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Urinary adiponectin excretion is increased in patients with overt diabetic nephropathy

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Abstract

Adiponectin, a novel adipose-derived adipocytokine, has beneficial effects not only on improvement of insulin sensitivity but also on mitigation of vascular damage. To evaluate whether adiponectin is implicated in the pathogenesis of diabetic nephropathy characterized by microvascular damage, we examined urinary and serum adiponectin levels in type 2 diabetic patients with different stages of nephropathy. We first confirmed adiponectin is excreted into urine through Western blot analysis, followed by measurements of urinary and serum adiponectin levels by radioimmunoassay. Interestingly, urinary adiponectin excretion levels were markedly increased in patient group with overt nephropathy relative to the groups without nephropathy and with incipient nephropathy. Surprisingly, serum adiponectin levels were also elevated in patient group with overt nephropathy. Increased urinary adiponectin excretion may result from elevations in circulating adiponectin levels and enhanced filtration of circulating adiponectin through the damaged kidney. Furthermore, adiponectin synthesis in adipose tissue and its secretion into circulating blood may be enhanced to mitigate microvascular damage in the advanced stage of diabetic nephropathy.

Keywords: Urinary adiponectin excretion; Serum adiponectin level; Diabetic nephropathy; Type 2 diabetes; Western blot analysis; Radioimmunoassay

Human adiponectin and its murine homolog Acrp30 are novel adipose-derived adipocytokines that have been independently identified by several groups [1–4]. In humans, adiponectin is one of the most abundant gene transcript proteins in adipose cells. Its plasma levels account for 0.01–0.03% of total plasma protein [5]. Previous works have shown that decreased circulating adiponectin levels are closely related to obesity, insulin resistance, and type 2 diabetes [5,6]. Moreover, circulating adiponectin levels have been reported to be negatively correlated with body mass index and the degree of hyperinsulinemia in humans [7,8]. Interestingly, administration of recombinant adiponectin to obese mice improves insulin resistance and lowers blood glucose levels [9,10]. These lines of evidence suggest that a reduction in adipose and circulating adiponectin levels in obesity may greatly contribute to the development of insulin resistance, and that adiponectin may be working as a key regulator of insulin sensitivity. Thus, the associations of the adiponectin levels with obesity and insulin resistance have been understood.

On the other hand, it is not well known whether adiponectin is implicated in vascular diseases, similar to other adipocytokines such as tumor necrosis factor-α (TNF-α) and plasminogen activator inhibitor-1 (PAI-1). However, two recent studies show interesting findings regarding the associations of adiponectin with vascular diseases. One study indicated that plasma adiponectin levels were lower among hemodialysis patients with cardiovascular events than among those without the events, and therefore suggests that plasma adiponectin levels are an inverse predictor of cardiovascular outcomes among patients with end-stage renal disease [11]. The other study showed that plasma adiponectin levels were markedly increased in patients with nephritic

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syndrome and strongly related to the degree of proteinuria, suggesting that adiponectin may work to mitigate vascular damage in patients with chronic renal diseases [12]. Based on these findings, adiponectin may have beneficial effects not only on improvement of insulin resistance but also on mitigation of vascular damage. In addition, adiponectin is expected to have some protective effects also on the development and progression of diabetic nephropathy characterized by microvascular damage.

Adiponectin is a protein with small molecular weight, 30 kDa. Therefore, it is expected for adiponectin to appear in urine. However, this has not been reported so far. If adiponectin is excreted into urine, it will be intriguing to investigate whether its urinary levels are associated with diabetic nephropathy. In the present study, we first examined whether urinary adiponectin is detectable in type 2 diabetic patients with different stages of nephropathy. Next, we measured urinary and serum adiponectin levels in these patients and evaluated the association of adiponectin with diabetic nephropathy.

Materials and methods

Study population. We recruited 38 male type 2 diabetic patients with different stages of nephropathy (18 with normoalbuminuria, 7 with microalbuminuria, and 13 with macroalbuminuria). The stage of diabetic nephropathy was diagnosed by measuring the albumin-to-creatinine ratio in two random spot urine samples collected over a 2- to 4-month period. Normoalbuminuria, microalbuminuria, and macroalbuminuria were regarded as being present if urinary albumin excretion was <30, 30–300, and > 300 μg/g creatinine, respectively. Patients with normoalbuminuria were defined as being without nephropathy and those with microalbuminuria were regarded as having incipient nephropathy. The diagnostic criteria for overt nephropathy were as follows: presence of persistent macroalbuminuria, absence of urinary tract infection, and presence of diabetic retinopathy. Patients treated with thiazolidinediones were not included in this study, as these drugs are known to increase plasma adiponectin levels [13–15]. Informed consent was obtained from all study participants. Serum and urine samples were collected from all study subjects and stored at −85 °C before testing.

Detection of adiponectin excreted into urine by Western blot analysis. Aliquots of urine samples (15 μl) from patients with macroalbuminuria were separated by SDS–PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA). To confirm whether the band detected in urine sample was adiponectin itself or unknown cross-reactive substances, the membranes were incubated with either 1:100,000 dilution of mouse anti-human adiponectin monoclonal antibody (Chemicon International, Temecula, CA) or 1:100,000 dilution of the same monoclonal antibody pretreated with 2 μg of recombinant human adiponectin (R&D Systems, Minneapolis, MN) at room temperature for 12 h, and then incubated with 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G antibody (DAKO, Glostrup, Denmark) at room temperature for 1 h. After incubation, the membranes were soaked in chemiluminescence solution using ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were exposed to the same X-ray film, and thus adiponectin protein in the urine was visualized. Western blot analysis for urinary adiponectin was also employed on urine samples of 6 patients with macroalbuminuria. These samples were adjusted to the same albumin concentration (300 μg/ml) by dilution with saline. Urinary albumin concentrations were measured by radioimmunoassay (RIA) as described previously [16].

Measurement of urinary and serum adiponectin levels. Urinary and serum adiponectin levels were measured using a commercially available RIA kit (Linco Research, St. Louis, MO). After urinary adiponectin concentrations were determined, urinary adiponectin excretion levels were expressed as the ratio of urinary adiponectin concentration to grams of urinary creatinine. To confirm whether adiponectin detected in urine by RIA is adiponectin itself or other cross-reactive substances, adiponectin levels in serially diluted urine samples of patients with macroalbuminuria were also measured by this RIA kit.

Statistical analysis. Statistical analysis was performed using SPSS software (Chicago, IL). Data are presented as means ± SD. Statistical significance was evaluated with ANOVA. Post hoc comparisons of group pairs were performed by Scheffe's multiple comparison test after ANOVA had revealed significant differences among groups. The correlations between serum and urinary adiponectin levels and urinary albumin levels were analyzed by Spearman's rank-order correlation test. The frequencies of hypertension among the groups were compared by χ^2 test. A P value <0.05 was considered statistically significant.

Results

Clinical characteristics of study subjects

Clinical characteristics of study subjects are shown in Table 1. The three patient groups with normoalbuminuria, microalbuminuria, and macroalbuminuria were well matched with regard to age and body mass index. Naturally, HbA1c levels of all the study subjects were above normal values. Serum creatinine levels and urinary albumin-to-creatinine ratios in patients with macroalbuminuria were significantly elevated compared to the other groups ($P < 0.0001$ for all comparisons). Hypertension was observed significantly more often in patients with macroalbuminuria compared to patients with normoalbuminuria ($P < 0.05$).

Detection of adiponectin in urine of patients with macroalbuminuria

Adiponectin protein was visualized by Western blot analysis in urine samples of patients with macroalbuminuria as a band of 30 kDa (Fig. 1). Because the band of adiponectin disappeared by incubating the protein-blotted membrane with anti-human adiponectin antibody pretreated with recombinant human adiponectin (Fig. 1A), we confirmed that the visualized band shows adiponectin protein. When albumin concentrations in urine samples of patients with macroalbuminuria were adjusted to the same level (300 μg/ml) by dilution with saline, adiponectin contents in these urine samples were not the same (Fig. 1B).

Table 1
Clinical characteristics in type 2 diabetic patients with normoalbuminuria, microalbuminuria, and macroalbuminuria

	Normoalbuminuria	Microalbuminuria	Macroalbuminuria
n	18	7	13
Age (years)	61 ± 7	62 ± 6	60 ± 8
Body mass index (kg/m^2)	23.2 ± 2.5	24.4 ± 1.7	23.8 ± 2.7
Hypertension (*n* (%))	10 (56)	6 (86)*	13 (100)*
HbA1c (%)	7.1 ± 0.5	8.2 ± 0.6	6.7 ± 0.7
Serum creatinine (mg/dl)	0.70 ± 0.10	0.80 ± 0.20	2.00 ± 1.20**
Serum adiponectin (μg/ml)	6.5 ± 2.1	7.9 ± 3.8	11.0 ± 5.5*
ACR (mg/g creatinine)	7.5 ± 4.3	65.1 ± 30.7	2154.0 ± 1285.0**
Retinopathy (N/S/P)	14/2/2	3/0/4	0/2/18

Data are presented as means ± SD or *n* (%). ACR, urinary albumin-to-creatinine ratio; retinopathy: N, normal; S, simple; and P, proliferative.
* $P < 0.05$ vs. normoalbuminuria.
** $P < 0.0001$ vs. normoalbuminuria, and microalbuminuria.

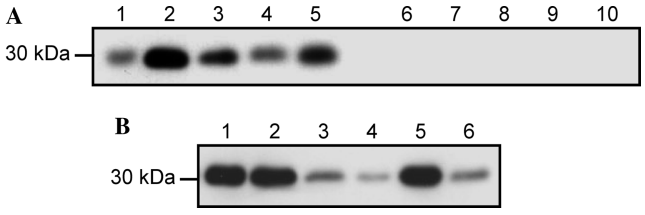

Fig. 1. Western blot analysis in urine samples of patients with type 2 diabetes with macroalbuminuria using anti-human adiponectin monoclonal antibody. Adiponectin was identified in urine as a 30 kDa protein. (A) Lanes 1 and 6, 1 ng of recombinant human adiponectin per a lane; lanes 2–5 and 7–10, 15 μl of urine sample per lane. The protein-transferred membrane of lanes 6–10 was incubated with the dilution of anti-human adiponectin antibody pretreated with recombinant human adiponectin. (B) Lanes 1–6, 15 μl of urine sample adjusted to albumin concentration of 300 μg/ml per a lane.

Urinary and serum adiponectin levels

Adiponectin levels of serially diluted urine samples in 5 patients with macroalbuminuria are shown in Table 2. Adiponectin levels were decreased by approximately half in urine samples diluted two times compared to adiponectin levels before dilution. Urinary adiponectin levels were significantly higher ($P < 0.05$ for all comparisons) in patients with macroalbuminuria (50.3 ± 55.8 μg/g creatinine) compared to those in patients with normoalbuminuria (4.9 ± 2.3 μg/g creatinine) and in patients with microalbuminuria (5.9 ± 2.4 μg/g creatinine) (Fig. 2A). Significant differences were not observed in urinary adiponectin levels between patients with normoalbuminuria and microalbuminuria. Urinary adiponectin levels were positively correlated with urinary albumin excretion levels only in patients with macroalbuminuria ($r = 0.816$, $P < 0.001$) (Fig. 2B). Serum adiponectin levels were significantly higher ($P < 0.05$) in patients with macroalbuminuria (11.0 ± 5.5 μg/ml) than in those with normoalbuminuria (6.5 ± 2.1 μg/ml). However, there was no significant difference in serum adiponectin levels between patients with macroalbuminuria and microalbuminuria (7.9 ± 3.8 μg/ml). As shown in Fig. 3, positive correlations between urinary and serum adiponectin levels were observed in patients with microalbuminuria ($r = 0.794$, $P < 0.05$) and in those with macroalbuminuria ($r = 0.762$, $P < 0.01$). In patients with normoalbuminuria, urinary adiponectin levels were not significantly correlated with serum adiponectin levels.

Table 2
Urinary adiponectin level (ng/ml) in each of diluted urine samples of patients with macroalbuminuria

Patient No.	Non-diluted	1:2	1:4	1:8	1:16
1	158.4	78.25	41.60	20.20	10.28
2	29.50	14.27	6.67	3.88	1.63
3	73.68	31.52	16.29	8.07	4.43
4	24.66	11.77	4.86	2.11	1.65
5	21.39	8.40	4.57	2.30	1.23

Discussion

In the present study, we first confirmed that adiponectin is excreted into urine through Western blot analysis. Given the facts that adiponectin is exclusively expressed in adipose tissue [1] and abundantly released into circulating blood [5] and that its expression is not observed in kidney [1], urinary adiponectin possibly originates not from kidney itself but from circulating adiponectin in blood. When diabetic nephropathy is caused by long-term hyperglycemia, it seems likely that urinary adiponectin excretion is affected by changes in circulating adiponectin levels and by renal damage. To examine the association of urinary adiponectin excretion with diabetic nephropathy, we next measured urinary and serum adiponectin levels in type 2 diabetic patients with different stages of nephropathy by RIA. Interestingly, urinary adiponectin levels were markedly higher in patient group with macroalbuminuria than in the other two groups with normoalbuminuria and microalbuminuria (Fig. 2A). Surprisingly, serum adiponectin levels were also significantly elevated in the group with

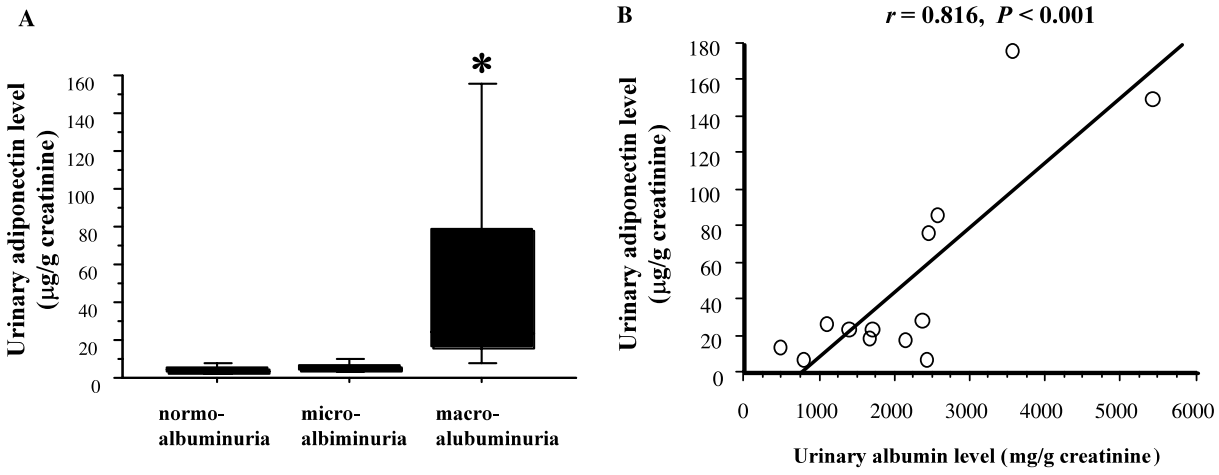

Fig. 2. (A) Urinary adiponectin levels in type 2 diabetic patients with normoalbuminuria, microalbuminuria, and macroalbuminuria. Data are presented as means ± SD. *$P < 0.05$ vs. normoalbuminuria and microalbuminuria. (B) Correlation between urinary levels of adiponectin and albumin in type 2 diabetic patients with macroalbuminuria.

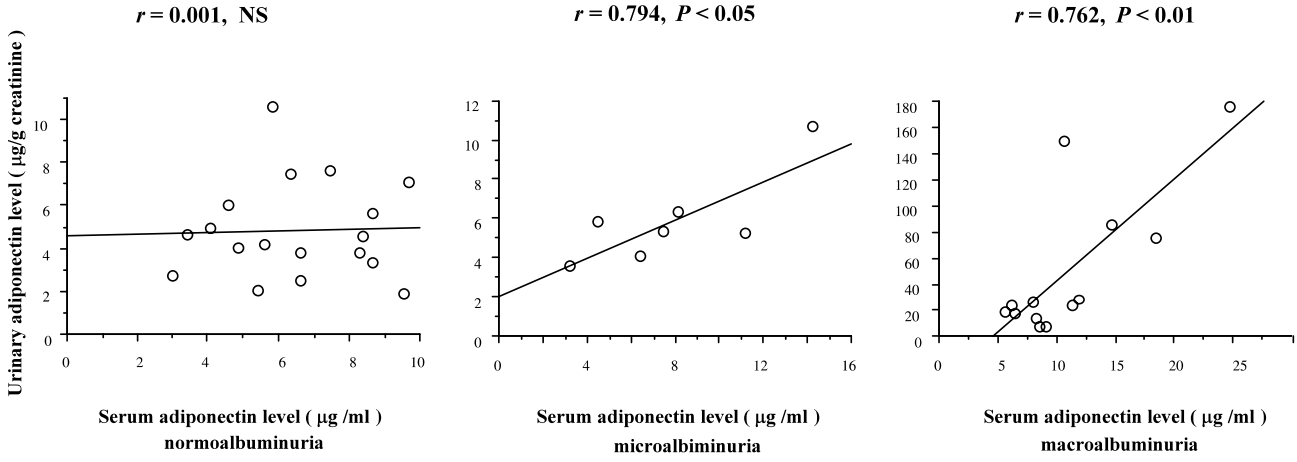

Fig. 3. Correlations between urinary and serum adiponectin levels in type 2 diabetic patient groups with normoalbuminuria, microalbuminuria, and macroalbuminuria. NS, not significant.

macroalbuminuria relative to the group with normoalbuminuria. There were no significant differences in urinary and serum adiponectin levels between patient groups with normoalbuminuria and microalbuminuria (Fig. 2A). Furthermore, positive correlations between urinary and serum adiponectin levels were observed in patient groups with microalbuminuria and macroalbuminuria (Fig. 3). Thus, we found that the patients with macroalbuminuria, i.e., advanced diabetic nephropathy, show increased urinary adiponectin excretion and have elevated circulating adiponectin levels, and that urinary adiponectin levels are positively correlated with serum adiponectin levels in patients with incipient and advanced diabetic nephropathy. Therefore, the increase in urinary adiponectin excretion may result from elevations in circulating adiponectin levels and enhanced filtration of circulating adiponectin through the damaged kidney. Adiponectin is shown to play a protective role in vascular damage through its ability to suppress the attachment of monocytes to endothelial cells [17]. Considering this finding together with our result that the patients with advanced diabetic nephropathy have elevated circulating adiponectin levels despite increased urinary adiponectin excretion, we suggest that adiponectin synthesis in adipose tissue and its secretion into circulating blood may be enhanced to mitigate microvascular damage in the advanced stage of diabetic nephropathy. The mechanism by which urinary adiponectin excretion is increased in advanced diabetic nephropathy still remains unclear. However, increased urinary adiponectin excretion seems to be implicated in the pathogenesis of advanced diabetic nephropathy.

We also evaluated the correlation between urinary adiponectin and albumin levels in this study. Urinary adiponectin levels were positively correlated with urinary albumin levels in patient group with macroalbuminuria

($r = 0.816$, $P < 0.001$), whereas significant correlations between urinary adiponectin and albumin levels were not observed in patient groups with normoalbuminuria and microalbuminuria. These findings indicate that analysis for urinary adiponectin in patients with diabetes should be undergone by dividing patients with diabetic nephropathy into two stages of premacroalbuminuria and macroalbuminuria. Furthermore, a positive correlation between urinary adiponectin and albumin levels in patients with macroalbuminuria supports the suggestion that a major part of urinary adiponectin may be attributed to the leakage of circulating adiponectin through the damaged kidney in these patients. On the other hand, the present study provided an interesting finding that adiponectin contents in urine samples adjusted to the same albumin concentration by dilutions were different in each of patients with macroalbuminuria (Fig. 1B). This finding suggests that a part of adiponectin detected in urine is not increased in proportion to an increase in urinary albumin in these patients. Therefore, urinary adiponectin excretion may be partly modulated by unknown factors in the advanced stage of diabetic nephropathy.

Finally, we here report that adiponectin excreted into urine is detectable by Western blot analysis and measurable by RIA, and that its urinary levels are markedly elevated in type 2 diabetic patients with advanced nephropathy. In particular, urinary adiponectin excretion seems to be implicated in the pathogenesis of the advanced stage of diabetic nephropathy. Further studies are required to clarify the role of urinary adiponectin in the pathogenesis of diabetic nephropathy.

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