

# Plasma Ghrelin and Desacyl Ghrelin Concentrations in Renal Failure

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**Abstract.** Ghrelin is a novel hormone that possesses growth hormone (GH)-releasing, cardiovascular, and metabolic activities. Ghrelin is a unique acylated polypeptide, and the naked peptide, desacyl ghrelin, does not have the activity. This study examines plasma ghrelin concentrations in 41 patients with mild to severe renal diseases. Two kinds of radioimmunoassays were used: amino-terminal immunoreactivity represents ghrelin alone (N-IR), and carboxyl-terminal immunoreactivity corresponds to the sum of both ghrelin and desacyl ghrelin (C-IR). In all subjects, the plasma N-IR was much smaller than the C-IR, indicating that desacyl ghrelin predominates over ghrelin in the circulation. The plasma C-IR, but not N-IR, was significantly correlated with the serum creatinine level and was increased 2.8-fold in patients with end-stage renal disease

compared with those in patients with normal renal function. The plasma GH concentration was significantly correlated with the plasma N-IR and the C-IR, as well as with the serum creatinine level. Bilateral nephrectomy in mice caused marked increase in the plasma C-IR without significant changes in the local C-IR and ghrelin mRNA level in the stomach, which is the main site of ghrelin production. These findings suggest that circulating ghrelin concentrations play a role in the regulation of blood GH concentrations and that the kidney is an important site for clearance and/or degradation of desacyl ghrelin. Furthermore, elevation of blood GH levels in renal failure seems to be caused by a mechanism other than alteration in the circulating ghrelin concentration.

Patients with end-stage renal disease (ESRD) have a variety of complications, including hypertension, heart failure, appetite loss, and malnutrition. Children with ESRD also suffer from growth disturbance and are occasionally treated with growth hormone (GH) (1).

Ghrelin is a novel 28-amino-acid polypeptide hormone with a unique acylated structure, which was identified as an endogenous ligand for an orphan receptor termed GH secretagogue receptor (2–4). The peptide without addition of a fatty acid moiety at Ser3, desacyl ghrelin, possesses no activity (2). The sizes of both ghrelin and desacyl ghrelin are approximately 3 kD. The most abundant source of ghrelin production is the stomach (2), but we have shown that various tissues, including the kidney, synthesize ghrelin (5,6). Peripheral administration of ghrelin or GH secretagogue causes not only GH release from the pituitary gland (2,4,7–9), but it also causes decrease in BP, improvement in cardiac

function (9), increase in food intake (10), fat accumulation, and body weight gain (11), and enhancement of gastric acid secretion and stomach motility (12), which all appear to be exerted independently of GH. These findings suggest that circulating ghrelin plays various important physiologic roles. Ghrelin is also synthesized in the brain and possesses central effects (2,10,11,13,14). Circulating ghrelin concentrations have been reported to be elevated after fasting and normalized after refeeding (11,15,16), and lean people tend to have high circulating ghrelin concentrations (16). However, antibodies used in previous works to measure ghrelin concentrations in the human blood, which were prepared by ourselves or by Phoenix Pharmaceuticals (Belmont, CA; <http://www.phoenixpeptide.com/Catalog%20Files/Ghrelin%20Section/ghrelin.htm>), do not distinguish ghrelin and desacyl ghrelin. Therefore, very little is known about the ratio at which ghrelin and desacyl ghrelin exist in the blood, how circulating ghrelin and desacyl ghrelin are metabolized, what factors other than nutrition affects the circulating ghrelin concentration, and what is the clinical implication of circulating ghrelin.

To address these issues in the present study, we used two kinds of radioimmunoassays (RIA) specific for ghrelin (2,5,6): amino-terminal immunoreactivity representing ghrelin (N-IR) and carboxyl-terminal immunoreactivity corresponding not only to ghrelin but also to desacyl ghrelin (C-IR). Here, we examined plasma ghrelin and desacyl ghrelin concentrations in patients with mild-to-severe renal diseases and analyzed their

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association with renal function and the plasma GH level. To explore the mechanism for altered plasma ghrelin concentrations in renal failure, we also examined the effects of hemodialysis in humans and nephrectomy in mice upon plasma ghrelin concentrations.

## Materials and Methods

### Subjects

Forty-one patients who were admitted to Kyoto University Hospital for diagnosis and treatment of renal diseases were analyzed in the present study. The patients included 20 men aged 21 to 87 yr (mean  $59 \pm 4$  yr) and 21 women aged 22 to 77 yr ( $48 \pm 4$  yr). Allowing overlaps, they were diagnosed to have diabetes ( $n = 17$ ), hypertension ( $n = 22$ ), primary glomerular disease ( $n = 11$ ), autoimmune/rheumatic disease ( $n = 5$ ), hypokalemia ( $n = 2$ ), and liver disease ( $n = 2$ ). Their creatinine clearances were normalized to  $1.73 \text{ m}^2$  body surface area, which was calculated by the Du Bois-Du Bois formula (17). The present study was conducted under written informed consent and approved by the ethical committee on human research in Kyoto University Graduate School of Medicine.

### Measurement of Plasma, Tissue, and Urine Ghrelin Concentrations

Blood samples were collected with 2 mg/ml EDTA-2Na (Nacalai Tesque, Kyoto, Japan) and 500 KIU/ml aprotinin (Wako, Osaka, Japan) at 8 a.m. before breakfast. All patients with ESRD (six men and four women) were receiving hemodialysis, and blood was collected similarly at 8 a.m. before hemodialysis and also after hemodialysis. Peptide was extracted from plasma by Sep-Pak C18 cartridge (Waters, Milford, MA). Ghrelin concentrations in peptide samples were measured with RIA using polyclonal rabbit antibodies raised against the amino-terminal (amino acid positions 1 to 11 with *O*-*n*-octanoylation at Ser3) or carboxyl-terminal fragments (amino acid positions 13 to 28) of ghrelin (2,6). The mouse stomach was boiled in water to inactivate intrinsic proteases, and peptide extracted from homogenized tissue was similarly subjected to RIA. The mouse plasma was treated similarly as human plasma. The spot human urine was collected in the morning, and peptide was extracted immediately. Human and mouse ghrelin showed identical immunoreactivities in the RIA (5,6). The minimal detection limits of N-IR and C-IR were 0.4 and 8.0 fmol/tube, respectively. For N-IR and C-IR, the intra-assay coefficients of variation were 3.0% and 6.0%, and the inter-assay coefficients of variation were 6.0% and 9.0%, respectively.

### Measurement of Plasma GH Concentrations

In 18 men and 13 women, including 8 patients receiving hemodialysis, whose serum creatinine levels ranged from 0.6 to 11.3 mg/dl (mean  $2.3 \pm 0.4$  mg/dl), plasma GH concentrations were determined by an immunoradiometric assay kit (Daiichi Radioisotope Laboratories, Tokyo, Japan).

### Nephrectomy in Mice

Ten-week-old male C57BL/6 mice weighing 20 to 27 g were intraperitoneally anesthetized with pentobarbital during operation (30 mg/kg body wt; Sigma, St. Louis, MO). For heminephrectomy, the left kidney was removed through a dorsal incision, and both heminephrectomized and sham-operated control mice were fed *ad libitum* for 48 h until they were sacrificed under anesthesia with diethyl ether (Nacalai Tesque) to obtain the stomach and plasma. Bilaterally nephrectomized and control mice were given neither chow nor water for

16 h until they were killed. All animal experiments were conducted in accordance with guidelines for animal research in our institute.

### Northern Blot Analyses and Reverse-Transcription PCR of Mouse Ghrelin Gene Expression

Northern blot analyses were performed as described previously (18) to examine the ghrelin mRNA level in the mouse stomach. Total RNA was extracted from tissues using TRIzol reagent (Life Technologies, Grand Island, NY). In each lane, 20  $\mu\text{g}$  of total RNA was loaded. Hybridization was performed by [ $^{32}\text{P}$ ]dCTP-labeled cDNA probes for mouse preproghrelin (Kojima *et al.* GenBank accession number AB035701) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH, Clontech, Palo Alto, CA). The ghrelin mRNA level in the mouse kidney was determined by reverse-transcription PCR as described previously (5). Total RNA from the whole kidney was reverse transcribed using Superscript II (Life Technologies BRL, Grand Island, NY) and subjected to PCR reaction with the following primers: ghrelin sense, 5'-agcatgctctggatggacatg-3'; ghrelin antisense, 5'-aggcctgctccgtgtactgt-3';  $\beta$ -actin sense, 5'-aacgagcctccgctgtcc -3'; and  $\beta$ -actin antisense 5'-aatcttgatcttcctggtgc -3'. The annealing temperature was 60°C, and the cycles were 40 for ghrelin and 30 for  $\beta$ -actin, respectively. Aliquots of PCR products, corresponding to 20 ng of total RNA, were electrophoretically separated on 1.2% agarose gel and stained by ethidium bromide. Specificities of PCR products were confirmed by nucleotide sequence determination. The mean ghrelin mRNA level in the control stomach or kidney was arbitrarily defined as 100%, and the relative mRNA level in the tissue of nephrectomized mouse was normalized by the G3PDH or  $\beta$ -actin mRNA level.

### Statistical Analyses

All values were expressed as mean  $\pm$  SEM. Methods used for statistics are described in the Results section.  $P < 0.05$  was considered statistically significant.

## Results

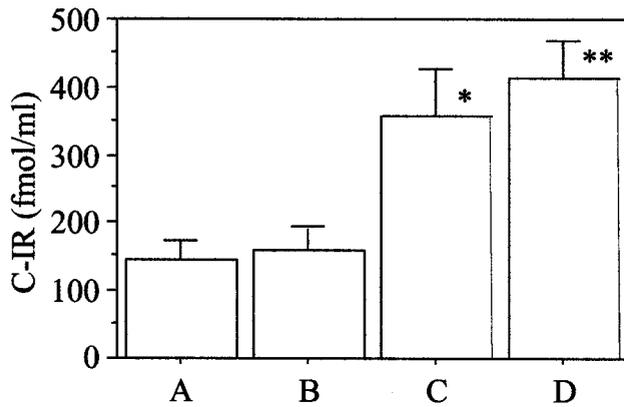
### The Predominant Ghrelin Immunoreactivity in the Human Blood Is Desacyl Ghrelin

The plasma concentration of genuine ghrelin was measured as N-IR, and the total amount of ghrelin and desacyl ghrelin was measured as C-IR. In patients with normal renal function (serum creatinine,  $\leq 0.8$  mg/dl;  $n = 11$ ), the plasma N-IR and C-IR were  $9.1 \pm 2.3$  and  $147 \pm 27$  fmol/ml, respectively. These findings indicate that circulating ghrelin and desacyl ghrelin exist physiologically at a ratio of approximately 6% and 94%, respectively.

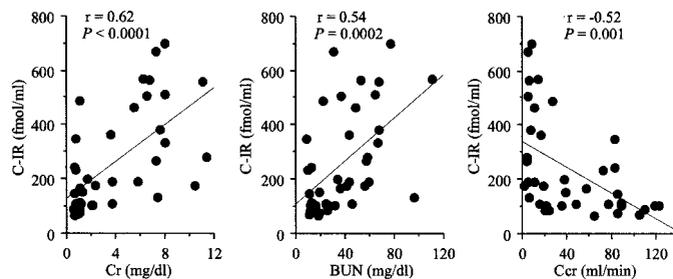
### The Plasma C-IR but not the N-IR Is Proportional to Severity of Renal Failure

Patients with mild-to-severe renal diseases were divided into four groups on the basis of their serum creatinine (Cr) levels, and the mean plasma C-IR was compared (Figure 1). As compared with the mean C-IR in patients with normal renal function, it was increased by 2.4-fold in those with severe renal failure (Cr  $> 3.0$  mg/dl, without hemodialysis,  $P = 0.004$  by Dunn's procedure as a multiple comparison procedure) and 2.8-fold in those with ESRD (all with hemodialysis,  $P = 0.0002$ ).

As shown in Figure 2, the plasma C-IR was correlated positively with the serum Cr level ( $r = 0.62$ ;  $P < 0.0001$ ;  $n =$



**Figure 1.** Plasma carboxyl-terminal ghrelin immunoreactivities in patients with renal failure. A, normal renal function (creatinine [Cr]  $\leq$  0.8 mg/dl;  $n = 11$ ); B, mild renal failure ( $0.8 < \text{Cr} \leq 3.0$ ;  $n = 12$ ); C, severe renal failure ( $\text{Cr} > 3.0$ ;  $n = 8$ ); D, end-stage renal disease (mean Cr,  $8.1 \pm 2.1$ ;  $n = 10$ ). C-IR, carboxyl-terminal immunoreactivity = concentration of ghrelin plus desacyl ghrelin. \* $P < 0.01$ ; \*\* $P < 0.0005$  versus A and B.



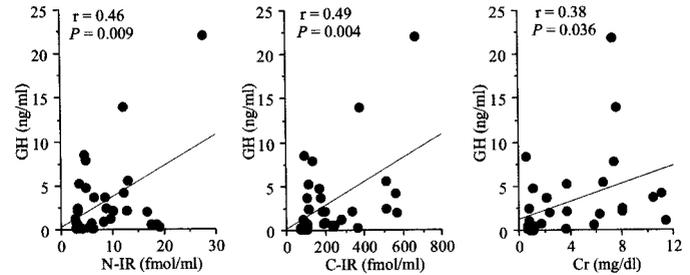
**Figure 2.** Correlations between the plasma C-IR and the serum Cr level, the blood urea nitrogen level, and Cr clearance.

41), the blood urea nitrogen level ( $r = 0.54$ ;  $P = 0.0002$ ), and negatively with Cr clearance ( $r = -0.52$ ;  $P = 0.001$ ) by Pearson's rank correlation. These findings indicate that the plasma C-IR is proportional to the severity of renal failure.

On the other hand, the plasma N-IR was not correlated significantly with the serum Cr level ( $r = 0.21$ ;  $P = 0.19$ ;  $n = 41$ ). The N-IR and C-IR were correlated significantly with each other ( $r = 0.53$ ;  $P = 0.0003$ ;  $n = 41$ ).

#### Plasma Ghrelin Concentrations Are Correlated with Plasma GH Concentrations

We also investigated the correlation between the plasma GH level and the N-IR or the C-IR, and found that the GH con-



**Figure 3.** Correlations between the plasma growth hormone (GH) concentration and the plasma N-IR and C-IR and the serum Cr level. N-IR and C-IR, amino- (ghrelin alone) and carboxyl-terminal ghrelin immunoreactivities.

centration was correlated significantly with the N-IR (Figure 3;  $r = 0.46$ ;  $P = 0.009$ ;  $n = 31$ ) and with the C-IR ( $r = 0.49$ ;  $P = 0.004$ ) by Pearson's rank correlation. GH concentrations were also significantly correlated with Cr levels ( $r = 0.38$ ;  $P = 0.036$ ). These findings suggest that the plasma GH level is affected by at least two factors, the circulating ghrelin level and renal function.

#### Effects of Hemodialysis in Humans and Nephrectomy in Mice

We found that  $53.3 \pm 8.3\%$  of the plasma C-IR ( $P = 0.0005$ ;  $n = 8$  by paired  $t$  test),  $73.8 \pm 4.0\%$  of the N-IR ( $P = 0.004$ ), and  $90.4 \pm 1.9\%$  of the GH level were removed from the blood by single course of hemodialysis (Table 1). Furthermore, we revealed that heminephrectomy in mice led to 1.3-fold increase ( $P = 0.15$ ;  $n = 5$  by unpaired  $t$  test) and bilateral nephrectomy led to 3.1-fold increase ( $P = 0.0009$ ;  $n = 5$ ) in the plasma C-IR (Table 2). In the stomach, neither the C-IR content (Table 2) nor the ghrelin mRNA level (Figure 4;  $117 \pm 8\%$  of control after nephrectomy;  $P = 0.11$ ;  $n = 4$ ) was altered significantly by bilateral nephrectomy. In the kidney, the ghrelin mRNA level tended to be decreased by heminephrectomy, but not significantly (Figure 5;  $75 \pm 12\%$  of control after nephrectomy;  $P = 0.21$ ;  $n = 4$ ). These findings suggest that the kidney plays an important role in metabolism, rather than production, of circulating desacyl ghrelin.

#### Urinary Ghrelin Concentrations

We examined whether ghrelin is excreted in the urine (Table 3). In fresh urine collected from patients with normal renal function, substantial amount of ghrelin immunoreactivity was

**Table 1.** Effects of hemodialysis in patients on the plasma C-IR, N-IR, and GH level<sup>a</sup>

	C-IR (fmol/ml)	N-IR (fmol/ml)	GH (ng/ml)	Cr (mg/dl)	BUN (mg/dl)
Pre HD	$411.9 \pm 66.6$	$12.6 \pm 2.5$	$5.2 \pm 2.5$	$8.3 \pm 0.8$	$61.6 \pm 11.2$
Post HD	$192.5 \pm 34.3^b$	$3.3 \pm 0.5^b$	$0.5 \pm 0.1^d$	$3.7 \pm 0.3^c$	$28.4 \pm 7.8^b$

<sup>a</sup>N-IR and C-IR, amino- (ghrelin alone) and carboxyl-terminal ghrelin immunoreactivities (ghrelin + desacyl ghrelin). HD, hemodialysis.  $n = 8$ .

<sup>b</sup> $P < 0.005$ ; <sup>c</sup> $P < 0.05$ ; <sup>d</sup> $P > 0.05$  versus pre-HD.

Table 2. Effects of nephrectomy in mice upon the plasma and stomach C-IR<sup>a</sup>

	Plasma C-IR (fmol/ml)	Stomach C-IR Contents & Concentrations	
		(pmol/stomach)	(fmol/mg stomach)
Heminephrectomy	602 ± 64 <sup>c</sup>	ND	ND
Control	448 ± 70	ND	ND
Bilateral nephrectomy	1677 ± 185 <sup>b</sup>	592 ± 50 <sup>c</sup>	3670 ± 317 <sup>c</sup>
Control	542 ± 20	617 ± 18	3111 ± 173

<sup>a</sup> C-IR, carboxyl-terminal ghrelin immunoreactivity = ghrelin plus desacyl ghrelin. *n* = 5 for each group. ND, not determined.

<sup>b</sup> *P* < 0.001; <sup>c</sup> *P* > 0.05 versus each control.

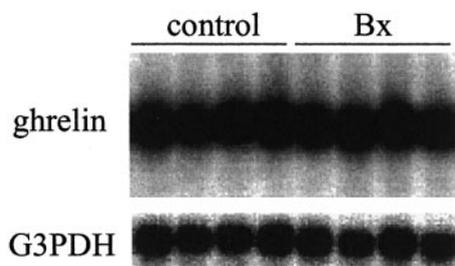


Figure 4. Effects of nephrectomy in mice on stomach ghrelin gene expression. Bx, bilateral nephrectomy.

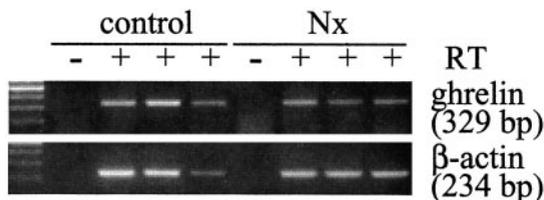


Figure 5. Effects of nephrectomy in mice on kidney ghrelin gene expression. Nx, heminephrectomy; RT + and -, with and without reverse transcription. Size markers: 500, 400, 300, and 200 bp from above.

detected, suggesting that ghrelin is filtered in the glomeruli or secreted by nephrons.

### Discussion

In the present study, we first determined plasma ghrelin levels (N-IR) and the sum of ghrelin and desacyl ghrelin levels (C-IR) and revealed that the genuine ghrelin concentration accounts for less than 10% of total circulating ghrelin plus desacyl ghrelin immunoreactivities. We have also demonstrated that the plasma C-IR in patients with renal diseases is increased in parallel with the severity of renal damage.

Furthermore, we have elucidated that plasma ghrelin concentrations, both N-IR and C-IR, are correlated with plasma GH concentrations. Plasma GH concentrations are also correlated with serum Cr levels, which is consistent with a previous finding that GH concentrations are elevated in chronic renal failure (1). Taken together with our recent work showing that

intravenous injection of ghrelin causes marked elevation in the circulating GH concentration in humans (7), the present study suggests that the GH level is influenced not only by renal function but also by the circulating ghrelin concentration. The plasma C-IR, but not N-IR, is correlated significantly with the serum creatinine level. Therefore, elevation of blood GH levels in renal failure seems to be caused not by alteration in the circulating ghrelin concentration, but rather by reduced renal elimination of circulating GH, as has been presumed (1).

Why is the plasma C-IR (the concentration of ghrelin plus desacyl ghrelin) elevated in patients with renal failure? We have revealed that approximately half of the plasma C-IR, as well as half of the serum Cr or blood urea nitrogen, is removed from the blood by single course of hemodialysis and that bilateral nephrectomy in mice causes marked increase in the plasma C-IR. At the same time, nephrectomy does not increase steady state ghrelin contents and ghrelin mRNA levels in the stomach. These findings suggest that increased C-IR in renal failure results from decreased clearance or degradation in the kidney. However, there is still a possibility that overproduction of ghrelin in organs other than the stomach (5,6) may contribute to higher plasma concentrations.

To note, approximately one fifth of ghrelin-like immunoreactivity is detected in the urine compared with the plasma ghrelin concentration in subjects with normal renal function, suggesting that ghrelin is filtered from the blood or actively secreted into the urine. The N-IR/C-IR ratio in the urine (26.6%) is much higher than that in the plasma. Ghrelin might be more efficiently filtered or secreted into the urine compared with desacyl ghrelin, or ghrelin might be more stable in the urine than in the circulation. As described above, apart from GH secretion (2,4,7–9), ghrelin possesses a variety of biologic actions: it decreases BP, improves cardiac function, and increases food intake, fat accumulation, and body weight (9–11). Thus, administration of ghrelin to patients with ESRD might be superior to GH administration, which is now clinically performed to obtain catch up growth in children with this disease (1).

In conclusion, we demonstrate that plasma desacyl ghrelin concentrations are markedly increased in patients with renal failure. The present study brings a new insight into our understanding of clinical implication of circulating ghrelin.

Table 3. Comparison of ghrelin immunoreactivities in the plasma and in the urine<sup>a</sup>

	C-IR (fmol/ml)	N-IR (fmol/ml)	Cr (mg/dl)	N/C ratio (%)
Plasma	162.0 ± 33.5	14.7 ± 5.9	0.6 ± 0.0	8.3 ± 2.0
Urine	33.9 ± 3.8	9.0 ± 1.4	104 ± 48	26.6 ± 2.5

<sup>a</sup> N/C ratio, N-IR/C-IR. n = 4.

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