kidney and glomerulus. We also show by reverse-phase high performance liquid chromatography coupled with radioimmunoassay that the mouse kidney does produce ghrelin. The ghrelin immunoreactivity in the mouse kidney is 6.79 ± 0.48 fmol/mg ($n = 5$), which is much more abundant than that in the mouse plasma of 0.339 ± 0.029 fmol/μl ($n = 6$). Furthermore, prepro-ghrelin gene is expressed in cultured rat mesangial cells, fibroblast-like NRK-49F cells and mouse podocytes, but not in rat epithelial cell-like NRK-52E cells. Ghrelin receptor gene is also expressed in the rat kidney. These findings demonstrate that the kidney, glomerulus and renal cells express prepro-ghrelin gene and ghrelin is produced locally in the kidney, and suggest the endocrine and/or paracrine roles of ghrelin in the kidney.

Key words: Ghrelin; Acylation; Growth hormone; Growth hormone secretagogue; Receptor; Kidney

1. Introduction

Growth hormone (GH) and insulin-like growth factors (IGFs) play a number of important physiological and pathophysiological roles in the kidney [1]. GH stimulates IGF-1 secretion from the liver, and both GH and IGF-1 increase renal blood flow, glomerular filtration rate, tubular phosphate reabsorption, and possibly tubular sodium reabsorption [1–6]. They are also involved in normal kidney growth as well as renal hypertrophy observed in nephrectomy and diabetes [1,7,8]. Ghrelin is a novel 28 amino acid peptide with an *O*-*n*-octanoyl ($C_7H_{15}CO$) moiety, which has potent activity to stimulate GH release from the pituitary gland in rats and humans [9,10]. The unique modification in ghrelin peptide, or acylation, is essential for its biological action. This kind of modification for peptides has never been reported, suggesting the presence of a novel mechanism for post-translational modification [1]. Ghrelin was identified in the stomach as an endogenous ligand for an orphan receptor which has so far been called GH secretagogue (GHS) receptor [11,12]. Prepro-ghrelin gene is expressed in the oxyntic gland of the stomach and the arcuate nucleus of the hypothalamus, but its expression in other tissues is not well understood [9]. Whether acylation of ghrelin peptide occurs specifically in the stomach also remains unknown. Furthermore, actions of ghrelin except for stimulation of GH secretion have been implied based upon studies on the actions of GHS and the distribution of GHS receptor [9,13–15]. In the present study, we investigated prepro-ghrelin gene expression in the kidney, glomerulus and kidney-derived cultured cells, and also the gene expression of its receptor, GHS receptor, in the kidney. We also examined whether the kidney produces ghrelin.

2. Materials and methods

2.1. Animals

Kidneys and blood were collected from adult C57BL/6 mice (Shimizu Laboratory Supplies, Kyoto, Japan) and adult Wistar-Kyoto rats (Shionogi Research Laboratories, Osaka, Japan) under anesthesia with diethyl ether (Nacalai Tesque, Kyoto, Japan). Glomeruli were isolated from kidney cortices by the differential sieving method [16].

2.2. Kidney and blood samples for radioimmunoassay (RIA)

For peptide extraction, mouse kidneys were boiled for 5 min in 10-fold volume of water to inactivate intrinsic proteases, adjusted to 1 M acetic acid/20 mM HCl after cooling, and homogenized with Polytron mixer (Kinematica GmbH Kriens, Luzern, Switzerland). The supernatant obtained after 30 min centrifugation at 11 000 rpm was loaded onto Sep-Pak C18 cartridge (Waters, Milford, MA, USA), which was pre-equilibrated with 0.1% trifluoroacetic acid (TFA). The cartridge was washed with 5% CH_3CN/0.1% TFA and eluted with 60% CH_3CN/0.1% TFA. The eluate was lyophilized and subjected to RIA for ghrelin.

Whole blood samples were collected with 2 mg/ml EDTA-2Na (Nacalai Tesque) and 500 KIU/ml aprotinin (Wako, Osaka, Japan). Plasma was diluted with an equal volume of 0.9% saline before loading onto Sep-Pak C18 cartridge.

2.3. RIA for ghrelin

Two kinds of polyclonal antibodies were raised against the carboxyl- (Gln^{13}–Arg^{28}) and amino-terminal fragments (Gly^1–Lys^{11} with *O*-*n*-octanoylation at Ser^3) of rat ghrelin in rabbits as previously described [9,17]. Mouse ghrelin has a completely identical structure with rat ghrelin (manuscript submitted). Ghrelin and its fragments were first synthesized as fully protected peptides by a peptide synthesizer (433A, Applied Biosystems, Foster City, CA, USA), and further acylated with *n*-octanoic acid at Ser^3 as previously reported [9]. RIA was carried out in duplicate at 4°C. Each RIA incubation mixture was

*Corresponding author. Fax: (81)-75-771 9452.
E-mail: keyem@kuhp.kyoto-u.ac.jp

Abbreviations: GH, growth hormone; IGF, insulin-like growth factor; GHS, growth hormone secretagogue; TFA, trifluoroacetic acid; RIA, radioimmunoassay; RP-HPLC, reverse-phase high performance liquid chromatography; RT-PCR, reverse transcription-PCR; C-IR, carboxyl-terminal ghrelin immunoreactivity; N-IR, amino-terminal ghrelin immunoreactivity

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composed of 100 μl of standard ghrelin or unknown sample and 200 μl of antiserum diluted with RIA buffer (50 mM sodium phosphate buffer (pH 7.4), 0.5% bovine serum albumin, 0.5% Triton X-100, 80 mM NaCl, 25 mM EDTA-2Na and 0.05% NaN_3) containing 0.5% normal rabbit serum. Two antisera recognizing the carboxyl- and amino-termini of ghrelin were used at final dilutions of 1:20 000 and 1:6 000 000, respectively. After incubation for 12 h, 100 μl of ^{125}I-labeled tracer (15 000 cpm) was added. After an additional 36 h incubation, 100 μl of anti-rabbit IgG goat serum was added and further incubated for 24 h. Free and bound tracers were separated by centrifugation at 3000 rpm for 30 min. The radioactivity in the pellet was counted with a gamma counter (ARC-600, Aloka, Tokyo, Japan).

2.4. Reverse-phase high performance liquid chromatography (RP-HPLC) coupled with RIA

Peptide extract from mouse kidneys (20 mg wet tissue) was loaded onto Sep-Pak C18 cartridge and eluate was fractionated by RP-HPLC on μBondasphere C18 (3.9×150 mm, Waters) as previously described [9]. Solvent system was a linear gradient of CH_3CN from 10% to 60% in 0.1% TFA for 40 min and flow rate was 1 ml/min. An aliquot of each fraction (0.5 ml) was lyophilized and subjected to RIA for ghrelin.

2.5. Cell culture

Establishment of cultured mesangial cells from rat glomeruli has been described elsewhere [16]. Normal rat kidney-derived NRK-52E and NRK-49F cells were obtained from American Type Culture Collection (Rockville, MD, USA). A conditionally immortalized mouse podocyte clone, MPC-5, was kindly provided by Dr. Peter Mundel (Albert Einstein College of Medicine, NY, USA) [18]. Podocytes were cultured either with 10 U/ml mouse recombinant γ-interferon (Gibco BRL, Grand Island, NY, USA) at 33°C (permissive condition) or without γ-interferon at 37°C (non-permissive condition). Mesangial cells, NRK-52E, and NRK-49F were grown in Dulbecco's modified Eagle's medium (Gibco BRL) and podocytes in RPMI 1640 (Nihon-seiyaku, Tokyo, Japan), both with 10% fetal calf serum (Gibco BRL).

2.6. Reverse transcription (RT)-PCR for prepro-ghrelin gene expression

Total RNA was extracted from tissues and cells using TRIzol reagent (Gibco BRL) as described previously [19]. RT-PCR was performed with oligo $(dT)_{15}$ primer, SuperScript II reverse transcriptase (Gibco BRL), and rTaq (Takara, Tokyo, Japan). The following primers were used: mouse prepro-ghrelin sense, 5′-AGCATGCTCTG-GATGGACATG-3′ (nucleotides (nt) 55–75) (manuscript submitted); mouse prepro-ghrelin antisense, 5′-AGGCCTGTCCGTGGTTA-CTTGT-3′ (nt 383–362); rat prepro-ghrelin sense, 5′-TTGAGCCCA-GAGCACCAGAAA-3′ (nt 112–132) [9]; rat prepro-ghrelin antisense, 5′-AGTTGCAGAGGAGGCAGAAGCT-3′ (nt 458–437); β-actin sense, 5′-AACGAGCGCTTCCGCTGTCC-3′ (nt 754–773) [20]; β-actin antisense, 5′-AATCTTGATCTTCATGGTGC-3′ (nt 987–968). The annealing temperature was 60°C and cycles of PCR were 40 for ghrelin and 30 for β-actin. In the case of rat prepro-ghrelin, Southern blot hybridization was performed as previously described with 5′-end labeled internal oligonucleotide probe, 5′-AAAGGAATCCAA-GAAGCCACCAGC-3′ (nt 144–167) [9,21]. Specificities of the products were confirmed by direct sequencing with BigDye Terminator cycle sequencing kit FS and 310 Genetic Analyzer (Applied Biosystems).

2.7. RT-PCR for ghrelin receptor gene expression

As the receptor for ghrelin, we examined rat GHS receptor type 1a [9,12]. The following primers were used: sense, 5′-GCTGAGCGTC-GAGCGCTACTTCG-3′ (nt 405–427); antisense, 5′-GTTGCAG-TACTGGCTGATCTGAGC-3′ (nt 2943–2920). The reaction profile was identical with that of ghrelin.

3. Results

3.1. Prepro-ghrelin gene expression in the kidney and glomerulus

By use of RT-PCR, we examined prepro-ghrelin gene expression in the mouse kidney and glomerulus. The prepro-ghrelin cDNA, 329 bp in size, was amplified both in the kidney and glomerulus (Fig. 1). β-Actin cDNA, 234 bp in size, was also amplified to verify that reverse transcription was successfully done for all materials. In negative control experiments, where all the procedures were similar except that reverse transcriptase was omitted, no bands were observed. These findings prompt us to examine the content of ghrelin in the kidney.

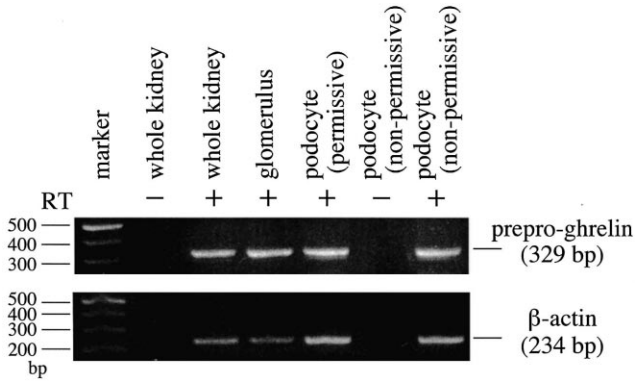

Fig. 1. Prepro-ghrelin gene expression in mouse kidney, glomerulus, and podocyte. RT + and −, with and without reverse transcription.

3.2. Detection of ghrelin in the kidney and blood

By RIA using an antiserum recognizing both the carboxyl-termini of ghrelin and the desacyl peptide, carboxyl-terminal ghrelin immunoreactivity (C-IR) in the mouse kidney extract was measured as 6.79 ± 0.48 fmol/mg wet tissue (mean ± S.E.M., $n = 5$). The amino-terminal ghrelin immunoreactivity (N-IR), which was assayed using another antiserum specific for the amino-terminus of ghrelin but not for the desacyl peptide, was 0.282 ± 0.074 fmol/mg wet tissue ($n = 5$). To further analyze the specificity of these ghrelin immunoreactivities, we fractionated the kidney extract by RP-HPLC and measured ghrelin immunoreactivities again (Fig. 2). C-IR was eluted as a major peak with a retention time of 13 min by 13.2 fmol/fraction, which was identical with the elution position of synthetic desacyl ghrelin [9]. The major peak of N-IR appeared with a retention time of 22 min by 1.3 fmol/fraction, which co-migrated with synthetic ghrelin. These findings confirmed the specificity of C-IR and N-IR in the mouse kidney. The minor peak of N-IR with a retention time of 24 min might correspond to an unidentified form of ghrelin. We also examined ghrelin concentration in the mouse plasma and found that C-IR and N-IR were 339 ± 29 and 6.80 ± 1.00 fmol/ml, respectively ($n = 6$). These findings indicated that the mouse kidney and plasma contain both ghrelin and the desacyl peptide, the desacyl peptide predominates ghrelin, and the ghrelin content in the kidney is much more abundant than that in the plasma.

3.3. Prepro-ghrelin gene expression in cultured renal cells

We next investigated prepro-ghrelin gene expression in kidney-derived cultured cells. We examined rat mesangial cells, non-glomerular epithelial cell-like NRK-52E cells and fibroblast-like NRK-49F cells. Rat prepro-ghrelin cDNA, 347 bp in size, was amplified in mesangial cells and NRK-49F cells, but not in NRK-52E cells (Fig. 3). We further examined mouse podocytes. In permissive conditions these cells exhibit cobblestone-like appearances corresponding to undifferenti-

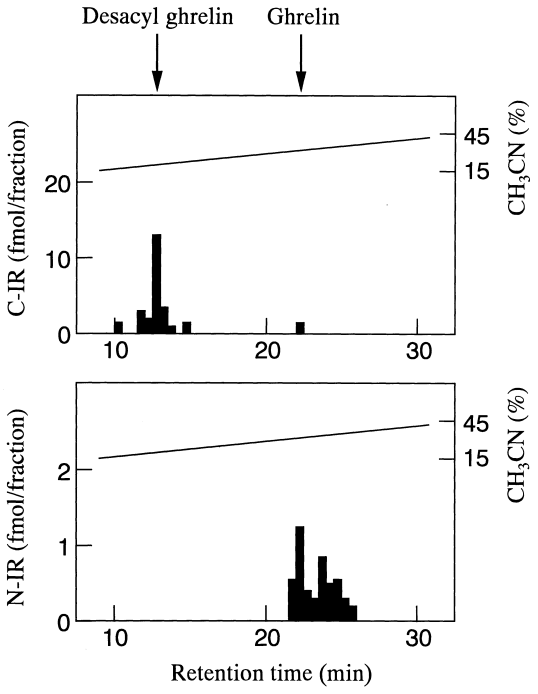

Fig. 2. Representative RP-HPLC profiles of ghrelin immunoreactivity in mouse kidney tissue extract. C-IR and N-IR, carboxyl- and amino-terminal ghrelin immunoreactivity; arrows, elution positions of ghrelin and desacyl ghrelin.

ated status, whereas in non-permissive conditions they show arborized shapes corresponding to differentiated or physiologic status [18]. Podocytes in non-permissive conditions as well as in permissive conditions expressed prepro-ghrelin gene (Fig. 1).

3.4. Ghrelin receptor gene expression in the kidney

Ghrelin receptor (or GHS receptor) cDNA, 511 bp in size, was amplified in the rat kidney (Fig. 4). The amplification was observed only when reverse transcriptase was included in the reaction.

4. Discussion

In the present study, we have demonstrated that prepro-ghrelin gene is expressed in the kidney and glomerulus. This

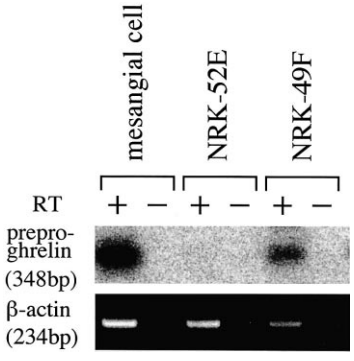

Fig. 3. Prepro-ghrelin gene expression in rat kidney-derived cell lines. For prepro-ghrelin cDNA, Southern blot hybridization was performed.

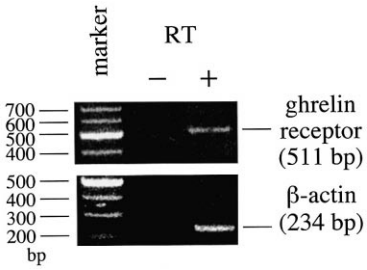

Fig. 4. Ghrelin receptor gene expression in rat kidney.

is the first report to show prepro-ghrelin gene expression in any tissue other than the gastrointestinal tract and brain [9]. We have also demonstrated that the kidney contains both ghrelin and the desacyl peptide by RP-HPLC coupled with RIA. Since ghrelin immunoreactivities (both C-IR and N-IR) in the kidney are more than 10 times abundant than those in the plasma, we can conclude that ghrelin is produced in the kidney. The C-IR in the kidney is approximately 1/140 of that in the mouse stomach (manuscript submitted). Since ghrelin is unstable and tends to be converted into the desacyl peptide in vivo when administered exogenously (unpublished data), a small amount of ghrelin produced locally may have physiological functions if the receptor exists in the proximity. With respect to N-IR/C-IR ratio, or the proportion of ghrelin in total ghrelin immunoreactivities, the kidney and plasma have values of 4.2% and 2.0%, respectively. These findings seem to be consistent with a short half-life of ghrelin. The present study also elucidates that acylation of ghrelin peptide does occur not only in the stomach [9] but also in the kidney, implying that molecules required for this reaction, which are not identified yet, might be shared among different tissues.

We have further revealed the gene expression in cultured mesangial cells and podocytes, suggesting that these cells are the cellular components expressing prepro-ghrelin gene in the glomerulus in vivo. Moreover, the gene of its receptor, GHS receptor, is also expressed in the kidney. In previous reports, GHS receptor gene expression was detected in the brain and several peripheral tissues such as the hypothalamus, pituitary gland, hippocampus, heart, pancreas, intestine and adipose tissue [9,15]. The present study adds the kidney as one of possible targets of direct ghrelin action, which should be independent of GH release [14]. Furthermore, prepro-ghrelin gene expression is observed in fibroblast-like NRK-49F cells. Fibroblast accumulation and fibrosis have been well known to play important roles in tubulointerstitial renal diseases, which strongly affect renal function [22]. Thus, ghrelin might not only have physiological but also pathophysiological significance in the kidney. Further studies on the intrarenal localization of ghrelin and its receptor, and on the direct actions of ghrelin upon the kidney and cultured renal cells are ongoing in our laboratory.

Here, we describe the gene expression of prepro-ghrelin and ghrelin receptor in the kidney. These findings suggest possible roles of ghrelin in the kidney as endocrine and/or paracrine system. The present study provides new insights into our understanding of the physiological and clinical implications of ghrelin.

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