Fig. 1 Serum creatinine concentration that would indicate referral to nephrologists. *Hatched bar* shows the percentage of nephrologists who replied "177 µmol/l or below". *$P < 0.0001$
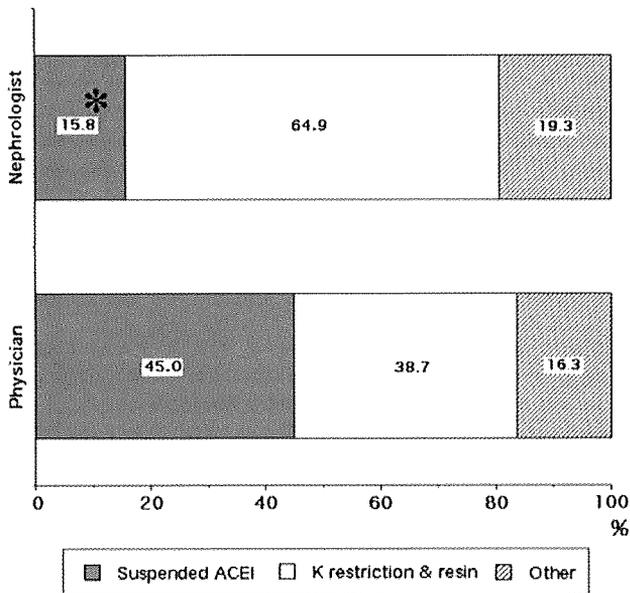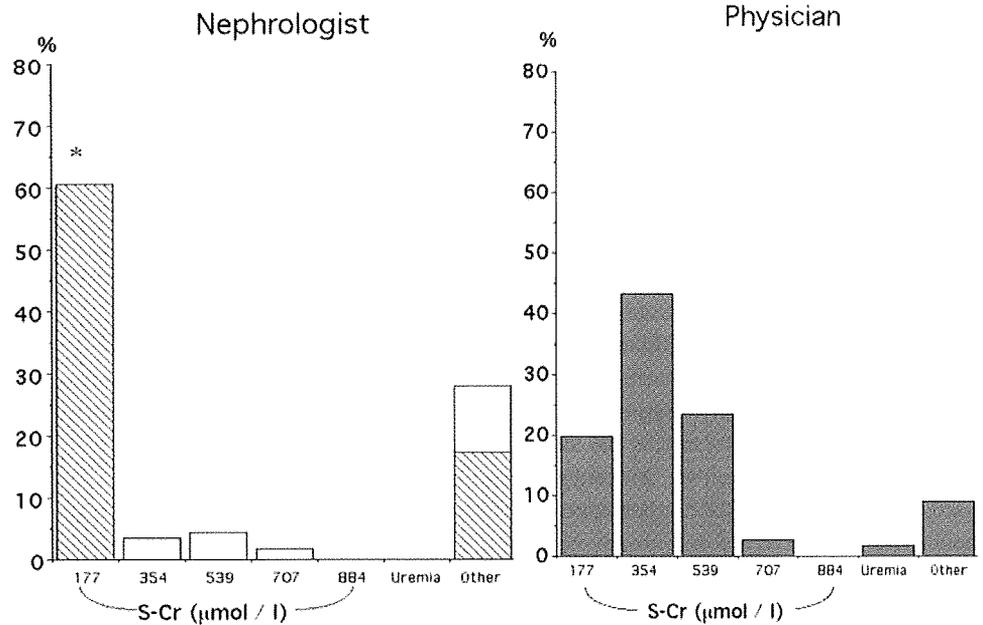

Fig. 2 Decision about ACEI continuance in case of mild increase of serum creatinine and serum potassium due to the drug. *$P < 0.0001$

guidelines for chronic kidney disease (CKD) published by the National Kidney Foundation, consultation and/or co-management with a kidney disease team is recommended for patients with stage 3 disease, and referral to a nephrologist is recommended for patients with stage 4 disease [5]. Stage 3 is defined as creatinine clearance between 30 and 59 ml/min/1.73 m^2 and stage 4 is defined as creatinine clearance between 15 and 29 ml/min/1.73 m^2. The guideline for CKD treatment was also issued by the Japanese Society of Nephrology in 2007 [9]. From the point of renal function, physicians are recommended to

consult nephrologists for treatment of patients with GFR less than 50 ml/min/1.73 m^2. However, as can be seen from Table 1, most Japanese doctors used serum creatinine as a marker of renal function in 2004. Only about one-third of physicians in Japan use creatinine clearance tests for outpatients. Moreover, many physicians work in areas with small populations compared with areas covered by nephrologists. The average age of inhabitants of small towns in Japan is generally much older than that of inhabitants of large cities. As shown by Cockcroft's equation, renal function deteriorates with age, even if serum creatinine level does not change [10]. Therefore, renal dysfunction might be considerably underestimated by physicians in Japan who use serum creatinine as a marker of renal function. In other words, the referral gap between nephrologists and physicians might be larger than shown based on figures for serum creatinine alone.

Although there are some limitations to our study, the results clearly show that there is a difference between decisions made by nephrologists and by general physicians in Japan regarding treatment of renal insufficiency. Results of previous studies also suggest that such a difference exists in other countries. Kalra et al. [11], a nephrologist, reported inappropriate use of ACEIs by general practitioners in the UK. De Lusignan et al. [12] reported that there have been many cases of overlooked renal insufficiency in the UK, based on results of analysis of data from medical databases. Akbari et al. [13] reported that the rate of detection of renal disease by family doctors was increased by 22.4% by using the equation for estimating creatinine clearance. These findings suggest a similar difference between nephrologists and physicians in other countries.

Recently, several studies have shown that serum cystatin C is a better indicator of renal function than not only serum creatinine but also creatinine clearance [14, 15]. However, the superiority of cystatin C over creatinine is controversial [16]. Moreover, the use of serum cystatin C as a marker of renal function has not become widespread due to the high cost of the test and lack of information.

Classical powerful tools such as calculated creatinine clearance (CCr) and new renal markers such as cystatin C can alert physicians to the possibility of renal dysfunction. However, improvements in the sensitivity of methods for detecting renal dysfunction will be meaningless without a change in the perception of physicians. As we have shown here, perception of physicians is significantly different from that of nephrologists. Moreover, the recent CKD guidelines also state the importance of proteinuria and hematuria as referral condition to nephrologist: (1) spot urine protein-to-creatinine ratio 0.5 g/g or positive dipstick test of proteinuria (2+ or greater), (2) positive dipstick test (1+ or greater) of both proteinuria and hematuria. Thus, vigorous promotion of the CKD guidelines is essential. In conclusion, there is significant difference between decisions made by physicians and nephrologists regarding treatment for patients with serum creatinine concentration of 177 μmol/l. Further evaluation of the effectiveness of CKD guidelines is needed.

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Original Paper

Microscopic Hematuria and Diabetic Glomerulosclerosis – Clinicopathological Analysis of Type 2 Diabetic Patients Associated with Overt Proteinuria

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Abstract
Background/Aims: The information available concerning the qualitative and quantitative clinical variables in cases with pathologically defined diabetic glomerulosclerosis (DGs) has been insufficient so far. In addition, the prevalence and composition of nondiabetic renal disease (NDRD) among proteinuric diabetics still remain to be delineated. *Methods:* The glomerular pathology, clinical correlates, and the prevalence of NDRD were retrospectively analyzed in 50 type 2 proteinuric diabetics who underwent a renal biopsy between 1990 and 2006. The patients were divided into two groups according to clinical and pathological features. Thereafter, the diagnostic contribution of the laboratory and clinical variables that were significant between the two groups were determined by logistic regression analysis. *Results:* There were 34 cases with pure DGs and 15 cases (30%) had NDRD with or without DGs. Although the difference in the prevalence of microscopic hematuria between these two groups was significant, it was no longer statistically significant when the patients were limited to nephrotic cases. We identified 14 hematuric cases with pathologically defined DGs, and they all had a significantly lower renal func-
tion than nonhematuric patients with DGs. The prevalence of nephrotic syndrome and retinopathy were significantly higher in the cases with hematuric DGs than in the cases with nonhematuric DGs. Based on a logistic regression analysis, the presence of nephrotic syndrome and known duration of diabetes were identified to be significant predictors for hematuria with DGs. *Conclusions:* Our observations suggest that the presence of hematuria may be a common feature for DGs with nephrotic syndrome.

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Introduction

Diabetic nephropathy (DN) is classically defined as a progressive increase in urine protein excretion, accompanied by a rising blood pressure and a persistent decline in glomerular filtration, culminating ultimately in end-stage renal failure [1]. Although there are no definitive and specific morphological diagnostic hallmarks for DN, diabetic glomerulosclerosis (DGs), including diffuse and nodular lesions, tends to be the major pathological characteristic of DN [2, 3]. In diabetics with overt proteinuria, a diagnosis of DN could be made without pathological confirmation by renal biopsy in the appropriate clinical setting, such as the presence of diabetic retinopathy, a long duration of diabetes, and hypertension [1]. There-

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fore, the information available concerning the qualitative and quantitative renal morphology in type 2 DM is sparse, and the relationship to clinical renal parameters is still insufficient [2–4].

On the other hand, performing renal biopsy has usually been considered when the presence of renal disease other than DN is suggested by several clinical signs, such as rapid deterioration of renal function, microscopic or macroscopic hematuria, and proteinuria in newly diagnosed diabetics without retinopathy or neuropathy [5–9]. A large number of renal biopsy studies have shown the concurrent presence of nondiabetic renal disease (NDRD) in diabetics; however, there were great variations in the prevalence and the composition of NDRD among the diabetic patients who underwent renal biopsy [5–9].

In the present study, we investigated the clinical and pathological records obtained from the 50 overt proteinuric type 2 diabetics who underwent renal biopsy because they felt that the underlying pathological lesions were not likely to be DGs and evaluated the prevalence of NDRD. The relationships between clinical features and renal morphology in the diabetics with pathologically defined DGs were also analyzed.

Patients and Methods

This study included 50 type 2 DM patients (29 males and 21 females) admitted to Jichi Medical University Hospital for a renal biopsy between 1990 and 2006. The onset of DM was defined as the time when the presence of glycosuria or DM was first diagnosed by a physician, and the duration of DM was defined as the period between the time of onset and renal biopsy. Of 50 patients, no information was available about a previous history of type 2 DM in 9 cases. In these cases, the renal disorder and DM were detected at the same time and the duration of DM was defined as zero. All the patients manifested overt proteinuria (>0.5 g/day). A percutaneous renal biopsy was performed because they were suspected to have NDRD. The reasons for performing a biopsy included short or obscure duration of DM, and/or absence of retinopathy, and/or rapid decline in renal function, and/or presence of microscopic hematuria. Tissue samples were routinely processed for light microscopy, immunofluorescence and electron microscopy. The biopsies were stained with hematoxylin and eosin, periodic acid-Schiff and periodic acid-silver methenamine for light microscopy. The biopsy samples were also analyzed immunohistochemically for the presence of IgG, IgA, IgM, complement C3, C1q and fibrinogen using immunofluorescence with frozen tissue.

DN was diagnosed pathologically based on the presence of DGs. DGs was classified into two types: namely, nodular and diffuse [2, 3]. A Kimmelstiel-Wilson nodule was defined as a nodular-type DGs. The nodular type was characterized by the unequivocal presence of acellular nodules surrounded by a rim of cell nuclei. The mesangial nodules were surrounded by patent glomerular capillaries. The diagnosis of diffuse type was established by the absence of a nodular lesion and a diffuse segmental or global increase in the mesangial matrix. Other glomerular features, including a thickening of the basement membrane, exudative glomerular lesions such as hyaline caps and capsular drops, arteriolar hyalinosis, and linear deposits of IgG in glomerular basement membranes (GBM) on immunohistochemistry helped identify DN. However, these findings were not useful in making a diagnosis in the absence of DGs. Other renal abnormalities were diagnosed using the standard pathological criteria [10].

The diabetic patients were divided into two groups: the group that had typical diabetic lesions without other renal disease (DGs group) and the group that had NDRD with or without diabetic lesions (other ± DGs group). The patients were also subdivided according to the presence or absence of nephrotic syndrome, retinopathy, and microscopic hematuria. The clinical parameters recorded for each patient on admission for renal biopsy included sex, age, hemoglobin A1c (HbA1c), systolic and diastolic blood pressure, urine protein (UP), creatinine clearance (Ccr), serum creatinine (sCr), serum albumin (sAlb), presence or absence of retinopathy and hematuria. Diabetic retinopathy was diagnosed using direct opthalmoscopy, with pupils dilated by an ophthalmologist, and graded as absent, simplex (SDR), or proliferative (PDR). Microscopic hematuria was defined as urine with >10 red blood cells/μl.

The data are expressed as the mean ± SD. The differences between the groups were analyzed using either the χ^2 test or nonpaired Student's t test. Clinical parameters that were significant at the 0.05 levels were entered into a stepwise forward multivariate logistic regression analysis in which the odds ratio and the 95% CI were determined to evaluate their contributions to the pathological diagnosis of DGs in the nephrotic cases. Their contributions to the presence of hematuria or diabetic retinopathy in the cases with DGs alone were also explored. The statistical analysis was performed using the StatView software program 5.0 for Windows (SAS Institute, Cary, N.C., USA).

Results

Patients' Profile at Renal Biopsy

There were a total of 50 cases analyzed, with 34 patients having isolated DGs and 16 patients having NDRD, either alone or in combination with DGs. The histological findings, deposits of immunoglobulins and complement fractions obtained by renal biopsy are shown in table 1. Nineteen patients suffered from nodular DGs and 18 had diffuse DGs. Among 15 patients (30%) found to have NDRD, 4 (8%) had membranous nephropathy (MN), 3 (6%) had IgA nephropathy (IgA-N), 2 (4%) had nephrosclerosis (NSc), and the remaining 3 patients had membranoproliferative glomerulonephritis (MPGN), lupus nephritis (LN) and focal segmental glomerulosclerosis (FSGS), respectively. Two patients (4%) had MN and 1 (2%) had IgA-N associated with diffuse DGs. One patient showed no glomerular lesion.

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Table 1. Renal pathology in 50 type 2 diabetic patients with overt proteinuria

Patient No.	Light and electron microscopy		Immunofluorescence microscopy		
	diabetic glomerulosclerosis	non-diabetic glomerular lesion	immunoglobulins	complement-fractions	location in glomeruli
1	nodular	absent	no tissue		
2	absent	mesangial proliferative glomerulonephritis	IgA	C3	mesangium
3	diffuse	mesangial proliferative glomerulonephritis	IgA	C3	mesangium
4	nodular	absent	none	none	
5	diffuse	membranous nephropathy	IgG, IgM	C3	capillary
6	nodular	absent	IgG	none	capillary
7	diffuse	absent	none	none	
8	nodular	absent	none	none	
9	absent	membranous nephropathy	IgG, IgM	none	capillary
10	absent	focal segmental glomerulosclerosis	IgM	none	capillary
11	absent	membranoproliferative glomerulonephritis	IgM, IgG	C3	mesangium, capillary
12	absent	membranous nephropathy	IgG, IgA	C3	capillary
13	nodular	absent	none	none	
14	nodular	absent	IgA, IgM	C1q	capillary
15	absent	nephrosclerosis	none	none	
16	nodular	absent	none	none	
17	diffuse	absent	IgG, IgA	none	capillary
18	absent	lupus nephritis type V	IgG	C3	mesangium
19	nodular	Absent	IgG	none	capillary
20	nodular	absent	IgG	none	capillary
21	diffuse	absent	IgA, IgM	C3	capillary
22	nodular	absent	none	none	
23	nodular	absent	none	none	
24	diffuse	absent	none	none	
25	diffuse	absent	none	none	
26	diffuse	absent	none	none	
27	nodular	absent	IgG, IgM	C3	capillary
28	diffuse	absent	IgM	none	capillary
29	diffuse	absent	IgM	none	capillary
30	absent	membranous nephropathy	IgG, IgM	C3	capillary
31	absent	minor	none	none	
32	diffuse	absent	IgM	none	capillary
33	nodular	absent	none	none	
34	diffuse	absent	IgM	none	capillary
35	nodular	absent	IgG	none	capillary
36	absent	mesangial proliferative glomerulonephritis	IgA	C3	mesangium
37	diffuse	absent	IgM	none	capillary
38	absent	mesangial proliferative glomerulonephritis	IgA	C3	mesangium
39	nodular	absent	none	none	
40	nodular	absent	IgA	none	capillary
41	diffuse	membranous nephropathy	IgG, IgM	C3	capillary
42	nodular	absent	IgM	none	capillary
43	diffuse	absent	none	none	
44	diffuse	absent	none	none	
45	diffuse	absent	IgG	none	capillary
46	diffuse	absent	IgA	none	capillary
47	absent	nephrosclerosis	none	none	
48	absent	membranous nephropathy	IgG	none	capillary
49	nodular	absent	none	none	
50	diffuse	absent	IgM	none	capillary

Table 2. Clinical data in 50 type 2 DM with overt proteinuria

	Glomerulopathy		p value
	diabetic glomerulosclerosis	other ± diabetic glomerulosclerosis	
Number, sex	12 F/22 M	9 F/7 M	
Age, years	52 ± 12	55 ± 10	
Hemoglobin A1c	7.3 ± 2.3	6.7 ± 1.2	
Known duration of diabetes, years	5.8 ± 5.4 (0–20)	7.6 ± 5.6 (0–18)	
Systolic blood pressure, mm Hg	162 ± 28	151 ± 15	
Diastolic blood pressure, mm Hg	89 ± 15	84 ± 11	
Urine-protein, g/day	5.3 ± 3.6	4.6 ± 4.7	
Creatinine clearance, ml/min	67.7 ± 41.7	71.3 ± 37.0	
Serum creatinine, mg/dl	1.1 ± 0.4	1.2 ± 0.7	
Serum albumin, g/dl	3.0 ± 0.8	3.1 ± 0.9	
Retinopathy, %	61	43	
Hematuria, %	41	75	<0.05

Table 3. Clinical characteristics of the type 2 diabetics with nephrotic syndrome grouped as DGs and other ± DGs

	Glomerulopathy		p value
	DGs	other ± DGs	
Number, sex	6 F/12 M	3 F/3 M	
Age, years	55 ± 12	56 ± 8	
Hemoglobin A1c	7.4 ± 3.0	6.9 ± 1.1	
Known duration of diabetes, years	7.4 ± 3.0 (0–20)	7.7 ± 5.0 (0–18)	
Systolic blood pressure, mm Hg	169 ± 30	142 ± 15	
Diastolic blood pressure, mm Hg	92 ± 17	78 ± 8	
Urine-protein, g/day	7.2 ± 2.9	8.1 ± 6.4	
Creatinine clearance, ml/min	45.9 ± 17.9	67.4 ± 23.3	<0.05
Serum creatinine, mg/dl	1.3 ± 0.5	0.9 ± 0.4	
Serum albumin, g/dl	2.4 ± 0.4	2.1 ± 0.7	
Retinopathy, %	83	33	<0.05
Hematuria, %	72	75	

The clinical characteristics and pertinent laboratory findings of the 50 type 2 diabetics are shown in table 2. There were no differences between the two groups, i.e. DGs and other ± DGs, with respect to the gender ratio, age, HbA1c, known duration of diabetes, systolic and diastolic blood pressure, UP, Ccr, sCr, sAlb, and the prevalence of diabetic retinopathy. However, hematuria was noted in 41% of the DGs group and in 75% of the other ± DGs group (p < 0.05). All cases with hematuria were microscopic and no case with macroscopic hematuria was observed.

Among the 34 patients with DGs alone and the 16 patients with other renal disease with or without DGs, nephrotic syndrome was revealed in 18 patients (53%) and 6 patients (38%), respectively. The clinical parameters of these groups were then compared between the two groups (table 3). In nephrotic patients, no significant difference was observed in the prevalence of hematuria; however, a significant difference was observed in Ccr and the prevalence of diabetic retinopathy. According to a stepwise forward multivariate logistic regression analysis, only the presence of retinopathy was identified to be an indepen-

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Table 4. Relative risks for the presence of hematuria among patients with DGs

Variables	Odds ratio	95% CI	p value
Presence of nephrotic syndrome	139.67	3.642–5356.386	0.0079
Known duration of diabetes, years	1.52	1.008–2.286	0.0458

Odds ratio of each variable was determined by a multivariate logistic regression analysis.

dent predictor of DGs (p = 0.0318), and the odds ratio was 10.0 (95% CI 1.222–81.828).

Relationship between DGs and Hematuria

Next, we analyzed about the differences in the clinical characteristics between the 14 hematuric and 20 nonhematuric patients with DGs alone. The gender ratio, age, HbA1c, systolic and diastolic blood pressure, and UP, were similar in both groups (data not shown). However, when compared to the nonhematuric DGs, the hematuric DGs had a longer known duration of DM (9.0 ± 5.8 vs. 3.6 ± 3.8 years, p < 0.01) and a higher level of sCr (1.4 ± 0.5 vs. 0.9 ± 0.4 mg/dl, p < 0.01). Conversely, when compared to the non-hematuric DGs, the hematuric DGs had lower level of Ccr (45.2 ± 14.9 vs. 83.3 ± 47.8 ml/min, p < 0.01) and sAlb (2.5 ± 0.4 vs. 3.2 ± 0.8 g/dl, p < 0.01). Significant increases in the prevalence of nephrotic syndrome (72 vs. 6%, p < 0.01) and retinopathy (57 vs. 15%, p < 0.01) were also found in the cases with hematuric DGs but not in those with nonhematuric DGs. The prevalence of nodular lesion was numerically higher in the patients with hematuria (71%) than in the patients without hematuria (45%); however, this difference did not reach statistical significance.

Using the variables that were significant between the cases with hematuric DGs and those with nonhematuric DGs, we then further evaluated their contributions in hematuria. A stepwise forward multivariate logistic regression analysis identified the presence of nephrotic syndrome and the duration of diabetes to be significant predictors for hematuria with DGs (table 4).

Impact of Diabetic Retinopathy on Renal Functional and Structural Characteristics in Type 2 Diabetics with Overt Proteinuria

In the overt proteinuric type 2 diabetics with retinopathy (7 female/15 male), DGs were pathologically confirmed in 75% of cases and the remaining 25% were grouped as other ± DGs. On the other hand, 59% were confirmed to have DGs alone and the remaining 41%

were grouped as other ± DGs in the cases without retinopathy (14 female/14 male). There were no significant differences in the prevalence of DGs and other renal disease with or without DGs between the patients with retinopathy and those without retinopathy. However, when compared to the patients without retinopathy, the patients with retinopathy had a significantly higher prevalence of nephrotic syndrome (60 vs. 31%, p < 0.05) and a significantly lower value of Ccr (54.6 ± 26.0 vs. 86.9 ± 47.2 ml/min, p < 0.01). None of these parameters, except for Ccr, showed an association with the presence of retinopathy in diabetics with overt proteinuria (odds ratio 0.984 [95% CI 0.970–0.999], p = 0.0354).

Next, the patients whose renal lesion was limited to DGs alone were selected and the differences in the clinical characteristics of the patients with or without retinopathy were further analyzed. As shown in table 5, the prevalence of nephrotic syndrome and hematuria were significantly higher in the patients with retinopathy. On the other hand, Ccr and sAlb were significantly higher in the patients without retinopathy. The prevalence of nodular lesions was significantly higher in the patients with retinopathy (81%) than in the patients without retinopathy (9%, p < 0.01). Among these five variables, the presence of a nodular lesion and Ccr was identified as independent predictors for the presence of diabetic retinopathy in the cases with DGs (table 6).

Discussion

Although the presence of hematuria has been considered one of the atypical features indicating the presence of NDRD among proteinuric diabetics [11], the clinical significance of hematuria on the overall course of DN may need to be evaluated more profoundly. Indeed, there have been several reports suggesting that hematuria could be a sign of DN [12, 13]. However, there has been no available information about the relationship between hematuria and renal function. In the present study, we

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Table 5. Clinical characteristics of the cases with pathologically diagnosed DGs

	With retinopathy	Without retonopathy	p value
Number, sex	8 F/13 M	4 F/9 M	
Age, years	54 ± 11	47 ± 14	
Hemoglobin A1c	7.4 ± 2.7	7.0 ± 1.5	
Known duration of diabetes, years	7.1 ± 6.1 (0–10)	3.6 ± 3.4 (0–10)	
Systolic blood pressure, mm Hg	170 ± 29	148 ± 20	<0.01
Diastolic blood pressure, mm Hg	91 ± 17	85 ± 8	
Urinary protein, g/day	6.2 ± 2.8	3.7 ± 4.5	
Creatinine clearance, ml/min	48.3 ± 19.6	98.6 ± 49.2	<0.01
Serum creatinine, mg/dl	1.2 ± 0.4	0.9 ± 0.5	
Serum albumin, g/dl	2.6 ± 0.4	3.4 ± 1.0	<0.01
Nephrotic syndrome, %	71	23	<0.01
Hematuria, %	57	15	<0.05

Table 6. Multivariate logistic regression analysis of relative risks for the presence of retinopathy among the patients with DGs

Variables	Odds ratio	95% CI	p value
Presence of nodular lesion	11.737	1.465–94.039	0.0204
Creatinine clearance, ml/min	0.97	0.942–0.998	0.0373

demonstrated for the first time that the cases with hematuric DGs had a more greatly reduced renal function than the cases with nonhematuric DGs. Furthermore, the presence of nephrotic syndrome and a long history of diabetes were identified as significant predictors for hematuria among the patients with pathologically defined DGs. Since a reduced renal function and proteinuria in the nephrotic range are common features of advanced DN [14, 15], our results suggest that hematuria may therefore be a common feature for the patients with late-stage glomerular damage due to diabetes, and it might not be a useful predictor for NDRD among such cases. Alternatively, the association of hematuria and reduced renal function also suggest the possibility that the patients who have hematuria at the onset or early stage of DGs might be at greater risk for reduction in renal function.

The diabetics with retinopathy included in the present study were more likely to have a reduced renal function in comparison to diabetics without retinopathy. This difference was also the case in the selected patients whose renal lesions were limited to DGs alone. These observations might be related to the previous findings that retinopathy is a significant predictor of the progression of renal damage in type 2 diabetics with proteinuria [16]. Similarly, Rossing et al. [17] also found that the degree of diabetic retinopathy independently predicted the rate of decline in renal function in a longitudinal analysis.

The greater rate of reduction in renal function in type 2 diabetics with retinopathy may be due to a more complicated glomerular and the interstitial lesion, since there are several observations indicating a positive correlation between the presence of retinopathy and renal structure abnormalities and functional impairment [4, 18, 19]. In the present study, the presence of a nodular lesion was identified as one of the significant variables for the prediction of retinopathy. Similar observations showing retinopathy to be associated with nodular lesions, but not with mesangial sclerotic lesions, have also been reported [3]. From these findings including our results, there might be a latent relationship between nodular lesions and the advanced stage of DGs. Indeed, the renal function of the patients with nodular lesions was significantly lower than those with diffuse lesions (data not shown).

Although the present study provides new information on the presumable relationship between hematuria and diabetic nephropathy, our results should be interpreted within the context of its limitations. First, the number of patients included in our study was relatively small. The small sample size likely meant that the study might be underpowered to detect the clinical significance of several parameters such as hematuria or retinopathy. For in-

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stance, although the prevalence of hematuria was significantly higher in the cases with retinopathy than in the cases without retinopathy, we failed to identify the presence of hematuria as a significant predictor for retinopathy in the cases with pathologically diagnosed DGs. We also found a significant difference in the prevalence of retinopathy between the cases with hematuric and nonhematuric DGs; however, the relative risk of the retinopathy for the hematuric DGs was not statistically significant. Nevertheless, these observations led us to propose the presence of a latent relationship between hematuria and retinopathy. Obviously, further detailed analysis including a higher number of cases with pathologically defined DGs should be required.

Second, as observed in previous studies [5–9], the renal biopsies in the present study were not performed on the basis of research protocols but for clinical indications, thereby involving an intrinsic selection bias. Therefore, our findings that 15 of 50 type 2 DM patients (30%) with overt proteinuria had various types of NDRD might be overestimated, since the lower prevalence of NDRD (23%) has been reported by a prospective study in which no special selection criteria were applied [20]. Interestingly, Mazzucco et al. [21] have demonstrated that the policies applied in nephrology centers for the selection of diabetic patients to undergo a renal biopsy are related to the discrepancies in the frequency of various types of glomerulopathy reported in previous studies. A renal biopsy has also been recommended for proteinuric type 2 diabetics without retinopathy, since a separation between diabetic and nondiabetic glomerulopathy was not possible in such patients based on demographic, clinical and laboratory findings [22]. Indeed, we could identify nondiabetic glomerular lesions in 41% of diabetics without retinopathy and confirm the presence of DGs in 59% of the cases in the present study. On the other hand, several recent studies have suggested that a morphological analysis of the kidney would be a valuable diagnostic procedure for the overall management of patients with DN [2, 7, 23]. Therefore, the clinical significance of a renal biopsy in the overall assessment for proteinuric diabetics should be evaluated more carefully.

Acknowledgement

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Regulation of Megalin Expression in Cultured Proximal Tubule Cells by Angiotensin II Type 1A Receptor- and Insulin-Mediated Signaling Cross Talk

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Impairment of proximal tubular endocytosis of glomerular-filtered proteins including albumin results in the development of proteinuria/albuminuria in patients with chronic kidney disease. However, the mechanisms regulating the proximal tubular function are largely unknown. This study aimed to investigate the role of angiotensin II type 1A receptor ($AT_{1A}R$)- and insulin-mediated signaling pathways in regulating the expression of megalin, a multiligand endocytic receptor in proximal tubule cells (PTCs). Opossum kidney PTC-derived OK cells that stably express rat $AT_{1A}R$ but are deficient in endogenous angiotensin II receptors ($AT_{1A}R$-OK cells) were used for this study. Treatment of the cells with angiotensin II suppressed mRNA and protein expression of megalin at 3- and 24-h incubation time points, respectively. Cellular uptake and degradation of albumin and receptor-associated protein, megalin's endocytic ligands were suppressed 24 h after angiotensin II treatment. The $AT_{1A}R$-mediated decrease in megalin expression was partially prevented by ERK inhibitors. Insulin competed with the $AT_{1A}R$-mediated ERK activation and decrease in megalin expression. Inhibitors of phosphatidylinositol 3-kinase (PI3K), a major component of insulin signaling, also suppressed megalin expression, and activation of the insulin receptor substrate (IRS)/PI3K system was prevented by angiotensin II. Collectively the $AT_{1A}R$-mediated ERK signaling is involved in suppressing megalin expression in the OK cell line, and insulin competes with this pathway. Conversely, the insulin-IRS/PI3K signaling, with which angiotensin II competes, tends to stimulate megalin expression. In conclusion, there is $AT_{1A}R$- and insulin-mediated competitive signaling cross talk to regulate megalin expression in cultured PTCs. (*Endocrinology* 150: 871–878, 2009)

Proximal tubule cells (PTCs) reabsorb glomerular-filtered proteins including albumin via receptor-mediated endocytosis (1, 2). Proteinuria is generally assumed to be a result of increased transit of serum proteins (mostly, albumin) through glomeruli. However, it is also attributed to altered reabsorption of the proteins by PTCs because it is estimated that in healthy adults, PTCs reabsorb 3–6 g of albumin on a daily basis (2). Progressive proximal tubular impairment associated with chronic kidney disease is likely to result in increased proteinuira/albuminuria. Also, even at the early stages of diabetic nephropathy, albumin reabsorption by PTCs has been found to be impaired (3, 4), which is primarily associated with the mechanism

Abbreviations: Ang II, Angiotensin II; ATR, angiotensin II receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; IRS, insulin receptor substrate; JAK, Janus kinase; JNK, Jun N-terminal kinase; MEK, MAPK or ERK kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PTC, proximal tubule cell; RAP, receptor-associated protein; RAS, renin-angiotensin system; STAT, signal transducer and activator of transcription; TCA, trichloroacetic acid.

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of microalbuminuria. The endocytic function of PTCs is likely to be mediated by a variety of factors that act on the cells via specific membrane receptors. Among these factors, angiotensin II (Ang II) and insulin are the most likely to affect the function of the cells.

Ang II acts on the kidney via types 1 and 2 Ang II receptors (AT_1R and AT_2R), both of which are expressed in PTCs (5). These receptors work in a counterbalanced manner, although AT_1R is more involved in pathological actions (6). In diabetes, renal AT_2R is down-regulated (7); thus, Ang II-mediated pathological actions in the kidney become more evident via AT_1R. In rodents, there are two AT_1R subtypes ($AT_{1A}R$ and $AT_{1B}R$), but $AT_{1A}R$ is recognized as the primary subtype responsible for Ang II actions (8). It has been found that AT_2R-mediated protein kinase B activation modulates albumin endocytosis in PTCs (9). However, the role of AT_1R, the major Ang II receptor acting in the diabetic kidney, and its signaling pathways involved in the endocytic functions of PTCs have not been thoroughly identified, despite the well-established effectiveness of AT_1R blockers for reduction of albuminuria in patients with diabetic nephropathy (10).

Insulin-mediated signaling pathways also appear to play an important role in the mechanisms of albuminuria because albuminuria is a well-known clinical characteristic of patients with insulin resistance (11). Insulin-mediated signaling pathways are likely to be involved in regulating endocytic functions of PTCs because these cells are a major target of insulin (12, 13). Furthermore, there is evidence for competitive cross talk between Ang II- and insulin-mediated signaling pathways, which may be associated with the pathogenesis of insulin resistance (14). However, the molecular mechanisms of Ang II- and insulin-mediated signaling interactions involved in endocytic functions of PTCs are undetermined.

Megalin, a high-molecular-mass (~600 kDa) member of the low-density lipoprotein receptor family (15), is a major endocytic receptor involved in proximal tubular uptake of glomerular-filtered proteins including albumin (1). At clathrin-coated pits, megalin internalizes its ligands into endocytic compartments and is recycled to the cell surface. Megalin also plays a critical role in vitamin homeostasis by metabolizing vitamin-binding proteins, such as vitamin D-binding protein and retinol-binding protein (16). Megalin knockout mice display symptoms of low-molecular-weight proteinuria and albuminuria (17, 18). Patients with Donnai-Barrow and faciooculoacousticorenal syndromes, caused by mutations in the megalin gene, were also found to show massive urinary excretion of albumin and low-molecular-weight proteins (19). Decreased expression of megalin in PTCs has been found in the early stages of diabetes in experimental animals (20). It is also suggested that the functions of megalin are likely to be impaired in patients in the early stages of diabetes because low-molecular-weight proteinuria, as well as albuminuria, are frequently observed in patients at these stages (21, 22). However, the mechanisms regulating megalin expression have been largely unknown to date.

To investigate specific functions of AT_1R solely, a kidney cell line derived from opossum PTC has been established (23). This cell line is deficient in endogenous Ang II receptors but manually incorporated with functional rat $AT_{1A}R$. The characteristic of

this cell line would allow us to examine the role of the type 1 receptors without interference by the function of type 2 receptors. In this study, we took advantage of the nature of this cell line to analyze $AT_{1A}R$- and insulin-mediated signaling mechanisms that regulate the expression of megalin *in vitro*.

Materials and Methods

Materials

Ang II and insulin were purchased from Sigma-Aldrich (St. Louis, MO). A rabbit polyclonal antibody was raised against the rat megalin cytoplasmic tail as described previously (24). A monoclonal antibody to β-actin was purchased from Abcam (Cambridge, MA). Antibodies to phosphorylated ERK1/2 (Thr202/Thy204) and (total) ERK1/2 were obtained from Cell Signaling Technology (Beverly, MA). Antiinsulin receptor substrate (IRS) 1, anti-IRS2 and antiphosphatidylinositol 3-kinase (PI3K) p85 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). A monoclonal antibody to phosphorylated tyrosine, p-Tyr, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). LY 294002, U0126, and SB 203580 were purchased from Promega (Madison, WI). Wortmannin, PD 98059, bisindolylmaleimide I, SP600125, and AG490 were purchased from Calbiochem (La Jolla, CA). The AT_1R blocker CV-11974 was kindly provided by Takeda Chemical Industries, Ltd. (Osaka, Japan).

Cell culture

A subline of OK cells that do not express endogenous Ang II receptors but stably express transfected cDNA encoding rat $AT_{1A}R$ (designated as $AT_{1A}R$-OK cells in this study) were maintained in DMEM/F12 (17.5 mM glucose) supplemented with 10% fetal calf serum and 200 μg/ml G418 (Sigma-Aldrich) at 37 C and 5% CO_2 (23). The cells were washed twice with DMEM/F12 and serum starved for 24 h. Cells were then treated with Ang II and/or insulin and incubated for specified time periods under serum-free culture conditions to investigate the expression of megalin or examine Ang II- and insulin-mediated signaling pathways. Cell culture reagents were obtained from Invitrogen (Carlsbad, CA) except where indicated. All signaling-pathway inhibitors were added to culture media, 1 h before Ang II and/or insulin treatment, at the standard concentrations used for experiments with OK cells in previous reports (25, 26).

SDS-PAGE and immunoblotting

Cultured cells and rat kidneys were solubilized in lysis buffer [0.5% Triton X-100, 20 mM HEPES, 150 mM NaCl, 1× complete protease inhibitor (Roche, Basel, Switzerland) (pH 7.4)] and centrifuged at 1500 × g at 4 C for 30 min. Protein concentrations were determined using the bicinchoninic acid assay kit (Pierce, Rockford, IL). Samples were resolved by SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were first blocked in a buffer containing 25 mM Tris-HCl (pH 7.4), 137 mM NaCl, 2.7 mM KCl, 0.05% Tween 20, and 5% fetal calf serum for 1 h and then incubated with primary antibodies for either 2 h at room temperature or overnight at 4 C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h. Immunoreactive proteins were detected by enhanced chemiluminescence (Super Signal; Pierce). Immunoblots were quantitated using β-actin expression as an internal control with National Institutes of Health ImageJ software (available at http://rsb.info.nih.gov/nih-image/, last accessed June, 2007).

Preparation of glutathione S-transferase (GST) fusion proteins

Rat receptor-associated protein (RAP) was prepared as a fusion protein with GST as described previously (27). Also, cDNA encoding the rat

megalin cytoplasmic tail was prepared by RT-PCR using rat kidney RNA, cloned in the pGEX-KG vector, and expressed as a GST-fusion protein, as described previously (28).

RNA extraction and real-time RT-PCR

RNA was extracted from $AT_{1A}R$-OK cells following the standard ISOGEN method (Nippon Gene Co., Ltd., Tokyo, Japan) and resuspended in autoclaved diethylpyrocarbonate-treated water. Extracted RNA concentrations were determined using GeneQuant (Biochrom Ltd., Cambridge, UK) and equalized to 1 μg/μl. Two separate amplicons, located on the megalin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) loci, were reverse transcribed and quantified using the same template RNA for relative quantification analysis, using a one-step real-time RT-PCR kit (RNA-direct SYBR, Green Realtime PCR master mix; Toyobo Co., Ltd., Osaka, Japan) and LineGene (BioFlux, Tokyo, Japan). The final reaction mixture (20 μl total volume) contained 2.5 mM $Mn(OAc)_2$, 10 μl recombinant *Thermus thermophilus* DNA polymerase, deoxynucleotide triphosphates, and SYBR Green I in a single master mix, 500 nM each of forward and reverse primers (for the megalin amplicon: forward, 5'-aggctcccttctgccatctcttc-3' and reverse, 5'-gcagaatct-ggtccaaaacctgacac-3'; for the GAPDH amplicon: forward, 5'-gagatgct-ggagccgagta-3' and reverse, 5'-gtggttcacccccatcac-3') and 2 μg of RNA template. The enzyme was first activated at 90 C for 30 sec, followed by reverse transcription at 61 C for 20 min. After denaturation of the synthesized cDNA from the template RNA strand at 95 C for 30 sec, 40 amplification cycles of 95 C for 15 sec and 55 C for 15 sec were performed. The fluorescence signal data were collected at 74 C for 30 sec at the end of each amplification cycle. The melting curve analysis followed the amplification cycles, from 74 C to 94 C with a step rate of 0.5 C. All cycling steps were performed with a ramp rate of 4 C/sec. Each set of runs included a megalin subset and its parallel GAPDH subset, with a corresponding standard curve for each amplicon. The resulted outcome was normalized for each sample subset with the corresponding standard curve, using the LineGene software (BioFlux) with the second derivative method. Each sample was tested in duplicates.

Immunoprecipitation

Cultured cells were solubilized in lysis buffer [0.5% Triton X-100, 20 mM HEPES, 150 mM NaCl, 10 mM NaF, 1 mM sodium orthovanadate, 1× complete protease inhibitor (Roche) (pH 7.4)] and centrifuged at 15,000 × g at 4 C for 30 min. Nonspecific binding proteins were precleared by incubating approximately 500 μg of each cell lysate with 30 μl Protein G Plus/protein A agarose beads (Calbiochem) for 30 min. The supernatant from this preclearing precipitation was then incubated with 4 μg anti-IRS1 IgG or 4 μl anti-IRS2 IgG at 4 C overnight. Another 30 μl of Protein G Plus/protein A agarose beads were added to the same supernatant to bind antibodies and associated proteins, and the beads were washed three times with lysis buffer and twice with PBS. Bound immune complexes were eluted from the beads by denaturation in 1× Laemmli sample buffer at 95 C for 3 min and then resolved by SDS-PAGE, followed by immunoblotting with anti-PI3K p85 and tyrosine, p-Tyr antibodies. TrueBlot antirabbit IgG immunoprecipitation beads and horseradish peroxidase-conjugated antirabbit IgG system (eBioscience, San Diego, CA) were used when the anti-p85 antibody was applied to minimize nonspecific binding to the heavy and light chains of the antibodies used for immunoprecipitation.

Radioiodination

Rat albumin (Sigma-Aldrich) and GST-RAP were radioiodinated using 1 mCi Na-[125]I (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and one Iodo-Bead (Pierce) according to the manufacturer's instructions. Free Na-^{125}I was removed from the labeled proteins by binding to a PD-10 column (Bio-Rad Laboratories). Specific activities of the resulting purified ^{125}I-albumin and ^{125}I-RAP were 2.4 × 10^6 and 1.0 × 10^6 cpm/μg, respectively.

Cellular degradation assays

Cells were grown to confluence (1 × 10^5 cells/well) on 12-well tissue culture plates. The cells were washed twice with DMEM and serum starved for 24 h. Cells were then treated with Ang II (100 nM) or its vehicle and incubated for 24 h under serum-free condition. The culture media were then replaced with DMEM containing 0.1% ovalbumin and ^{125}I-albumin or ^{125}I-RAP (1.0 μg/ml). After 3 h incubation, the culture media were mixed with trichloroacetic acid (TCA) at a final concentration of 15% to precipitate the labeled proteins, and the radioactivity level of the TCA-soluble degradation products was quantified by γ-counting. To correct for iodine liberated from ^{125}I-labeled ligands, the level of TCA-soluble radioactivity in the medium incubated without cells was subtracted from that found in the samples.

Statistics

Data are expressed as means ± SD. The comparison between two experimental groups was made using Student's t test for unpaired data. For multiple comparisons, one-way ANOVA with Bonferroni/Dunn analysis was used. $P < 0.05$ was considered statistically significant.

Results

Ang II suppresses mRNA and protein expression and the endocytic function of megalin in $AT_{1A}R$-OK cells

Initially, the specificity of the polyclonal antibody raised against rat megalin cytoplasmic tail-derived peptide sequence was confirmed on the lysates of opossum PTC-derived OK cells stably expressing rat $AT_{1A}R$ ($AT_{1A}R$-OK cells), the parental OK cells and rat kidney by competitive assays with recombinant rat megalin cytoplasmic tail proteins (supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Protein expression of megalin was suppressed 24 h after treatment of $AT_{1A}R$-OK cells with Ang II that had been serum starved for 24 h, but the expression was not reduced in OK cells similarly treated (Fig. 1A). The Ang II-mediated decrease in megalin expression in $AT_{1A}R$-OK cells was reversed by incubation with CV-11974, an AT_1R blocker, indicating that the effect of Ang II was mediated via the $AT_{1A}R$ (Fig. 1B). No suppression of megalin protein expression was found at either 3- or 8-h incubation time points (data not shown). Such a long onset time for the effect of Ang II suggests that decreased megalin expression is likely to be regulated at the transcriptional level. In fact, RT-PCR analysis of the cells 3 h after treatment with Ang II showed that mRNA expression of megalin was decreased, indicating that Ang II suppressed megalin expression at the transcriptional level (Fig. 1C).

Twenty-four hours after treatment with Ang II, changes in the endocytic function of megalin in $AT_{1A}R$-OK cells were investigated by cellular degradation assays using the megalin endocytic ligands ^{125}I-labeled albumin and RAP. As shown in Fig. 2, degradation of these ligands in the cells was found to be significantly suppressed by Ang II treatment.

Ang II-mediated ERK activation is involved in the signaling pathway that suppresses megalin expression

Ang II has been shown to activate the ERK-mediated signaling pathway in vascular smooth muscle cells (29) and primary cultured PTCs (30). As shown in Fig. 3, A and B, Ang II was also
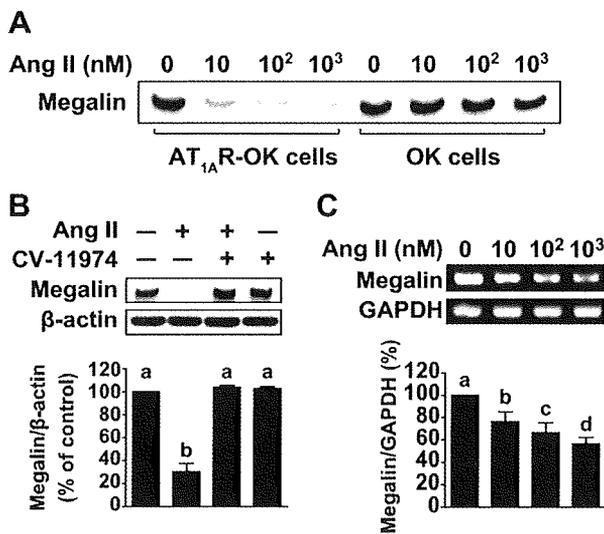
FIG. 1. Ang II suppresses protein and mRNA expression of megalin via $AT_{1A}R$ in $AT_{1A}R$-OK cells. A, Immunoblotting shows that protein expression of megalin was suppressed 24 h after treatment of 24-h serum-starved $AT_{1A}R$-OK cells with Ang II, whereas the expression level was unchanged in OK cells. B, CV-11974 (1 μM), an AT_1R blocker, abolished Ang II (100 nM)-mediated reduction of megalin protein expression in $AT_{1A}R$-OK cells, indicating that the Ang II effect is mediated via the $AT_{1A}R$. The *upper panel* shows representative immunoblotting results. In the *lower panel*, megalin bands were quantified and normalized with the endogenous β-actin control (n = 4). C, Real-time RT-PCR analysis of the cells after 3 h of incubation with Ang II showed a decrease in mRNA expression of megalin, indicating that Ang II suppressed megalin expression at the transcriptional level. The *upper panel* shows representative RT-PCR results on agarose gel electrophoresis. In the *lower panel*, the gene expression level of megalin was quantified with reference to that of the endogenous control GAPDH (n = 10). Values are expressed as mean ± SD. The differences between values associated with *different letters* are all statistically significant (P < 0.05).

found to activate the ERK-pathway in 24-h serum-starved $AT_{1A}R$-OK cells. We therefore tested whether Ang II-mediated ERK activation plays a role in the $AT_{1A}R$-mediated signaling that suppresses the expression of megalin. As shown in Fig. 3, C and E, an MAPK or ERK kinase (MEK)-1/2 inhibitor, U0126, significantly but not completely counteracted Ang II-mediated suppression of megalin mRNA and protein expression at the 3- and 24-h incubation time points, respectively. The counteraction to the Ang II-mediated suppression of megalin mRNA by PD98059, another MEK1/2 inhibitor, was also significant at both the 3- and 8-h incubation time points. However, this reversing effect was smaller at the 8-h incubation time point and was far from a complete setback (Fig. 3, F and G); this phenom-
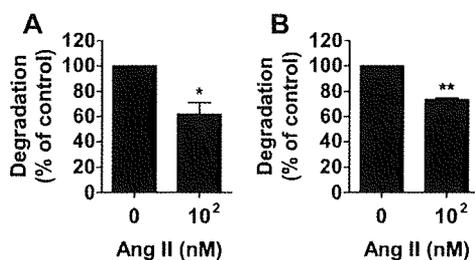

FIG. 2. Ang II suppresses the endocytic uptake and degradation of albumin and RAP in $AT_{1A}R$-OK cells. Treatment of 24-h serum-starved $AT_{1A}R$-OK cells with Ang II (100 nM) for 24 h decreased the degradation of endocytic megalin ligands, ^{125}I-lableled albumin (A), and RAP (B). Values are expressed as mean ± SD. *, P < 0.05, n = 6; **, P < 0.01, n = 4.
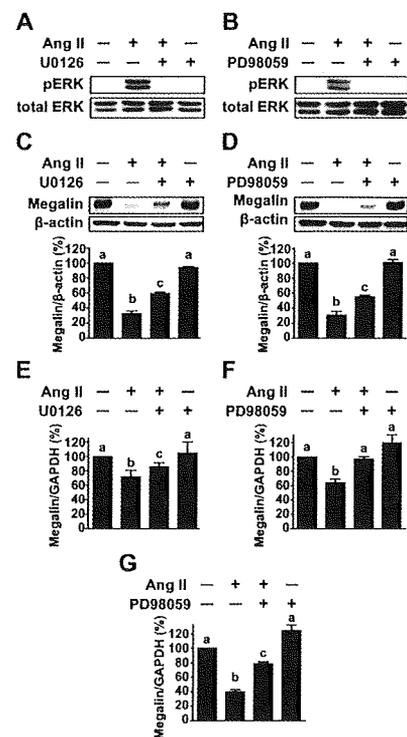

FIG. 3. Ang II-mediated ERK activation is involved in the signaling pathway that suppresses megalin expression in $AT_{1A}R$-OK cells. Treatment of 24-h serum-starved $AT_{1A}R$-OK cells with Ang II (100 nM) for 5 min was found to induce ERK activation, as shown by immunoblotting with anti-ERK1/2 (total ERK) and anti-phospho-ERK1/2 (pERK) antibodies. This effect was virtually abolished when the cells were treated with MEK1/2 inhibitors U0126 (10 μM) (A) or PD98059 (25 μM) (B) starting 60 min before the Ang II treatment. Suppression of megalin protein expression observed when the cells were treated with Ang II (100 nM) for 24 h was significantly but not completely reversed by the similar pretreatment of the cells with 10 μM U0126 (C, n = 3) or 25 μM PD98059 (D, n = 5). Suppression of megalin mRNA expression in the cells by Ang II treatment for 3 h was also significantly but not completely reversed by the pretreatment of the cells with 10 μM U0126 (E, n = 10), as shown by real-time RT-PCR. Similarly, the pretreatment of the cells with 25-μM PD98059 significantly reversed the effect of Ang II suppressing megalin mRNA expression after 3 h of incubation (F, n = 4), but the reversing effect attenuated in 8 h (G, n = 4). Values are expressed as mean ± SD. The differences between values associated with *different letters* are all statistically significant (P < 0.05).

enon might explain the significant but incomplete inhibition of Ang II-mediated suppression of megalin protein expression by PD98059 at the 24-h incubation time point (Fig. 3D). These data indicate that Ang II-mediated ERK activation is very likely to be involved at some point in the signaling pathway that suppresses megalin expression in $AT_{1A}R$-OK cells.

Ang II is also known to activate signaling pathways mediated via Jun N-terminal kinase (JNK), p38, protein kinase C (PKC), or Janus kinase (JAK)-signal transducer and activator of transcription (STAT) in vascular smooth muscle cells (29). However, inhibitors of these signaling pathways, used at standard experimental concentrations in OK cells (25, 26), did not alter the ability of Ang II to suppress megalin protein expression (supplemental Fig. 2). Collectively, the ERK signaling pathway is involved in Ang II-mediated suppression of megalin expression in $AT_{1A}R$-OK cells, but other Ang II-mediated signaling pathway(s) may be also present which are different from JNK, p38, PKC, and JAK-STAT pathways.

Insulin competes with the Ang II-ERK signaling pathway involved in suppression of megalin expression

We next investigated the effect of insulin on megalin expression in $AT_{1A}R$-OK cells because insulin-dependent signaling pathways are likely to be involved in the mechanisms of albuminuria and endocytic functions of PTCs. Treatment of 24-h serum-starved $AT_{1A}R$-OK cells with 100 nM insulin did not change megalin protein expression levels at the 24-h incubation time point (Fig. 4A). However, insulin reduced the inhibitory effect of Ang II on megalin protein expression after 24-h of incubation (Fig. 4A). The suppression of megalin mRNA expression by Ang II was also significantly inhibited by insulin at the 3-h incubation time point (Fig. 4B), indicating that insulin competes with the effect of Ang II at the level of regulating the megalin gene transcription. Insulin was also found to compete with Ang II-mediated ERK activation in the cells (Fig. 4C).

To unveil the reason that insulin alone did not change the level of megalin expression in $AT_{1A}R$-OK cells, we investigated the relationship between the glucose concentration in the culture media and the effect of insulin on the cells. Under the culture condition with a lower glucose concentration (7.5 mM), the baseline level of megalin protein expression in the cells was found to be low but significantly increased by the addition of 100 nM insulin (supplemental Fig. 3). These results suggest that the baseline megalin expression in the cells might have been at its maximal level under the original culture condition with the glucose concentration at 17.5 mM. The effect of insulin, provided that it

was to up-regulate megalin expression in the cells, could not have surpassed what was already abundantly expressed to its maximal amount, whereas such an effect was clearly observed when magalin expression had been suppressed by other factors such as Ang II.

Insulin is likely to compete with the Ang II-mediated ERK signaling pathway that suppresses megalin expression. It is also possible that insulin may compete with other undetermined signaling pathway(s) that may be involved in Ang II-mediated suppression of megalin expression in the cells.

The IRS/PI3K system, with which Ang II competes, acts to maintain megalin expression

To further elucidate the role of insulin-mediated signaling pathways in stimulating or maintaining megalin expression, we analyzed the function of the IRS/PI3K system, a major component of the insulin signaling pathway. As shown in Fig. 5, A and B, treatment of $AT_{1A}R$-OK cells with the PI3K inhibitors LY294002 and wortmannin suppressed megalin expression after 24 h of incubation. Furthermore, these inhibitors were found to suppress megalin mRNA expression at the 8-h incubation time point (Fig. 5, C and D), and these effects were competed by insulin. We also found that the treatment of $AT_{1A}R$-OK cells with Ang II suppressed insulin-mediated IRS/PI3K activation (Fig. 6).

These results indicate that the steady-state level of the IRS/PI3K activation in the cells plays a role in maintaining megalin gene expression under the culture condition. However, when the
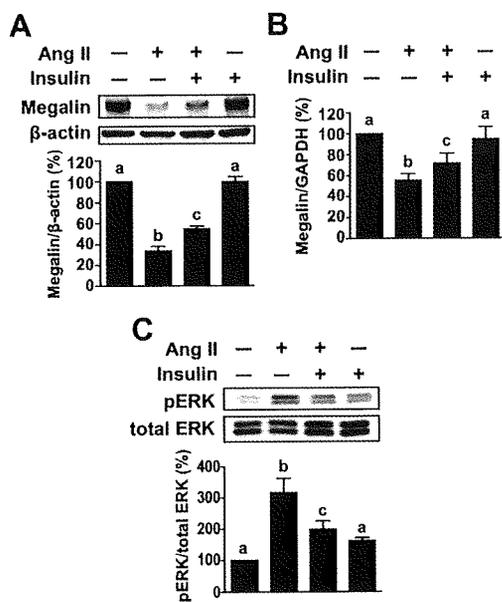

FIG. 4. Insulin competes with the Ang II-$AT_{1A}R$-ERK signaling pathway involved in suppression of megalin expression. $AT_{1A}R$-OK cells serum starved for 24 h were treated with 100 nM Ang II and/or 100 nM insulin. Insulin significantly reduced the inhibitory effect of Ang II on megalin protein expression at the 24-h incubation time point (A, n = 6) and on mRNA levels at the 3-h incubation time point (B, n = 8). Treatment of $AT_{1A}R$-OK cells with insulin (100 nM) did not change megalin protein or mRNA expression level at these time points. C, $AT_{1A}R$-OK cells serum starved for 24 h were treated with 100 nM Ang II and/or 100 nM insulin for 30 min. Insulin was also found to compete with Ang II-mediated ERK activation in the cells (n = 5). Values are expressed as mean ± SD. The differences between values associated with *different letters* are all statistically significant (*P* < 0.05).
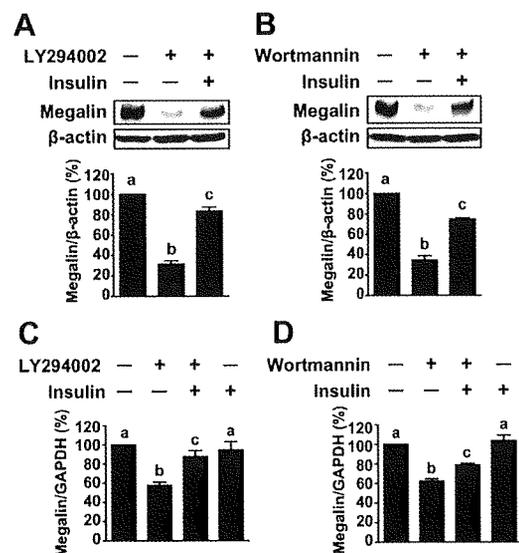
FIG. 5. Insulin reverses PI3K inhibitor-induced suppression of megalin expression in $AT_{1A}R$-OK cells. Suppression of megalin protein expression in 24-h serum-starved $AT_{1A}R$-OK cells was observed when the cells were treated for 24 h with 10 μM LY294002 (A, n = 3) and 100 nM wortmannin (B, n = 3), as shown by immunoblotting. The effects were significantly, but not completely, reversed by the treatment of the cells with insulin (100 nM) starting 30 min before the treatment with LY294002 or wortmannin. Suppression of megalin mRNA expression observed when the cells were treated for 8 h with 10 μM LY294002 (C, n = 4) and 10 μM wortmannin (D, n = 4) was significantly, but not completely, reversed by similarly preincubating the cells with insulin (100 nM), as shown by real-time RT-PCR. Values are expressed as mean ± SD. The differences between values associated with *different letters* are all statistically significant (*P* < 0.05).
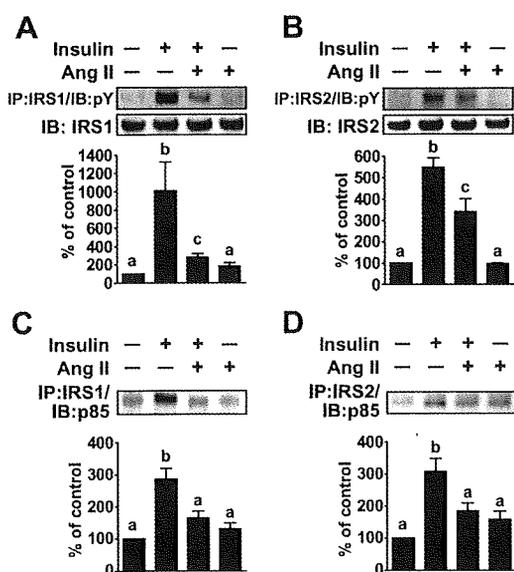
FIG. 6. Ang II suppresses insulin-mediated IRS/PI3K activation in $AT_{1A}R$-OK cells. $AT_{1A}R$-OK cells serum starved for 24 h were treated with either Ang II (100 nM) for 10 min, insulin (100 nM) for 7 min, or Ang II for 3 min followed by 7 min insulin treatment. Cell lysates were immunoblotted (IB) with either an anti-IRS (A) or IRS2 (B) antibody, or with an anti-pY antibody (A and B) after immunoprecipitation (IP) with either anti-IRS1 (A) or IRS2 (B) antibody. A, n = 5; B, n = 3. Similarly, the lysates were immunoblotted with an antibody against the p85 subunit of PI3K (p85) after IP with either anti-IRS1 (C) or IRS2 (D) antibody. C, n = 3; D, n = 5. Values are expressed as mean ± SD. The differences between values associated with different letters are all statistically significant (P < 0.05).

IRS/PI3K signaling is affected to suppress megalin expression, insulin competes with the effects and reverses suppressed megalin expression. Also, Ang II and insulin competitively regulate the levels of the IRS/PI3K activation, and the counterbalanced levels of the activated IRS/PI3K pathway are likely to regulate megalin expression.

The possible nature of the cross talk between Ang II- and insulin-mediated signaling pathways in the regulation of megalin expression in $AT_{1A}R$-OK cells is outlined in Fig. 7.

Discussion

We have found that Ang II suppressed mRNA and protein expression of megalin in an OK cell line that is deficient in endogenous Ang II receptors but stably expresses rat $AT_{1A}R$. This action of Ang II, which was completely suppressed by an AT_1R blocker, is mediated partly by ERK activation. Inhibitors of the JNK-, p38-, PKC- and JAK-STAT-mediated signaling pathways did not alter the ability of Ang II to lead decrease in megalin expression, suggesting that other undetermined Ang II-mediated signaling pathway(s) may be involved in suppressing megalin expression.

Insulin inhibited the $AT_{1A}R$-ERK-mediated suppression of megalin expression. As reported in previous studies using cultured PTCs (31, 32), we also found that insulin alone tended to stimulate ERK activation in $AT_{1A}R$-OK cells. However, the effect was not statistically significant, and insulin, adversely, suppressed Ang II-mediated ERK activation. This finding in our
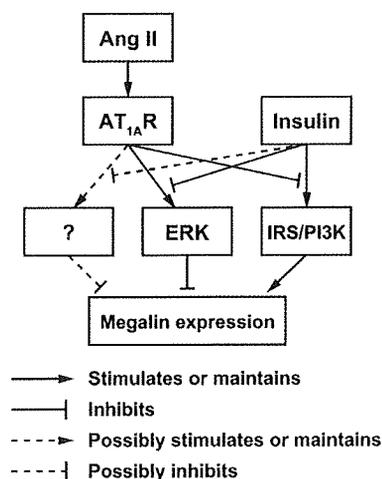

FIG. 7. Proposed scheme to illustrate the cross talk between Ang II-$AT_{1A}R$- and insulin-mediated signaling pathways in regulation of megalin expression. The Ang II-$AT_{1A}R$-ERK-mediated signaling pathway suppresses megalin expression in $AT_{1A}R$-OK cells, and insulin competes with this pathway. Other unknown Ang II-$AT_{1A}R$-mediated signaling pathway(s) may be present to suppress megalin expression, which may be also competed by insulin. Conversely, Ang II-$AT_{1A}R$-mediaed signaling competes with the insulin-mediated IRS/PI3K signaling pathway that acts to stimulate or maintain megalin expression.

study could be suggesting that insulin might intercept signaling events rather in the upstream part of Ang II-mediated ERK pathways in the cells, which may act more dominantly than insulin's own ERK activation signaling in $AT_{1A}R$-OK cells. A similar finding for such competitive cross talk between Ang II- and insulin-mediated ERK pathways in murine PTCs has been also reported, in which insulin-mediated ERK activation is suppressed by Ang II (32).

Treatment of the cells with insulin alone did not change the level of megalin expression under the original culture condition, but it significantly increased megalin expression at a lower glucose concentration. Also, PI3K inhibitors, which interfere with the IRS/PI3K system and work as antagonists of insulin-mediated signaling pathways, suppressed megalin expression, and the effects were reversed by insulin. These results indicate that insulin-IRS/PI3K signaling pathway has a stimulatory effect on megalin expression. Furthermore, Ang II was found to inhibit insulin-mediated IRS/PI3K signaling.

Collectively, cross talk occurs between the $AT_{1A}R$-ERK and insulin-IRS/PI3K signaling pathways to competitively regulate megalin expression in $AT_{1A}R$-OK cells. It is also possible that $AT_{1A}R$-mediated unknown signaling pathway(s) may be involved in suppressing megalin expression and may be interacted with insulin-mediated signaling pathways (Fig. 7). This study is the first demonstration of Ang II- and insulin-mediated signaling cross talk in kidney cells, which is involved in regulating an important membrane receptor in PTCs.

Impairment of PTC functions is evident even at the early stages of diabetes and often predisposes to apparent glomerular damage (33). Decreased reabsorption of glomerular-filtered proteins by PTCs was found in diabetic patients at the microalbuminuric stage (3). In diabetes, Ang II appears to be a crucial factor in phenotypic changes of PTCs (34), and the intrarenal renin-angiotensin system (RAS) is activated, whereas the circulating

RAS tends to stay normal or suppressed (35). PTCs play a central role in regulating the intrarenal RAS because these cells express all of the components required to synthesize Ang II, including renin, angiotensinogen, and angiotensin-converting enzyme (36). Our finding regarding the role of the Ang II-AT$_1$R-ERK system in the down-regulation of megalin expression makes a significant contribution to further understanding of the pathological actions of Ang II on PTCs and mechanisms of postglomerular albuminuria in diabetes.

Albuminuria is also a frequent symptom of patients with insulin resistance associated with metabolic syndrome (11). It has been suggested that in such patients, insulin-mediated signaling pathways are inhibited at the level of the IRS/PI3K system (37). Activation of RAS is likely to be involved in the process (14, 38), and Ang II thus may act to inhibit the insulin signaling to reduce megalin expression in PTCs of patients with metabolic syndrome. In models of insulin resistance, such as obese Zucker rats and those on a high-fat diet for several weeks, renal expression of the insulin receptor subunits was found to be down-regulated compared with respective controls (39). This also suggests that insulin signaling is altered in the kidney during insulin resistance, and this may also relate to the pathogenesis of impaired endocytic functions of PTCs and the mechanisms of postglomerular albuminuria. The manner of Ang II- and insulin-mediated signaling cross talk in the kidney remains to be further elucidated, such as how and when RAS activation and insulin resistance occur and interrelate in PTCs in the course of metabolic syndrome or type 2 diabetes.

Megalin has been also found to mediate lysosomal biogenesis in PTCs (40). Therefore, the deficiency in megalin function that is often found in diabetes may be involved in the hypertrophic changes of PTCs, which predispose to subsequent deterioration of kidney function (41). Also, megalin is known as an endocytic receptor for vitamin D-binding protein (17), and its impaired function is likely to be associated with vitamin D deficiency, especially in patients with diabetes (42). Therefore, treatment of diabetic patients with AT$_1$R blockers may be useful for maintaining various megalin actions, in addition to the reduction of albuminuria. The clinical efficacy of pioglitazone, an insulin-sensitizing peroxisome proliferator-activated receptor-γ agonist, in decreasing urinary excretion of albumin and liver-type fatty acid binding protein (43) (endocytic ligands of megalin) in type 2 diabetic patients (44) may also suggest that it could improve megalin function.

In conclusion, we have identified competitive cross talk between AT$_{1A}$R- and insulin-mediated signaling pathways in regulating megalin expression in cultured PTCs. The data obtained in this study may facilitate development of novel strategies for preventing the progression of proteinuria/albuminuria. Analysis of the megalin gene promoter and enhancer would be required to clarify the mechanisms of regulation of megalin gene expression.

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ARTICLE

Long-term effect of modification of dietary protein intake on the progression of diabetic nephropathy: a randomised controlled trial

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Abstract

Aims/hypothesis There is currently insufficient evidence to recommend a low-protein diet for type 2 diabetic patients with diabetic nephropathy. We assessed whether a low-protein diet could prevent the progression of diabetic nephropathy.

Methods This was a multi-site parallel randomised controlled trial for prevention of diabetic nephropathy progression among 112 Japanese type 2 diabetic patients with overt nephropathy. It was conducted in Japan from 1 December 1997 to 30 April 2006. The participants were randomly assigned using a central computer-generated schedule to either low-protein diet (0.8 g $kg^{-1}day^{-1}$) and normal-protein diet (1.2 g $kg^{-1}day^{-1}$), and were followed for 5 years. The participants and investigators were not blinded to the assignment. The primary outcomes were the annual change in estimated GFR and creatinine clearance, the incidence of doubling of serum creatinine and the time to doubling of baseline serum creatinine.

Other members of the Low-Protein Diet Study Group are listed in Electronic supplementary material

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