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Megalin is downregulated via LPS-TNF-α-ERK1/2 signaling pathway in proximal tubule cells

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ABSTRACT

Expression and function of megalin, an endocytic receptor in proximal tubule cells (PTCs), are reduced in diabetic nephropathy, involved in the development of proteinuria/albuminuria. Lipopolysaccharide (LPS) is chronically increased in diabetic sera, by the mechanism called metabolic endotoxemia. We investigated low-level LPS-mediated signaling that regulates megalin expression in immortalized rat PTCs (IRPTCs). Incubation of the cells with LPS (10 ng/ml) for 48 h suppressed megalin protein expression and its endocytic function. TNF-α mRNA expression was increased by LPS treatment, and knockdown of the mRNA with siRNA inhibited LPS-mediated downregulation of megalin mRNA expression at the 24-h time point. Incubation of IRPTCs with exogenous TNF-α also suppressed megalin mRNA and protein expression at the 24- and 48-h time points, respectively. MEK1 inhibitor PD98059 competed partially but significantly TNF-α-mediated downregulation of megalin mRNA expression. Collectively, low-level LPS-mediated TNF-α-ERK1/2 signaling pathway is involved in downregulation of megalin expression in IRPTCs.

1. Introduction

Diabetic nephropathy (DN) is a worldwide leading cause of end-stage renal disease. Proteinuria/albuminuria is an important clinical sign for the initiation and progression of DN [1,2] as well as a risk marker of cardiovascular disease (CVD) [3,4]. Although proteinuria has been generally assumed to be a result of increased permeability of serum proteins (mostly albumin) through glomeruli, it is also attributed to impaired reabsorption of the proteins by proximal tubule cells (PTCs) [5].

Megalin is a large (~600 kDa) glycoprotein member of the low-density lipoprotein receptor family [6] that is expressed abundantly at the apical membranes of PTCs [7]. Megalin plays a critical role in the reabsorption (endocytosis) of glomerular-filtered proteins including albumin and low-molecular-weight proteins [7]. Vitamin D binding protein is one of megalin's endocytic ligands, and megalin knockout mice display decreased utilization of vitamin D for its activation in PTCs [8,9]. Selenoprotein P, a major carrier of selenium, is also endocytosed by megalin and provides selenium required for PTCs to synthesize glutathione peroxidase 3 (GPx3), a circulating antioxidant [10].

Decreased megalin expression in PTCs is found in the early diabetic stages in experimental animal [11,12]. It is also suggested that the function of megalin is impaired in patients at the early stages of DN, since low-molecular-weight proteinuria are frequently observed in those patients [13,14]. Impairment of megalin function in patients with DN is also supported by the facts that the patients are often complicated with deficiencies of vitamin D and GPx3 [15,16], which are associated with the development of CVD [17,18]. Thus, regulation of megalin expression and its function in PTCs is a key determinant for the early prevention of proteinuria/albuminuria and the risk of CVD in diabetic patients [19]. However, the mechanisms of the regulation are not fully understood.

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria and acts as endotoxin by being re-

Abbreviations: AT_1R, angiotensin II type 1 receptor; CVD, cardiovascular disease; DN, diabetic nephropathy; GPx3, glutathione peroxidase 3; IRPTC, immortalized rat proximal tubule cell; LPS, lipopolysaccharide; PTC, proximal tubule cell; TCA, trichloroacetic acid.

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leased from lysed bacteria. In sepsis, the serum LPS level is increased, which induces severe inflammatory responses in multiple organs including kidney [20]. LPS is filtered by glomeruli and mainly targeted to PTCs via Toll-like receptor 4 located at the apical cell membrane [20,21]. The serum LPS level is also chronically elevated in subjects with obesity and type 2 diabetes; although it is lower than that in septic patients [22]. Such elavation in serum LPS in those subjects is named "metabolic endotoxemia" [23], which is considered to be consequences of changes in gut microbiota associated with high-fat diet and increased intestinal permeability of bacteria to circulation [24]. Chronic infectious complications such as periodontitis may be another cause of LPS increase in those subjects [25]. However, the effects of low-level LPS on the initiation and progression of DN, in particular, on the endocytic function of PTCs and the mechanisms of proteinuria/albuminuria in DN are not known.

In this study, we investigated low-level LPS-mediated effects on megalin expression and function in immortalized rat PTCs (IRPTCs), in order to elucidate the mechanism of megalin dysfunction that is involved in the development of proteinuria/albuminuria and the risk of CVD in diabetes.

2. Materials and methods

2.1. Cell culture

IRPTCs were kindly gifted by Dr. Julie R. Ingelfinger. The cells were maintained in DMEM (low-glucose) supplemented with 5% FCS, 1× non-essential amino acids and 25 mM HEPES at 37 °C and 5% CO_2 [26]. Cell culture reagents were obtained from Invitrogen (Carlsbad, CA) unless indicated. The cells were grown to confluence on 6- or 12-well tissue culture plates, washed twice with the culture medium without FCS and serum-starved for 24 h. The cells were then incubated with LPS (10–100 ng/ml) (Sigma–Aldrich, Saint Louis, MO) or its vehicle in serum-free media and subjected to immunoblotting and real-time RT-PCR. After serum-starved for 24 h, the cells were also treated with human TNF-α (1–100 ng/ml) (Sigma–Aldrich) in the presence or absence of an MEK1 inhibitor (PD98059, Calbiochem, La Jolla, CA) in order to block the downstream ERK1/2 signaling and analyze the effect on megalin expression.

2.2. Antibodies

Polyclonal anti-rat megalin antibodies were prepared as described previously [27]. A monoclonal antibody to β-actin was purchased from Abcam (Cambridge, MA, UK). Antibodies to phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2 were obtained from Cell Signaling Technology (Beverly, MA).

2.3. SDS–PAGE and immunoblotting

Cultured IRPTCs 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 15,000*g* at 4 °C for 15 min. Protein concentrations of the supernatants were determined using BCA Protein Assay Kit (Pierce, Rockford, IL). Phosphatase inhibitors (10 mM NaF and 1 mM Na_3VO_4) were added to the lysis buffer for immunoblotting with anti-phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2 antibodies. 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% FCS for 1 h, then incubated with primary antibodies for 2 h at room temperature, 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 NIH ImageJ software, available at http://rsb.info.nih.gov/nih-image/ (last accessed June, 2007).

2.4. Radioiodination

Human lactoferrin (Sigma–Aldrich) and rat albumin (Sigma–Aldrich) were radio-iodinated using 1 mCi Na–^{125}I (American Radiolabeled Chemicals Inc., Saint Louis, MO) and one Iodo-Bead (Pierce) according to the manufacturer's instructions. Free Na–^{125}I was removed from labeled protein using PD-10 columns (Bio-Rad Laboratories). The specific activities of ^{125}I-labeled lactoferrin and albumin were 2.3×10^6 and 2.2×10^6 cpm/μg, respectively.

2.5. Cellular uptake and degradation assay

IRPTCs were serum-starved for 24 h, and incubated with LPS (10–100 ng/ml) or its vehicle for 48 h. The culture media were replaced with serum-free media containing ^{125}I-labeled megalin ligand proteins (0.5 μg/ml). After 6-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 gamma counting and standardized by the cellular protein concentrations. To correct for iodine liberated from ^{125}I-labeled proteins, the level of TCA-soluble radioactivity in the medium incubated without cells was subtracted from that found in the samples.

2.6. RNA extraction and real-time RT-PCR

RNA was extracted from IRPTCs following the standard ISOGEN method (Nippon Gene Co., Ltd., Tokyo, Japan) and dissolved in autoclaved diethylpyrocarbonate-treated water. Extracted RNA concentrations were equalized at 10 ng/μl. Each RNA sample was reverse-transcribed and quantified for the target amplicons in the megalin, TNF-α and β-actin loci, using One Step SYBR® PrimeScript® PLUS RT-PCR Kit (TAKARA BIO Inc., Otsu, Japan) and Thermal Cycler Dice® Real Time System (TAKARA BIO Inc.). Each subset of samples was analyzed for relative quantification with β-actin. The final reaction mixture (25 μl total volume) contained 12.5 μl 2× One Step SYBR® RT-PCR Buffer 4, 1.5 μl *TaKaRa Ex Taq* HS Mix, 0.5 μl PrimeScript® PLUS RTase Mix, 1.0 μl each of forward and reverse primers (10 μM each) (for megalin: forward 5′-TAGCGATTTGGTTCTCCACC-3′, and reverse 5′-ACTTGTTGGCCTGCATAACC-3′, for TNF-α: forward 5′-AAATGGGCTCCCTCTCATCAGTTC-3′, and reverse 5′-TCTGCTTGGTGGTTTGCTACGAC-3′, for β-actin: forward 5′-CGAGTACAACCTTCTTGCAGC-3′, and reverse 5′-CATACCCACCATCACACCC-3′) and 50 ng of RNA template. Reverse transcription was carried out at 42 °C for 5 min, followed by denaturation of the synthesized cDNA from the template RNA strand at 95 °C for 10 s. PCR was then performed with 40 amplification cycles of 95 °C for 15 s and 60 °C for 30 s. The dissociation curve analysis followed the amplification cycles at 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. The outcome of each set of runs was analyzed with a corresponding standard curve for each amplicon, either internally included or imported, using the Thermal Cycler Dice® Real Time System software with the second derivative method. The primers for megalin and β-actin were designed based on rat mRNA sequences (NM_030827.1 for megalin; NM_031144.2 for β-actin) with Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers for TNF-α were designed as previously reported [28].

2.7. Knockdown of TNF-α mRNA in IRPTCs with siRNA

IRPTCs of the passage number under 10 were seeded at 5×10^4 cells per well in 12-well format 24 h prior to TNF-α siRNA transfection. The transfection complex in 100 μl Opti-MEM® I Reduced Serum Medium (Invitrogen) included a final concentration of 30 nM of pre-designed rat TNF-α siRNA (*Silencer*® Select Pre-designed siRNA, ID s128524, Applied Biosystems, Foster City, CA) and 3 μl of Lipofectamine™ RNAiMAX (Invitrogen) per well. Three different siRNA designs were tested against one another, and the one with the best knockdown efficiency was selected for our assay. Transfection media were removed from the culture after 24 h of incubation at 37 °C, by replacing the supernatant with fresh DMEM (low-glucose) without serum. After serum-starved for 24 h, the transfected cells were incubated with or without 10 ng/ml LPS in DMEM (low-glucose). The cells were harvested after 3 or 24 h after addition of LPS for RNA extraction (GenElute™ Total RNA Miniprep Kit, Sigma–Aldrich).

2.8. 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. A *p* value of less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Incubation of IRPTCs with low-concentration LPS for 48 h reduced megalin protein expression and its endocytic function

In previous studies with cultured renal epithelial cells, LPS was used at high concentrations (1–10 μg/ml) to study its effects on the cells, emulating the condition of septic shock [29,30]. In this study, however, we used LPS at a lower concentration of 10 (or 100 at most) ng/ml to investigate more chronic effects of LPS on PTCs, which simulated the condition associated with metabolic endotoxemia.

By incubating IRPTCs with LPS at the low concentration for 48 h, megalin protein expression was significantly decreased as shown by immunoblotting (Fig. 1). Cellular toxicities were not observed under the culture conditions using trypan-blue staining and LDH release assays (data not shown). The decrease in megalin protein expression was not evident when the cells were incubated for 24 h or less (data not shown), suggesting that the megalin downregulation is caused by LPS-mediated chronic cellular effects. Under the same culture conditions with LPS, the cellular activities for uptake and degradation of megalin's endocytic ligands (albumin and lactoferrin) were significantly decreased (Fig. 2), indicating that megalin-mediated endocytosis was also reduced by chronic actions of LPS.

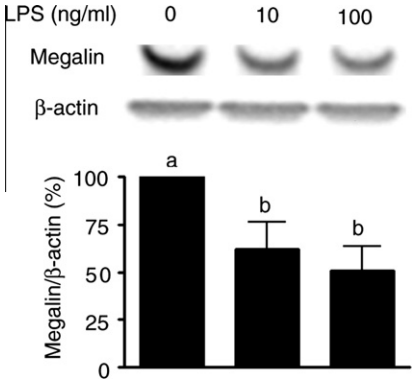

Fig. 1. LPS reduced megalin protein expression in IRPTCs. Treatment of IRPTCs with 10–100 ng/ml LPS for 48 h significantly reduced megalin protein expression. The upper panel shows representative immunoblotting results. In the lower panel, megalin bands were quantified and normalized with the endogenous β-actin control ($n = 8$). Values are expressed as mean ± SD. The differences between values associated with different letters (a and b) are all statistically significant ($p < 0.001$).

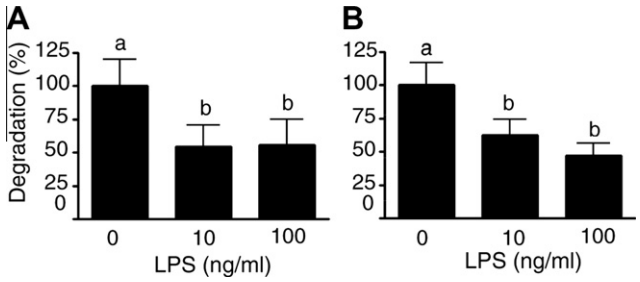

Fig. 2. LPS reduced megalin's endocytic function in IRPTCs. Treatment of IRPTCs with 10–100 ng/ml LPS for 48 h significantly reduced cellular uptake and degradation of ^{125}I-labeled megalin's endocytic ligand proteins, albumin (A) and lactoferrin (B) ($n = 6$). Values are expressed as mean ± SD. The differences between values associated with different letters (a and b) in A and B are all statistically significant ($p < 0.01$ and $p < 0.001$, respectively).

3.2. LPS-mediated upregulation of TNF-α was involved in suppressing megalin mRNA expression

Pro-inflammatory cytokines such as TNF-α were reported to be upregulated by LPS in various cells including cultured PTCs [31]. In fact, TNF-α is known to be upregulated in renal cells including PTCs in diabetic experimental models [32]. Using real-time RT-PCR, we quantitated TNF-α mRNA expression 1, 3, 5, 8 and 24 h after treatment with LPS (10–100 ng/ml). The mRNA expression for TNF-α started to increase at the 3-h time point and maintained the plateau through the 5 and 8-h time points, which decreased at the 24-h time point (data not shown). Megalin mRNA expression was

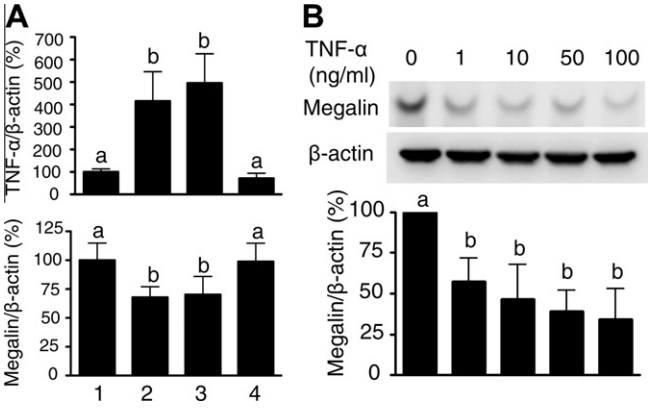

Fig. 3. LPS-mediated upregulation of TNF-α was involved in downregulating megalin in IRPTCs. (A) Treatment of IRPTCs with 10 ng/ml LPS upregulated TNF-α mRNA expression at the 3-h time point and the upregulation was suppressed significantly by TNF-α siRNA but not by negative control siRNA (upper panel). At the 24-h time point, megalin mRNA expression was significantly downregulated by LPS but the downregulation was competed with TNF-α siRNA but not with negative control siRNA (lower panel). (1) vehicle; (2) LPS (10 ng/ml); (3) LPS with negative control siRNA (30 nM) and (4) LPS with TNF-α siRNA (30 nM). (B) Incubation of IRPTCs with 1–100 ng/ml TNF-α directly suppressed megalin protein expression. Values are expressed as mean ± SD ($n = 5$ for A and 4 for B, respectively). The differences between values associated with different letters (a and b) are all statistically significant ($p < 0.05$).

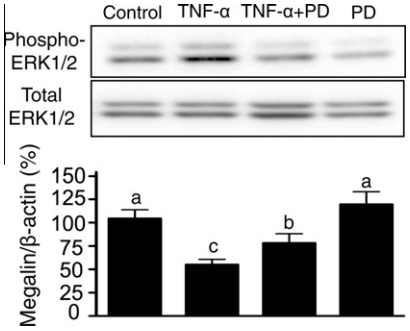

Fig. 4. TNF-α suppressed megalin mRNA expression via ERK1/2 pathway in IRPTCs. Treatment of IRPTCs with 50 ng/ml TNF-α was found to enhance phosphorylation of ERK1/2 at the 3-h time point and MEK1 inhibitor PD98059 (PD) suppressed the phosphorylation (upper panel). At the 24-h time point, megalin mRNA expression was suppressed by TNF-α but PD98059 partially but significantly competed TNF-α-mediated megalin mRNA suppression (lower panel). Values are expressed as mean ± SD ($n = 5$). The differences between values associated with different letters (a, b and c) are all statistically significant ($p < 0.05$).

suppressed with a time delay to the TNF-α increase, after 8 h of incubation in LPS, and the expression level continued to descend through till the 24-h time point (Fig. 3A). Knockdown of TNF-α mRNA with siRNA inhibited LPS-mediated suppression of megalin mRNA expression at the 24-h time point (Fig. 3A), suggesting that LPS-mediated upregulation of TNF-α is involved in suppressing megalin mRNA expression.

To confirm the direct effect of TNF-α for downregulating megalin expression in IRPTCs, the cells were incubated with TNF-α (1–100 ng/ml) for 48 h and subjected to immunoblotting. As shown in Fig. 3B, megalin protein expression was found to be decreased by the direct action of TNF-α on the cells. Megalin mRNA expression in the cells was also found to be significantly suppressed at the 24-h time point after incubation with TNF-α (50 ng/ml) (Fig. 4).

3.3. TNF-α suppressed megalin mRNA expression via ERK1/2 pathway

We further investigated TNF-α-mediated signaling mechanisms for suppressing megalin gene expression in IRPTCs. We found that ERK1/2 is activated by TNF-α in the cells and the MEK1 inhibitor PD98059 partially but significantly inhibited TNF-α-mediated megalin mRNA suppression (Fig. 4). We also found that TNF-α activates NFkB signaling but NFkB inhibitors did not inhibit TNF-α-mediated megalin mRNA suppression (data not shown). Collectively, TNF-α-ERK1/2 signaling pathway is involved in low-level LPS-mediated downregulation of megalin expression in IRPTCs.

We previously reported that megalin expression is downregulated in cultured PTCs by angiotensin II type 1 receptor (AT_1R)-mediated ERK1/2 signaling pathway, whereas it is upregulated by insulin-mediated IRS/PI3K signaling pathway [33]. In the report, we also demonstrated that there is competitive cross talk between AT_1R- and insulin-mediated signaling pathways in the regulation of megalin expression in the cells.

As intrarenal renin-angiotensin system is activated in diabetes [34], angiotensin II-mediated ERK1/2 signaling may be enhanced to suppress the expression of megalin in PTCs in the disease. Metabolic endotoxemia-related TNF-α activation in PTCs is likely to contribute to the additional augmentation of ERK1/2-mediated suppression of megalin expression.

Also, TNF-α is known to compete insulin-mediated cellular signaling in various organs and tissues, causing insulin resistance [35]. In diabetes, TNF-α may also suppress insulin signaling in renal cells including PTCs [32]. Therefore, LPS-mediated upregulation of TNF-α in PTCs would also augment a negative signaling balance for megalin expression by suppressing insulin signaling.

In conclusion, we demonstrated that low-level LPS suppresses megalin expression and its endocytic function in cultured PTCs. We also identified that LPS-mediated upregulation of TNF-α is involved in suppressing megalin gene expression partially via ERK1/2 pathway. These findings will be useful for future development of novel strategies for early diagnosis and treatment of LPS-mediated kidney injury in diabetes.

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