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Inhibition of mTOR signaling with rapamycin attenuates renal hypertrophy in the early diabetic mice

Masayoshi Sakaguchi [a], Motohide Isono [a], Keiji Isshiki [a], Toshiro Sugimoto [a,*], Daisuke Koya [b], Atsunori Kashiwagi [a]

[a] *Department of Medicine, Shiga University of Medical Science, Otsu, Shiga, Japan*
[b] *Division of Endocrinology and Metabolism, Kanazawa Medical University, Ishikawa 920-0293, Japan*

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Abstract

Early diabetic nephropathy is characterized by renal hypertrophy that is mainly due to proximal tubular hypertrophy. Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase, and its signaling has been reported to regulate protein synthesis and cellular growth, specifically, hypertrophy. Therefore, we examined the effect of mTOR signaling on diabetic renal hypertrophy by using the specific inhibitor for mTOR, rapamycin. Ten days after streptozotocin-induced diabetes, mice showed kidney hypertrophy with increases in the phosphorylation of p70S6kinase and the expression of cyclin kinase inhibitors, $p21^{Cip1}$ and $p27^{Kip1}$, in the kidneys. The intraperitoneal injection of rapamycin (2 mg/kg/day) markedly attenuated the enhanced phosphorylation of p70S6kinase, the increment of cyclin-dependent kinase inhibitors, and renal enlargement without any changes of clinical parameters, including blood glucose, blood pressure, and food intake. Overexpression of a constitutive active form of p70S6kinase resulted in increased cell size of cultured mouse proximal tubule cells; thus, activation of p70S6kinase causes hypertrophy of proximal tubular cells. Our findings suggest that activation of mTOR signaling causes renal hypertrophy at the early stage of diabetes.

Keywords: Diabetic nephropathy; Renal hypertrophy; Mammalian target of rapamycin; Rapamycin

Renal enlargement occurs in the early phases of human and experimental diabetes and, accompanied by glomerular hyperfiltration, contributes to the later development of overt diabetic kidney disease [1–5]. Renal enlargement arises very shortly after induction of hyperglycemia, and an increment of protein content and protein/DNA ratio has been shown in the early diabetic kidney, indicating that the renal enlargement is mainly due to renal cellular hypertrophy [6–8]. The precise biological mechanisms which regulate this process of renal hypertrophy, however, remain incompletely understood.

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that can directly control the functions of 70-kDa ribosomal protein S6 kinase (p70S6k) and eukaryotic initiation factor 4E-binding protein-1 (4EBP-1), which are important regulators of ribosome protein synthesis, ribosome biogenesis, and mRNA translation initiation [9]. Therefore, the activation of the mTOR signaling pathway is thought to lead to an increase in the cellular capacity for protein synthesis and cellular hypertrophy [10]. Rapamycin, an immunosuppressive macrolide, forms a complex with FK506-binding protein with a molecular weight of 12 kDa (FKBP12). This complex binds to mTOR and prevents the activation of mTOR signaling pathway [11,12]. Recently, rapamycin has been reported to attenuate pressure-overload-induced cardiac hypertrophy [13] and compensatory renal hypertrophy after unilateral nephrectomy [14], indicating that the mTOR signaling pathway might play an important role in organ hypertrophy under pathological conditions. However, there is no report as to

* Corresponding author. Fax: +81 77 543 3858.
E-mail address: toshiro@belle.shiga-med.ac.jp (T. Sugimoto).

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whether metabolic derangement in insulin-deficient streptozotocin (STZ)-induced diabetes may also cause renal hypertrophy through activation of the mTOR signaling pathway.

Thus, we examined whether the mTOR signaling pathway is activated in the kidney in mice with STZ-induced diabetes and whether its inhibitor, rapamycin, can reverse the renal hypertrophy in diabetes.

Methods

Experimental protocol. Male 8-week-old C57BL/6 mice (CLEA, Osaka, Japan) were made diabetic by intraperitoneal injection of STZ (150 mg/kg of body weight) (Sigma, St. Louis, MO) in 0.05 M citrate buffer (pH 4.5) for 2 days. Mice receiving an injection of citrate buffer were used as controls. Hyperglycemia was determined 2 days after injection, and mice with blood glucose levels >300 mg/dl were considered to have diabetes. Mice were divided into four groups: control mice, control mice treated with rapamycin, diabetic mice, and diabetic mice treated with rapamycin. Rapamycin (2 mg/kg/day) (LC Laboratories, Woburn, MA) or vehicle was administered intraperitoneally daily to control or diabetic mice [13]. Seven days after the induction of diabetes, individual mice were placed in metabolic cages for 24-h urine collection and measurement of food intake. Starting at 10 days after the induction of diabetes, body weight, blood glucose levels, hematocrit percentage, and systolic blood pressure were monitored. The blood pressure of conscious mice at a steady state was measured with a programmable tail-cuff sphygmomanometer (BP98-A; Softron, Tokyo, Japan). The blood was collected from the caudal vein of mice into heparin-coated microcapillary tubes. Hematocrit percentage was assessed using a hematocrit reader (Terumo, Tokyo, Japan). After mice were euthanized by cervical dislocation, the kidneys were decapsulated promptly. The heart and kidney weights were measured, and the kidneys were immediately frozen in liquid nitrogen. The Research Center for Animal Life Science of Shiga University of Medical Science approved all experiments.

Protein extraction and immunoblot analysis for mice kidneys. The kidneys were homogenized in 0.5 ml of ice-cold buffer (50 mM Tris–HCl, pH 7.6, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 1 mM EDTA, 150 mM NaCl, and 0.25% sodium deoxycholate), and Complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) with 1% Nonidet P-40. After sonication at 4 °C for 10 s, the homogenates were centrifuged at 15,000*g* for 10 min. Protein concentrations of the supernatants were measured with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). The kidney protein extracts (40 mg) were prepared in Laemmli sample buffer containing mercaptoethanol, boiled for 5 min, subjected to 12% or 15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred to a polyvinylidene difluoride (PVDF) filter (Immobilon; Millipore, Bedford, MA). The filters were blocked with Tris-buffered saline (10 mmol/L Tris–HCl, [pH 7.6], 150 mM NaCl)–0.1% Tween 20 (TBS-T) containing 5% non-fat milk at room temperature for 1 h. The filters were incubated with antibodies against phospho-p70S6k (Thr389, Thr421/Ser424), phospho-Akt (Ser473), and Akt (Cell Signaling Technology, Beverly, MA) at 1:1000 dilution in TBS-T with 5% bovine serum albumin, p70S6k at 1:500 dilution in TBS-T with 5% milk, $p21^{Cip1}$ and $p27^{Kip1}$ (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution in TBS-T with 5% milk, or β-actin (Sigma) at 1:5000 dilution in TBS-T with 5% milk. After multiple washes in TBS-T, the filters were incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG secondary antibody (phospho-p70S6k, p70S6k, phospho-Akt, Akt, and $p27^{Kip1}$) and anti-mouse IgG secondary antibody ($p21^{Cip1}$) for 1 h at 1:1000 dilution in TBS-T with 5% milk. After washing the filters several times with TBS-T, the immunoreactive proteins were detected using an enhanced chemiluminescence (ECL) system (Perkin-Elmer, Boston, MA). The density of the corresponding bands was measured quantitatively using Scion Image software (http://www.scioncorp.com/) and corrected by reference to the value for β-actin.

Cell culture, transfection, immunoblot analysis, and cell size analysis. Murine proximal tubular cells (mProx) (derivative, patent WO9927363, Japan, US, European Union), kindly provided by CMIC Co. Ltd., were cultured as previously described [15]. The cells on a 6-well plate were transfected with HA-tagged cDNAs expressing constitutive active [p70S6k CA, pRK7-HA-p70S6K1-active form (T389E, S411D, S418D, T421E, and S424D)], dominant negative p70S6k, [p70S6k KD, pRK7-HA-p70S6K1-KD (K100R)] (kindly provided by J. Blenis, Harvard Medical School, Boston, MA) [16] or empty vector (pcDNA 3.1) using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cells were harvested for immunoblot analysis and cell size analysis after 48-h transfection.

For immunoblot analysis, the cells were lysed with Laemmli sample buffer, sonicated for 10 s, and boiled at 95 °C for 5 min. After centrifugation at 15,000 rpm for 10 min, the supernatants were electrophoresed on 12% SDS–PAGE gel and transferred onto a PVDF filter. The filters were incubated with the indicated antibody (HA-tag, phospho-ribosomal S6 (Ser 235/236) and ribosomal S6 (Cell signaling technology, Beverly, MA) at 1:1000 dilution) and the immunoreactive proteins were detected as described above. To determine cell size and DNA content, FACS analysis (FACScan, Becton Dickinson, Mountain View, CA) was performed. The cells were washed once with phosphate-buffered saline (PBS) and incubated at 37 °C for 2 min in 1 ml of trypsin-EDTA, gently pipetted off the plate with PBS/10% FBS, transferred to 1.5-ml Eppendorf tubes, centrifuged for 3 min at 3000 rpm, and washed once with PBS/1% FBS, and the final cell pellets were resuspended with 0.2 ml PBS/1% FBS. Resuspended cells were fixed on ice by adding 0.6 ml of cold 100% ethanol (75% final). The fixed cells were centrifuged at 3000 rpm for 3 min, washed once with PBS/1% FBS, and incubated at 37 °C for 20 min in 0.5 ml PBS/1% FBS containing 0.1% Triton X-100 and 250 μg/ml RNase A. The cells were stained by propidium iodide (0.5 μg/ml) for FACS analysis to determine the size of cells in the G1-phase populations [17–19]. FACS analysis was used to obtain forward-angle light scatter (FSC) histograms (voltage: E-1; amplification gain: 4.00; threshold: 50 channel units based on FSC). FSC, which is proportional to relative cell size, for 10,000 cells in the G1 phase per sample was analyzed by using the CellQuest Pro software (Becton Dickinson Immunocytometry Systems, San Jose, CA) and gating on physical parameters to exclude cell debris. FSC measures cell diameter, and this measure was used to calculate cell volume for cells in the G1 phase of the cell cycle [20].

Statistical analysis. Results are expressed as means ± standard deviation (SD). Comparisons between two groups were analyzed by Student's unpaired *t* test. Comparisons among three or more groups were analyzed by one-way analysis of variance (ANOVA) followed by Scheffe's test to evaluate statistical in multiple comparisons. p values of <0.05 were defined as statistically significant.

Results

Effects of rapamycin on kidney weight in early diabetic mice

The characteristics of the four groups of mice at the end of the experimental period are presented in Table 1. The levels of blood glucose were significantly higher in diabetic mice than in control mice. The body weight of diabetic mice was significantly smaller than that of age-matched control mice, although the oral food intake was significantly increased in diabetic mice. On the other hand, the kidney-to-body weight ratio of diabetic mice was significantly larger than that of the control mice. The increment in the kidney-to-body weight ratio in the rapamycin-treated mice was less than one-half of that for the vehicle-treated diabetic mice. However, the rapamycin treatment did not have any effect on body weight,

Table 1
Characteristics of experimental mice

	Control	Control + rapamycin	Diabetes	Diabetes + rapamycin
n:	6	5	8	11
BW (g)	24.82 ± 0.82	23.99 ± 1.89	22.27 ± 1.91*	20.28 ± 1.40*
BS (mg/dl)	137 ± 26	144 ± 10	521 ± 61*	502 ± 86*
KW (g)	0.138 ± 0.010	0.131 ± 0.017	0.165 ± 0.009*	0.130 ± 0.011†
KW/BW (mg/g)	5.55 ± 0.51	5.45 ± 0.50	7.43 ± 0.51*	6.41 ± 0.60*,†
HW/BW (mg/g)	3.98 ± 0.22	3.80 ± 0.02	3.91 ± 0.39	3.94 ± 0.39
Ht (%)	45.3 ± 0.6	44.5 ± 0.7	43.5 ± 2.1	42.2 ± 0.8
Urine vol (ml)	0.96 ± 0.13	1.22 ± 0.1	11.82 ± 6.27*	10.04 ± 3.02*
Oral intake (g/day)	4.19 ± 0.54	4.23 ± 0.3	6.29 ± 0.99*	6.1 ± 1.07*
SBP (mmHg)	96.7 ± 3.5	97.0 ± 4.2	93.8 ± 6.0	95.5 ± 2.3

BW, body weight; BS, blood glucose level; KW, kidney weight; HW, heart weight; Ht, hematocrit; SBP, systolic blood pressure. Data are means ± SD.
* Significantly different vs. control ($p < 0.05$).
† Significantly different vs. diabetes ($p < 0.05$).

heart-to-body weight ratio, systolic blood pressure, blood glucose level or oral intake in either control or diabetic mice.

Effects of rapamycin on mTOR signaling pathway and expression of cyclin-dependent kinase inhibitors in early diabetic kidney

The phosphorylation of Akt, one of the upstream regulators of the mTOR signaling pathway, was not enhanced in diabetic mice. However, the phosphorylation of 70-kDa ribosomal protein S6 kinase (threonine 389 and threonine 421/serine 424), one of the substrates of mTOR, was enhanced in the diabetic kidneys (Fig. 1). The treatment with rapamycin attenuated this enhancement of p70S6k phosphorylation.

Furthermore, we also measured the expression of cyclin-dependent kinase inhibitors (CKI), $p21^{Cip1}$ and $p27^{Kip1}$, markers of G1-phase cell cycle arrest and cellular hypertrophy in the kidney [21]. The protein expression of both $p21^{Cip1}$ and $p27^{Kip1}$ of diabetic mice was significantly increased compared with the control, consistent with renal hypertrophy [22,23]. The treatment with rapamycin also significantly inhibited the expression of CKI in the diabetic kidneys (Fig. 2).

Effects of mTOR-p70S6k signaling on hypertrophy of the cultured renal proximal tubular cells

To evaluate the effects of mTOR-p70S6k signaling on renal hypertrophy, a constitutive active form, p70S6k CA, or a dominant negative form of p70S6k KD was overexpressed in the cultured mouse proximal tubular cells. The phosphorylation of ribosomal S6 protein, a substrate of p70S6k, was increased in the cells transfected with p70S6k CA but decreased in the cells transfected with p70S6k KD (Fig. 3A). To measure cell size, we performed the FACS analysis. FSC histogram of the cells transfected with p70S6k CA was shifted to the right. However, the histogram of cells transfected with p70S6k KD was shifted to the left (Fig. 3B). Calculated cell volume was significantly increased in the cells transfected with p70S6k CA, but was decreased in the cells transfected with p70S6k KD (Fig. 3C), indicating that the activation of p70S6k can induce cell hypertrophy in cultured renal proximal tubular cells.

Discussion

In the present study, we have demonstrated that the mTOR signaling pathway regulates renal hypertrophy in early diabetic mice. Ten days after induction of diabetes, the phosphorylation of threonine 389 in p70S6k, the specific phosphorylation site with mTOR [24,25], and the phosphorylation of ribosomal S6 protein were enhanced in the kidney, indicating that the mTOR signaling was activated and protein synthesis increased in the early diabetic kidney. The renal expression of the CKIs, $p21^{Cip1}$ and $p27^{Kip1}$, was increased in diabetic mice, indicating that G1/S arrest had occurred [22,26]. Therefore, the diabetic conditions induced an increase in protein synthesis without an increase in DNA synthesis in the kidney, suggesting that cell hypertrophy was the main cause of renal enlargement under our experiment conditions. The treatment with rapamycin, a specific inhibitor of mTOR, markedly attenuated the enhanced phosphorylation of p70S6k and ribosomal S6 protein, the increment of CKI expression, and renal enlargement without any changes of clinical parameters, including blood glucose, blood pressure, or food intake. These data suggest that mTOR is activated in the early diabetic kidney and that mTOR signaling causes renal hypertrophy through the regulation of phosphorylation of ribosomal S6 protein and the expression of cyclin-dependent kinase inhibitors.

As the overexpression of constitutive active form of p70S6k increased the cell size of the cultured mouse proximal tubular cells, the activation of p70S6k is sufficient to cause hypertrophy of proximal tubular cells. The renal enlargement in early diabetes has been reported to be mainly due to hypertrophy of proximal tubules [7]. This finding thus also suggests that the mTOR-p70S6k pathway might play an important role in the renal proximal hypertrophy in early diabetic mice.

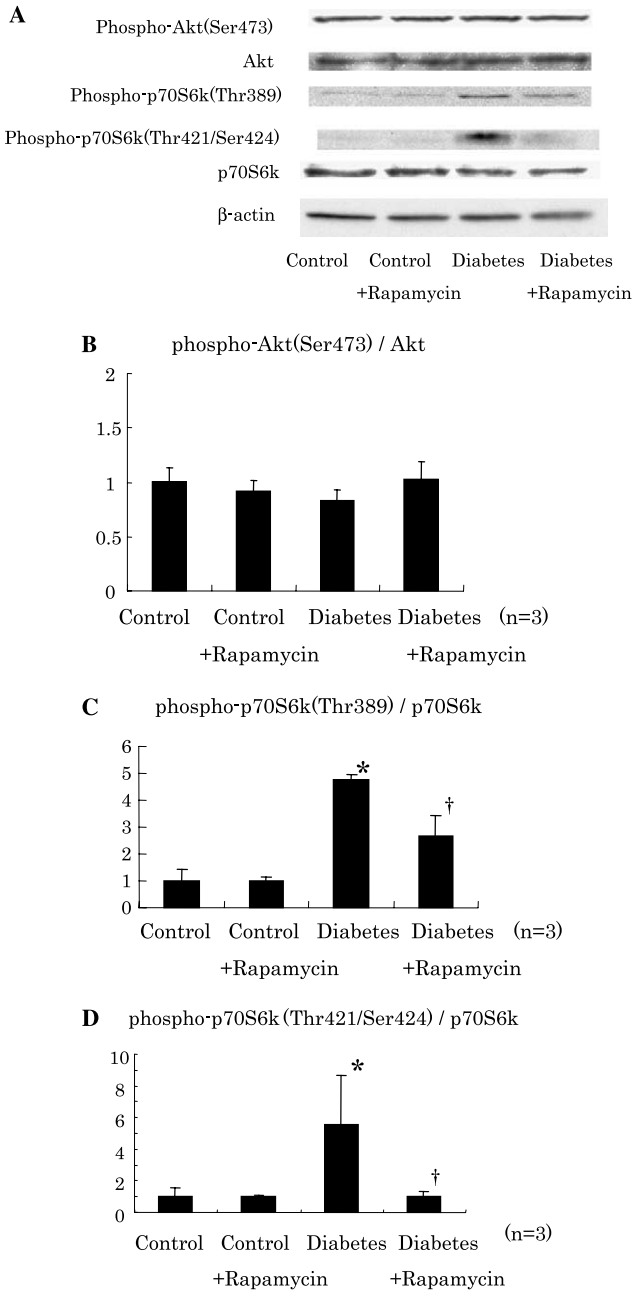

Fig. 1. The response of mTOR signaling pathway in early diabetic kidney. The immunoblot analyses were performed with the indicated antibodies, Akt, phospho-Akt, p70S6k or phospho-p70S6k using the diabetic mouse kidneys. (A) A representative set of results from among three sets of experiments is shown. The densitometric analyses for phospho-Akt (Ser473) (B), phospho-p70S6k (Thr389) (C), and phospho-p70S6k (Thr421/Ser424) (D). Data are means ± SD. *Significantly different vs. control ($p < 0.05$); †significantly different vs. diabetes ($p < 0.05$).

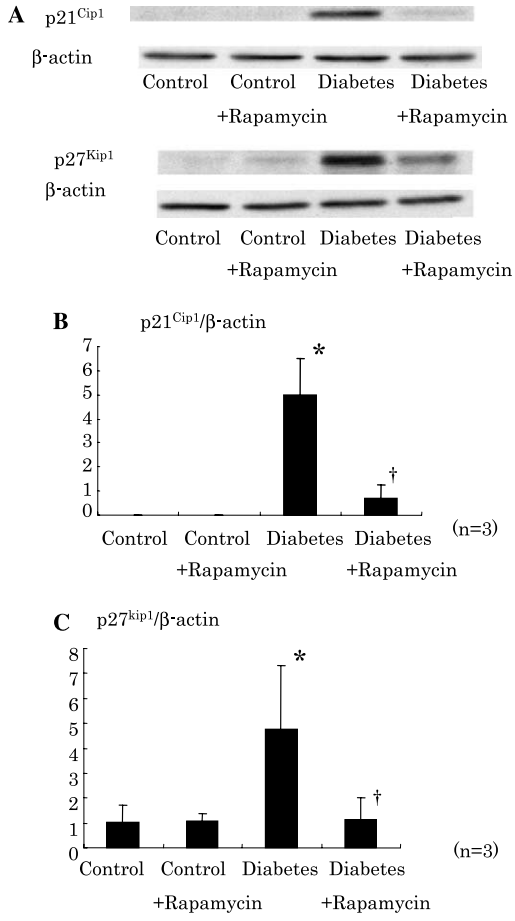

Fig. 2. The response of cyclin-dependent kinase inhibitors in early diabetic kidney. The immunoblot analyses were performed with the indicated antibodies using diabetic mouse kidneys. (A) A representative set of results from among three sets of experiments is shown. The densitometric analyses for $p21^{Cip1}$ (B) and $p27^{Kip1}$ (C) are presented. Data are means ± SD. *Significantly different vs. control ($p < 0.05$); †significantly different vs. diabetes ($p < 0.05$).

Several reports have described the role of mTOR signaling in the diabetic kidneys. One group reported that the phosphorylation of p70S6k was enhanced in rat glomeruli after 12 weeks of STZ-induced diabetes [27]. They also showed that PI3kinase/Akt was activated in the diabetic glomeruli. Thus, they concluded that a growth factor, growth arrest-specific gene 6, activated mTOR signaling and induced glomerular hypertrophy. Another group demonstrated that activation of the Akt/mTOR pathway in the renal cortex in diabetic db/db mice, a model of type II diabetes [28]. These reports suggest that mTOR signaling is enhanced by the effects of growth factors in the diabetic kidney. In the present study, however, the phosphorylation of Akt was not significantly increased in the mouse kidneys after 10 days of STZ injection, indicating that the mechanism other than growth factors might activate mTOR signaling in very early stage of diabetic kidneys, although renal hypertrophy was induced.

Recently, mTOR signaling was reported to be activated by nutrients, such as glucose or amino acids, independently of the PI3K/Akt signaling pathway; thus, mTOR is thought to integrate energy and amino acid-sensing pathways [19,29]. In early diabetes, an increase in proximal tubular reabsorption has been reported to accompany kidney hypertrophy [30,31]. In our diabetic mice, overload of nutrients including glucose and amino acids occurs in the renal proximal cells because of the increased food intake and blood glucose levels. Therefore, this metabolic

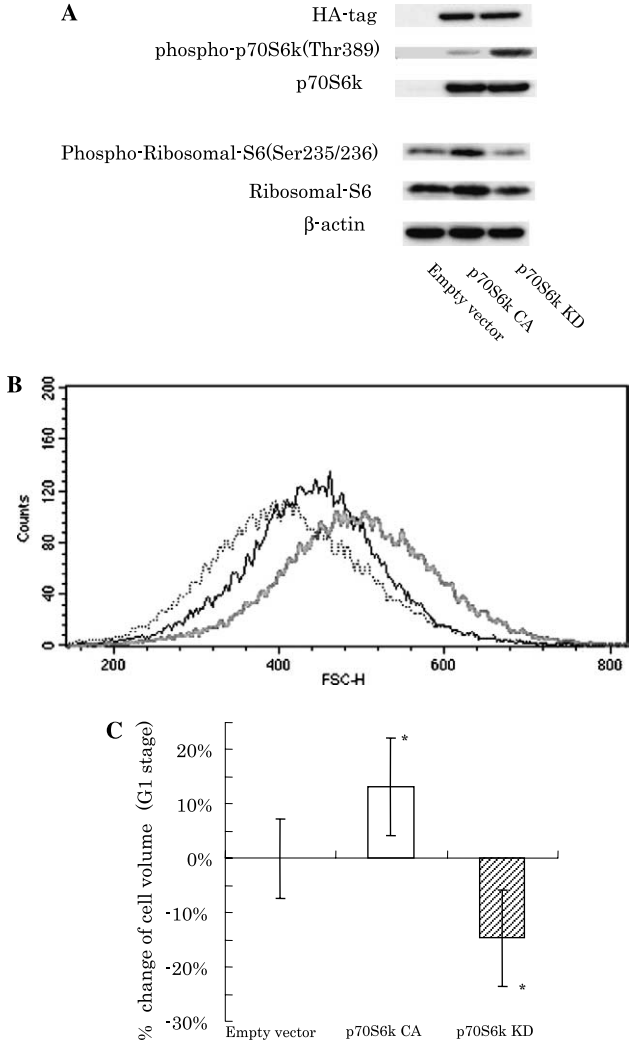

Fig. 3. Flow cytometric analysis of proximal tubular cell size. The mouse proximal tubular cells were transfected with p70S6k CA, p70S6k KD, and p70S6k, or the empty vector. (A) The immunoblot analyses were performed with the indicated antibodies using the transfected cells. (B) FSC histogram for the cultured mouse proximal tubular cells. The solid line is the FSC histogram of the cells transfected with empty vector, gray line that of the cells transfected with p70S6k CA, and the dotted line that of the cells transfected with p70S6k KD. (C) The data are expressed as percent change of cell volume compared with those of control (empty vector). Data are means ± SD. *Significantly different vs. cells transfected with empty vector ($p < 0.05$).

overload might cause the activation of mTOR signaling without the activation of PI3K/Akt pathway in the early diabetic kidneys. Indeed, the epithelial cell growth is seen in nephron segments downstream to site of diuretic action resulting from the extra reabsorptive burden [32], suggesting that increased loads for the renal tubule in early diabetes can cause renal cellular growth [31]. We have not, however, studied the precise temporal sequence connecting increased reabsorptive work to tubular growth in this study. Further studies are required to confirm this idea.

Diabetic nephropathy is a leading cause of end-stage renal disease, which is treated with dialysis therapy in Western and Asian countries [33–35]. At present, the standard treatment for diabetic nephropathy is glycemic control and blood pressure control, but we have not obtained satisfactory results for diabetic nephropathy with this standard treatment. Here, we have shown that rapamycin, a specific inhibitor of mTOR signaling, can attenuate renal hypertrophy in early diabetic kidneys. Our findings evoke a novel therapeutic concept for diabetic nephropathy, implying that pharmacological blockade for diabetic renal hypertrophy may suppress the progression of diabetic nephropathy. Therefore, modulation of mTOR activity might represent a novel approach for diabetic nephropathy and be a pathophysiological probe for diabetic renal hypertrophy for development of new therapeutic drugs.

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