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

Michihiro Hosojima, Hiroyoshi Sato, Keiko Yamamoto, Ryohei Kaseda, Taeko Soma, Asako Kobayashi, Akiyo Suzuki, Hideyuki Kabasawa, Aya Takeyama, Kenji Ikuyama, Noriaki Iino, Akira Nishiyama, Thomas J. Thekkumkara, Tetsuro Takeda, Yoshiki Suzuki, Fumitake Gejyo, and Akihiko Saito

Division of Clinical Nephrology and Rheumatology (M.H., K.Y., R.K., A.Su., H.K., A.T., K.I., T.T., F.G.) and Department of Applied Molecular Medicine (H.S., T.S., A.K., N.I., A.Sa.), Niigata University Graduate School of Medical and Dental Sciences, Niigata 951-8510, Japan; Center for Transdisciplinary Research (H.S.) and Niigata University Health Administration Center (Y.S.), Niigata University, Niigata 950-2181, Japan; Department of Pharmacology (A.N.), Faculty of Medicine, Kagawa University, Kagawa 761-0793, Japan; and Department of Pharmaceutical Sciences (T.J.T.), Texas Tech University Health Sciences Center, Amarillo, Texas 79106

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

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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.

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 facio-oculo-acousticorenal 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′-gcagaatctggtccaaaacctgacac-3′; for the GAPDH amplicon: forward, 5′-gagatgctggagccgagta-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

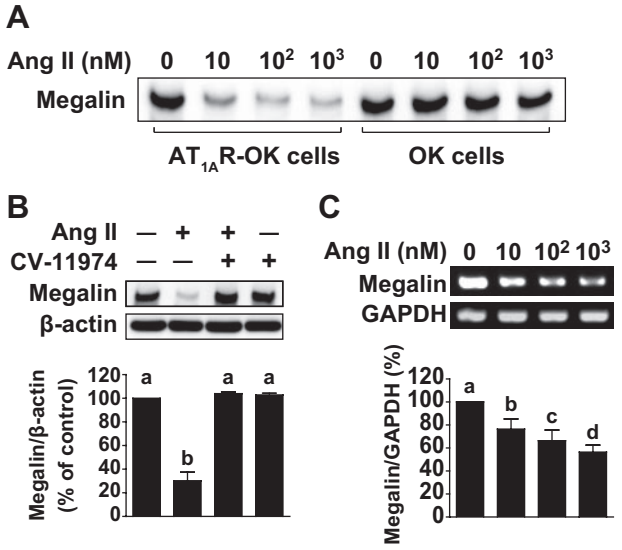

FIG. 1. Ang II suppresses protein and mRNA expression of megalin via $AT_{1A}R$ in $AT_{1A}R$-OK cells. A, Immunoblotting shows that protein expression of megalin was suppressed 24 h after treatment of 24-h serum-starved $AT_{1A}R$-OK cells with Ang II, whereas the expression level was unchanged in OK 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 phenomenon 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.

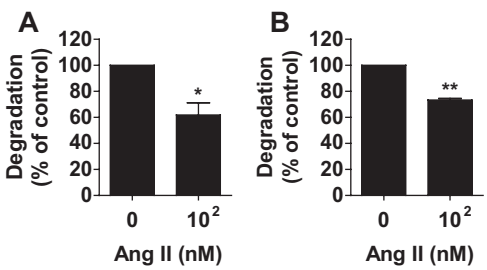

FIG. 2. Ang II suppresses the endocytic uptake and degradation of albumin and RAP in $AT_{1A}R$-OK cells. Treatment of 24-h serum-starved $AT_{1A}R$-OK cells with Ang II (100 nM) for 24 h decreased the degradation of endocytic megalin ligands, ^{125}I-labeled albumin (A), and RAP (B). Values are expressed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

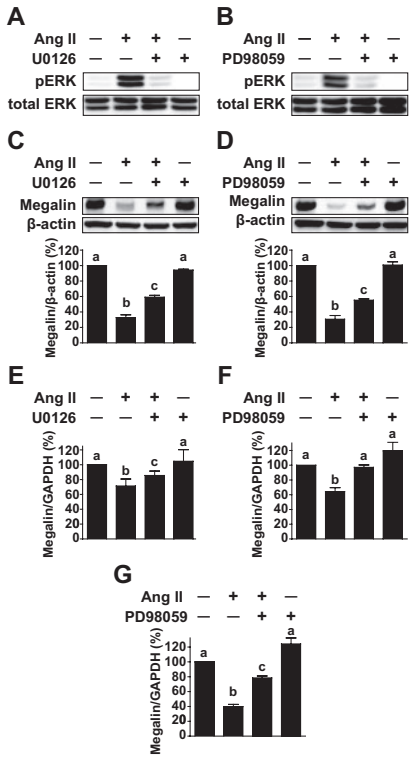

FIG. 3. Ang II-mediated ERK activation is involved in the signaling pathway that suppresses megalin expression in $AT_{1A}R$-OK cells. Treatment of 24-h serum-starved $AT_{1A}R$-OK cells with Ang II (100 nM) for 5 min was found to induce ERK activation, as shown by immunoblotting with anti-ERK1/2 (total 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 ($P < 0.05$).

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

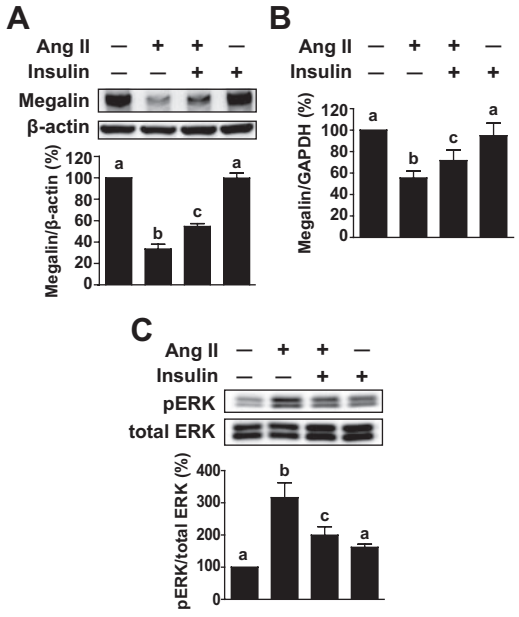

FIG. 4. Insulin competes with the Ang II-AT_{1A}R-ERK signaling pathway involved in suppression of megalin expression. AT_{1A}R-OK cells serum starved for 24 h were treated with 100 nM Ang II and/or 100 nM insulin. Insulin significantly reduced the inhibitory effect of Ang II on megalin protein expression 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 ($P < 0.05$).

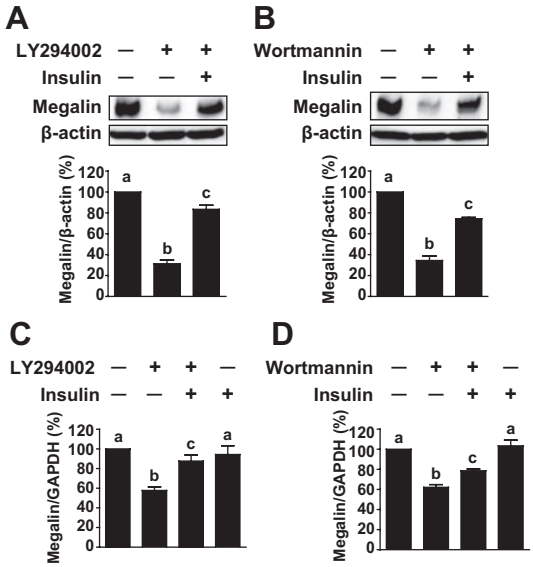

FIG. 5. Insulin reverses PI3K inhibitor-induced suppression of megalin expression in AT_{1A}R-OK cells. Suppression of megalin protein expression in 24-h serum-starved AT_{1A}R-OK cells was observed when the cells were treated for 24 h with 10 μM LY294002 (A, n = 3) and 100 nM wortmannin (B, n = 3), 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 ($P < 0.05$).

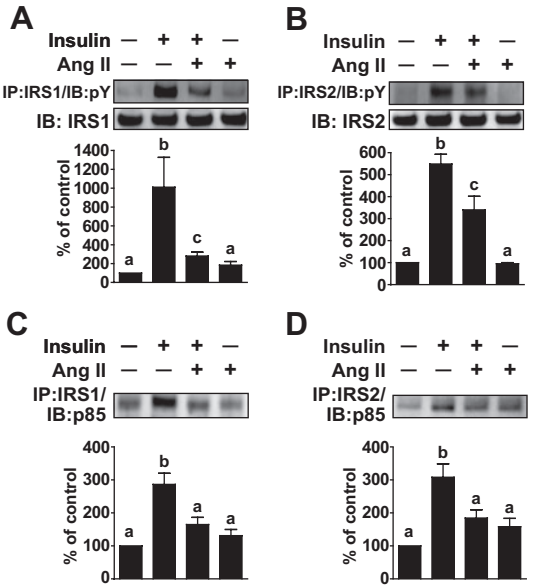

FIG. 6. Ang II suppresses insulin-mediated IRS/PI3K activation in $AT_{1A}R$-OK cells. $AT_{1A}R$-OK cells serum starved for 24 h were treated with either Ang II (100 nM) for 10 min, insulin (100 nM) for 7 min, or Ang II for 3 min followed by 7 min insulin treatment. Cell lysates were immunoblotted (IB) 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 ($P < 0.05$).

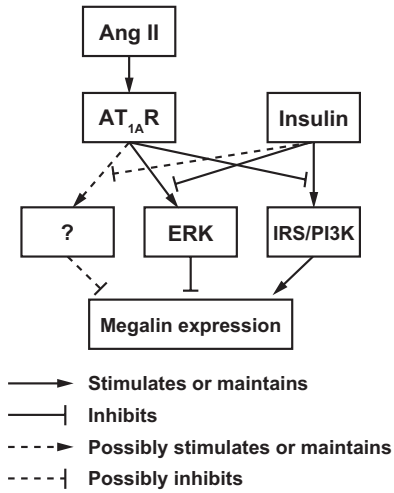

FIG. 7. Proposed scheme to illustrate the cross talk between Ang II-$AT_{1A}R$- and insulin-mediated signaling pathways in regulation of megalin expression. The Ang II-$AT_{1A}R$-ERK-mediated signaling pathway suppresses megalin expression in $AT_{1A}R$-OK cells, and insulin competes with this pathway. 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.

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 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_1R-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_1R 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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Address all correspondence and requests for reprints to: Akihiko Saito, M.D., Ph.D., Department of Applied Molecular Medicine, Niigata University Graduate School of Medical and Dental Sciences, 1-757, Asahimachi-dori, Chuo-ku, Niigata, 951-8510 Japan. E-mail: akisaito@med.niigata-u.ac.jp.

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