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Transcriptional suppression of nephrin in podocytes by macrophages: Roles of inflammatory cytokines and involvement of the PI3K/Akt pathway

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Abstract **Expression of nephrin, a crucial component of the glomerular slit diaphragm, is downregulated in patients with proteinuric glomerular diseases. Using conditionally immortalized reporter podocytes, we found that bystander macrophages as well as macrophage-derived cytokines IL-1β and TNF-α markedly suppressed activity of the nephrin gene promoter in podocytes. The cytokine-initiated repression was reversible, observed on both basal and inducible expression, independent of Wilms' tumor suppressor WT1, and caused in part via activation of the phosphatidylinositol-3-kinase/Akt pathway. These results indicated a novel mechanism by which activated macrophages participate in the induction of proteinuria in glomerular diseases.**

Keywords: Podocyte; Nephrin; Macrophage; IL-1β; TNF-α; Phosphatidylinositol-3-kinase

1. Introduction

Proteinuria is a typical clinical feature of glomerular diseases. Under physiologic situations, the glomerular filtration barrier consisting of the basement membrane and the slit diaphragm is maintained by podocytes and regulates passage of macromolecules in plasma into the urinary space [1]. Recent investigation identified nephrin, a podocyte-specific transmembrane protein, as a key regulator for maintaining the structure and function of the slit diaphragm [2]. Attenuated expression of nephrin mRNA is often observed in proteinuric glomerular diseases [3–5], whereas information is very limited regarding how expression of nephrin is down-regulated under pathologic circumstances.

Wilms' tumor suppressor WT1 is constitutively expressed in podocytes and contributes to basal expression of the nephrin gene [6,7]. The transacting potential of WT1 is dependent on its phosphorylation state; i.e., phosphorylation of WT1 by protein kinase C (PKC) or protein kinase A attenuates its trans-acting potential [8]. Downregulation of WT1 or its phosphorylation may be a possible mechanism for the repression of nephrin expression. Some reports indicated possible roles of the nuclear receptor family of molecules on the expression of nephrin gene. For example, recent reports showed that agonists of peroxisome proliferator-activated receptors upregulated expression of nephrin mRNA [9,10]. We identified that nuclear receptor ligands including retinoic acid, vitamin D_3 and glucocorticoid upregulate activity of the nephrin gene promoter and nephrin mRNA levels [11]. Although nuclear receptors and their endogenous ligands may contribute to basal expression of nephrin in podocytes, little is understood about their involvement in downregulation of nephrin under pathologic situations.

Infiltration of leukocytes, especially monocytes/macrophages, play a decisive role in the development of various proteinuric glomerular diseases [12]. Under pathologic conditions, activated macrophages secrete various inflammatory mediators and stimulate resident cells toward functional alteration. Depletion of macrophages or inhibition of macrophage infiltration attenuates glomerular injury and proteinuria, suggesting a critical role of macrophages in the development of proteinuria [13,14]. Recently, we found that podocytes exposed to macrophages exhibited down-regulation of nephrin mRNA. We hypothesized that, under pathologic situations, infiltrating local macrophages may repress nephrin expression, leading to proteinuria. The purpose of the present study is to investigate whether macrophages can suppress activity of the nephrin gene promoter in podocytes, and if so, how macrophage-derived factors regulate expression of the nephrin gene.

2. Materials and methods

2.1. Cells

Reporter podocytes REPON5.4 were established by stable transfection of murine podocytes with pN5.4-SEAP that introduces the secreted alkaline phosphatase (SEAP) gene under the control of the 5.4 kb murine nephrin gene promoter [11]. To prepare pN5.4-SEAP, the 5.4 kb promoter fragment was excised from p5.4N-nlacF (kindly provided by Dr. Lawrence B. Holzman, University of Michigan Medical School) [15] by digestion with *Kpn*I and *Nco*I. After blunting the *Nco*I site, those fragments were subcloned into multiple cloning sites

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Abbreviations: PKC, protein kinase C; SEAP, secreted alkaline phosphatase; FBS, fetal bovine serum; ATRA, all-*trans* retinoic acid; PI3K, phosphophatidylinositol-3-kinase; TPA, 12-*o*-tetradecanoylphorbol-1-3-acetate; MϕCM, macrophage-conditioned medium; LPS, lipopolysaccharide; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; REPON, reporter podocytes for nephrin; HGF, hepatocyte growth factor; RAR, retinoic acid receptor; RARE, retinoic acid response element

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(*Kpn*I and bluted *Xho*I sites) of pSEAP2-Basic (Clontech, Palo Alto, CA). As a control, murine podocytes were stably transfected with pSEAP2-Control (Clontech) that introduces the SEAP gene under the control of the simian virus 40 promoter [11]. Murine macrophage J774.1 cells were obtained from Riken Bioresource Center (Ibaraki, Japan) and used for co-culture and cross-feeding studies, as described later.

2.2. Pharmacological treatment

Reporter podocytes were seeded onto collagen-coated plates, pre-incubated for 48 h in RPMI-1640 containing 10% fetal bovine serum (FBS) and exposed to test reagents as follows; IL-1β (human recombinant, 0.02–200 ng/ml; Genzyme, Cambridge, MA), TNF-α (human recombinant, 0.1–100 ng/ml; Genzyme), 1,25-dihydroxyvitamin D_3 (100 nM; Chugai Pharmaceutical Co. Ltd., Tokyo, Japan), all-*trans* retinoic acid (ATRA, 1 μM; Genzyme), phosphophatidylinositol-3-kinase (PI3K) inhibitor LY294002 (50 μM; Sigma–Aldrich Japan, Tokyo), PKC inhibitor calphostin C (40 nM; Sigma–Aldrich) and 12-*o*-tetradecanoylphorbol-13-acetate (TPA, 50 nM; Sigma–Aldrich). Macrophage-conditioned medium (MϕCM) was prepared using J774.1 cells as described previously [16]. All assays were performed in the presence of 1% FBS.

2.3. Co-culture

J774.1 cells were activated with lipopolysaccharide (LPS, 1 μg/ml; Sigma–Aldrich) for 6 h. After washing twice, the macrophages (1×10^4 cells/well) were seeded onto confluent cultures of reporter podocytes (1×10^4 cells/well) in 96-well plates. After 24 h, culture media were subjected to SEAP assay, as described later.

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR analysis of nephrin gene expression was performed using Omniscript Reverse Transcriptase (Qiagen, Tokyo, Japan), as described before [11]. Expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

2.5. Northern blot analysis

Total RNA was extracted by a single-step method, and Northern blot analysis was performed as described previously [16]. Murine WT1 cDNA (kindly provided by Dr. Holger Scholz, Charite-Universitatsmedizen, Berlin) [17] was used to prepare a radio-labeled probe. Expression of GAPDH was used as a loading control.

2.6. Western blot analysis

Phosphorylation of Akt was evaluated by Western blot analysis [18] using anti-phospho-Akt Ab (1/200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). As a loading control, identical filters were re-probed for β-actin using anti-β-actin Ab (1/30000 dilution; Sigma–Aldrich).

2.7. Chemiluminescent assay

Activity of SEAP was evaluated by a chemiluminescent method using Great EscAPe SEAP detection kit (BD Bioscience, Palo Alto, CA) [19].

2.8. Formazan assay

The number of viable cells was assessed by a formazan assay using Cell Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan) [11].

2.9. Statistical analysis

Assays were performed in quadruplicate. Data are expressed as means ± S.E. Statistical analysis was performed using the non-parametric Mann–Whitney *U* test to compare data in different groups. *P* value <0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Repression of the nephrin gene promoter in podocytes by bystander macrophages

In glomerular diseases, activated macrophages play crucial roles in the induction of proteinuria. We hypothesized that

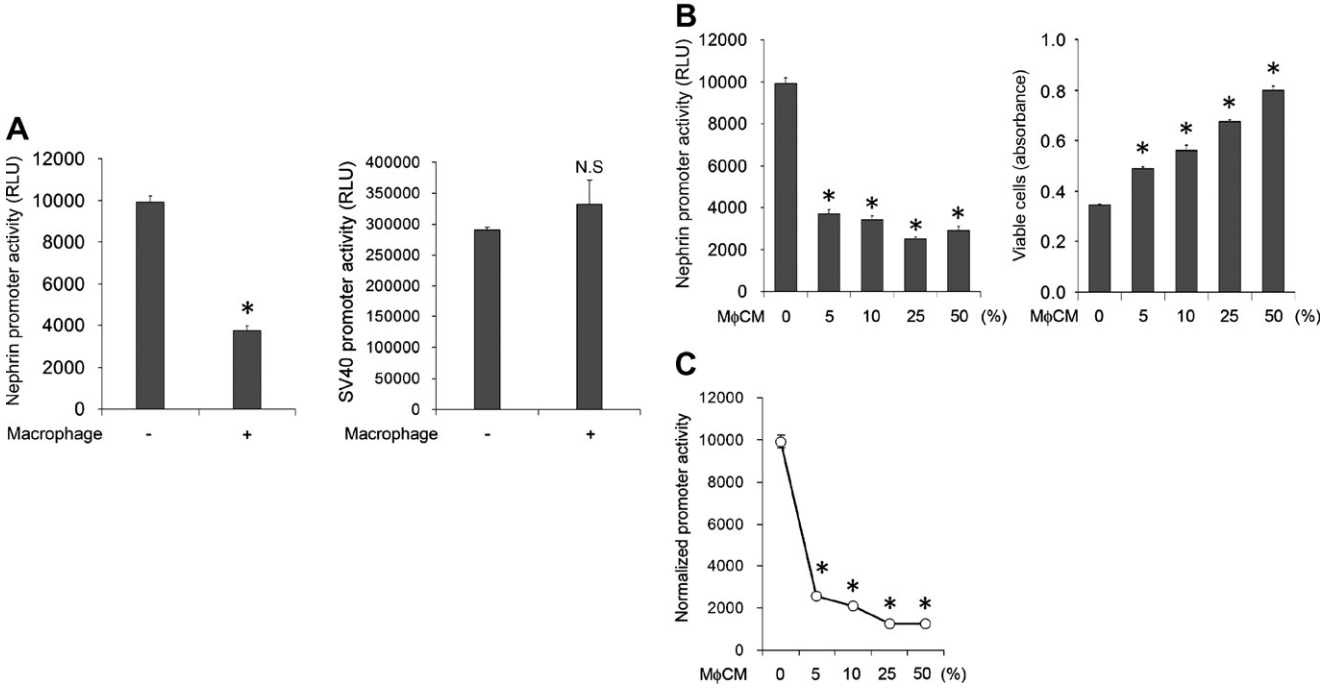

Fig. 1. Repression of the nephrin gene promoter in podocytes by bystander macrophages. (A) Reporter podocytes REPON (left) or podocytes producing SEAP under the control of the simian virus 40 promoter (right) were co-cultured with J774.1 macrophage pre-stimulated with lipopolysaccharide (LPS; 1 μg/ml) for 24 h, and culture media were subjected to secreted alkaline phosphatase (SEAP) assay. (B,C) REPON were treated for 24 h with conditioned media from activated J774.1 macrophages (MϕCM; 0–50 %), and culture media and cell were subjected to SEAP assay (B, left) and formazan assay (B, right). Activity of SEAP was normalized by the number of viable cells, and the resultant values are shown in (C). Data are expressed as means ± S.E., and asterisks indicate statistically significant differences ($P < 0.05$).

macrophages may affect podocyte function, especially nephrin expression, via direct contact or via elaborating inflammatory mediators. To examine these possibilities, we used murine reporter podocytes for nephrin (REPON) that secrete SEAP under the control of the murine nephrin gene promoter [11]. As the first step of investigation, REPON were co-cultured for 24 h with pre-activated murine macrophages J774.1, and culture media were subjected to chemiluminescent assay to evaluate SEAP activity. As shown in Fig. 1A (left), activity of the nephrin gene promoter in podocytes was significantly reduced to 37.8 ± 5.3% by bystander macrophages. In contrast, when control podocytes constitutively expressing SEAP under the control of a viral promoter were co-cultured with macrophages, the activity of SEAP was not declined (Fig. 1A, right). This result confirmed that the macrophage-triggered reduction of SEAP in the reporter cells was not due to cellular damage. To identify mechanisms involved in the effect of macrophages, cross-feeding studies were performed. REPON were exposed to serial concentrations of MϕCM derived from J774.1 cells, and activity of SEAP was evaluated. The result showed that MϕCM significantly repressed activity of the nephrin gene promoter (Fig. 1B, left). Even in the presence of 5% MϕCM, activity of the promoter was reduced to 37.2 ± 6.4%. In contrast, the number of viable cells was significantly increased by MϕCM in a dose-dependent manner (Fig. 1B, right). Normalization of SEAP activity by the number of viable cells revealed dose-dependent suppression of the nephrin promoter by MϕCM (Fig. 1C).

3.2. Repression of basal nephrin promoter activity in podocytes by inflammatory cytokines

Activated macrophages produce IL-1β and TNF-α abundantly [16,18], and these cytokines are involved in the pathogenesis of glomerular diseases [20]. We tested effects of IL-1β and TNF-α on the activity of the nephrin gene promoter in REPON. As shown in Fig. 2A, these cytokines repressed activity of the nephrin gene promoter in a dose-dependent manner. To exclude a possibility that the downregulation of SEAP by these cytokines might be caused by nonspecific damage of the reporter podocytes, the cells used for chemiluminescent assay were subsequently subjected to formazan assay to evaluate the number of viable cells. At any concentrations tested, cell viability was unaffected by IL-1β or TNF-α (Fig. 2B). Time-lapse experiments revealed that significant suppression of the nephrin gene promoter was observed within 8–12 h (Fig. 2C). The suppressive effects of IL-1β and TNF-α on the nephrin expression were further confirmed by RT-PCR. Consistent with the results shown in Fig. 2A, the basal level of nephrin mRNA was depressed by these cytokines (Supplemental Fig. 1).

To examine whether or not the repression of nephrin by cytokines was reversible, REPON were pretreated with IL-1β or TNF-α for 24 h and then incubated without cytokines for up to 48 h. During the initial 24 h after the treatment (0–24 h), suppression of SEAP was still observed (Fig. 2D, left). However, subsequent incubation for additional 24 h (24–48 h) did not show significant inhibition of SEAP activity in

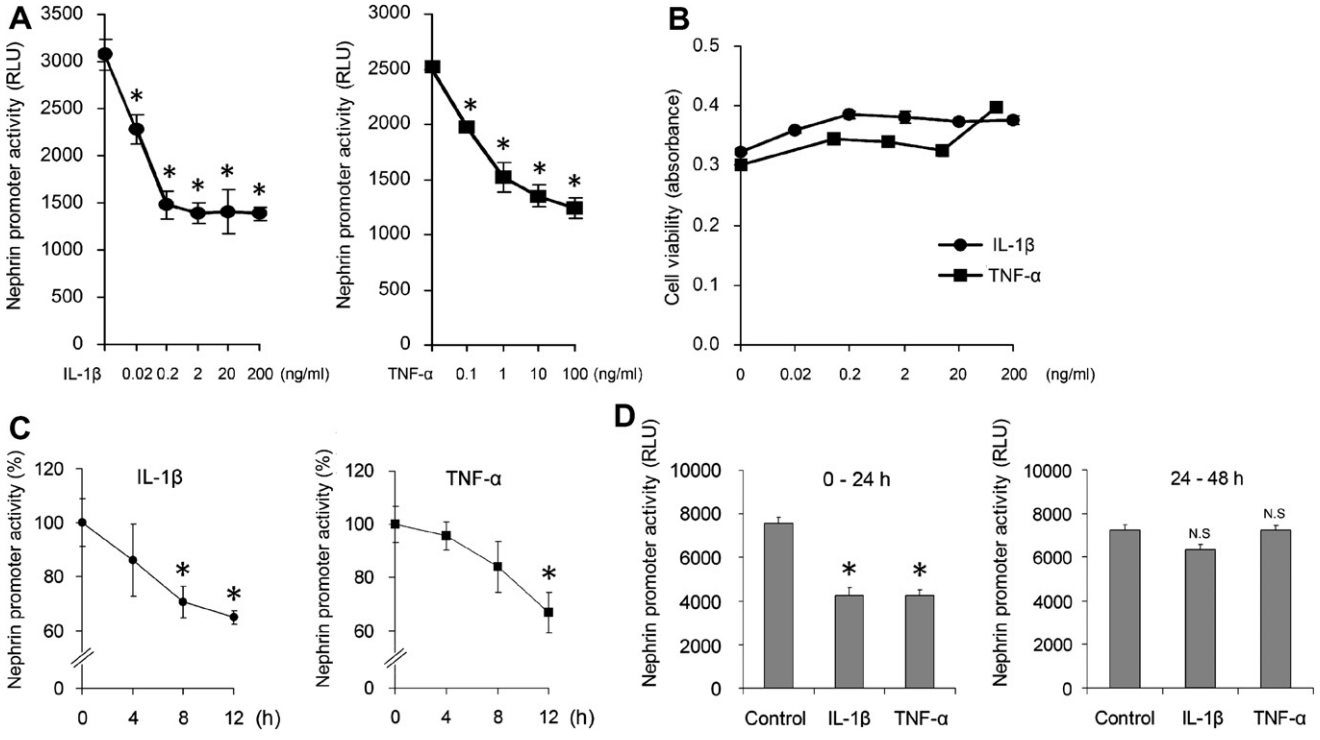

Fig. 2. Repression of basal nephrin promoter activity by inflammatory cytokines. (A,B) Reporter podocytes were treated for 24 h with IL-1β (0–200 ng/ml; left) or TNF-α (0–100 ng/ml; right), and culture media and cells were subject to SEAP assay (A) and formazan assay (B), respectively. (C) Reporter podocytes were exposed to IL-1β (20 ng/ml; left) or TNF-α (10 ng/ml; right) for up to 12 h, and culture media sampled every 4 h were subjected to SEAP assay. (D) Cells were pretreated for 24 h with IL-1β or TNF-α and then incubated without cytokines for up to 48 h. Activity of SEAP produced during initial 24 h and next 24 h was evaluated by chemiluminescent assay. Asterisks indicate statistically significant differences ($P < 0.05$). N.S, not statistically significant.

cytokine-treated cells (Fig. 2D, right). This result evidenced that the suppression of the nephrin gene promoter by cytokines is reversible.

3.3. Repression of inducible nephrin promoter activity in podocytes by inflammatory cytokines

We recently reported that 1,25-dihydroxyvitamin D_3 and ATRA upregulated activity of the nephrin gene promoter and nephrin mRNA levels in podocytes [11]. We next examined whether activation of the nephrin promoter by 1,25-dihydroxyvitamin D_3 or ATRA is also inhibited by inflammatory cytokines. REPON were treated with IL-1β or TNF-α in the presence of 1,25-dihydroxyvitamin D_3 or ATRA, and culture media were subjected to chemiluminescent assay. As shown in Fig. 3A, activation of the nephrin gene promoter by 1,25-dihydroxyvitamin D_3 was significantly attenuated by IL-1β or TNF-α. Similarly, activation of the nephrin gene promoter by ATRA was abrogated by the treatment with these cytokines (Fig. 3B). Consistently, induced expression of nephrin mRNA was downregulated by cytokines modestly in 1,25-dihydroxyvitamin D_3-treated cells (Fig. 3C, upper) and markedly in ATRA-treated cells (Fig. 3C, lower).

3.4. Molecular mechanisms involved in transcriptional suppression of nephrin by cytokines

Previous reports showed that WT1 is a crucial transcription factor that upregulates expression of nephrin in podocytes [6,7] and that the transacting potential is attenuated due to its phosphorylation by, for example, PKC [8]. Phosphorylation of WT1 by inflammatory cytokines may cause suppression of the nephrin promoter. Indeed, treatment of REPON with TPA, an activator of PKC, significantly suppressed the level of SEAP (Fig. 4A). However, treatment with calphostin C, an inhibitor of PKC, did not affect suppression of the nephrin promoter by IL-1β or TNF-α (Fig. 4B). Furthermore, Northern blot analysis revealed that treatment of the cells with IL-1β or TNF-α did not suppress but rather enhanced expression of WT1 mRNA (Fig. 4C). These results indicated lack of involvement of WT1 in the suppression of nephrin expression by inflammatory cytokines.

Recently, we found that hepatocyte growth factor (HGF), a putative de-differentiation factor of podocytes [21], markedly suppressed activity of the nephrin gene promoter (Supplemental Fig. 2A) as well as expression of nephrin mRNA (Supplemental Fig. 2B). HGF is a well-known activator of PI3K/Akt in various cells, including REPON (Supplemental Fig. 2C), and activation of PI3K/Akt by IL-1β and TNF-α is observed in some cell types [22,23]. We speculated that the PI3K/Akt pathway might be involved in the suppression of the nephrin gene in podocytes. Indeed, Western blot analysis revealed that phosphorylation of Akt was rapidly induced in podocytes after the treatment with IL-1β or TNF-α (Fig. 4D). Of note, IL-1β activated Akt to less extent and for shorter term than TNF-α. Interestingly, treatment with LY294002, a PI3K inhibitor, abrogated the suppressive effect of TNF-α, but not IL-1β (Fig. 4E). These results indicated differential involvement of the PI3K/Akt pathway in the suppression of nephrin gene expression by macrophage-derived, proinflammatory cytokines.

4. Discussion

In this report, we describe that bystander macrophages have the ability to suppress expression of nephrin in podocytes via production of inflammatory cytokines. The cytokine-initiated repression of nephrin was reversible, observed on both basal and inducible expression, and independent of Wilms' tumor suppressor WT1. In contrast to our present findings, a previous report indicated that, in A293 human embryonic kidney cells and primary culture of human podocytes, IL-1β and TNF-α upregulated nephrin gene expression [24]. The reason for the discrepancy is currently unclear, but regulation of nephrin in human podocytes could be different from that in murine podocytes, or different culture conditions such as serum concentrations might have affected the experimental outcome.

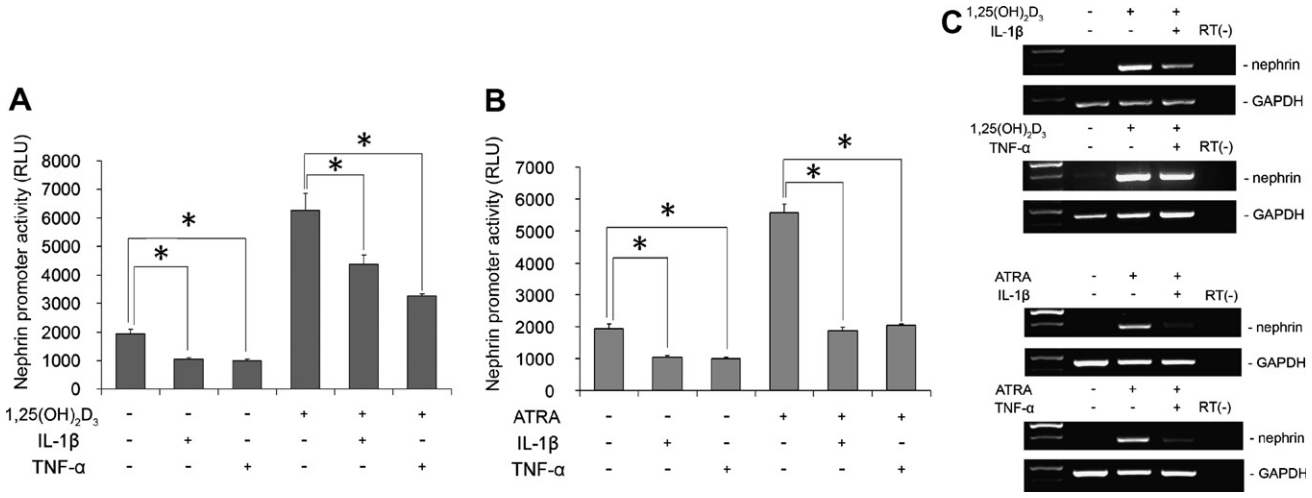

Fig. 3. Repression of inducible nephrin promoter activity by inflammatory cytokines. Reporter podocytes were treated with IL-1β (20 ng/ml) or TNF-α (10 ng/ml) in the absence (−) or presence (+) of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$; 100 nM] (A,C) or all-*trans* retinoic acid (ATRA; 1 μM) (B,C) for 24 h, and culture media and cells were subject to SEAP assay (A,B) and reverse transcriptase-polymerase chain reaction analysis of nephrin and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (C). Asterisks indicate statistically significant differences ($P < 0.05$). RT(−), reaction without reverse transcriptase.

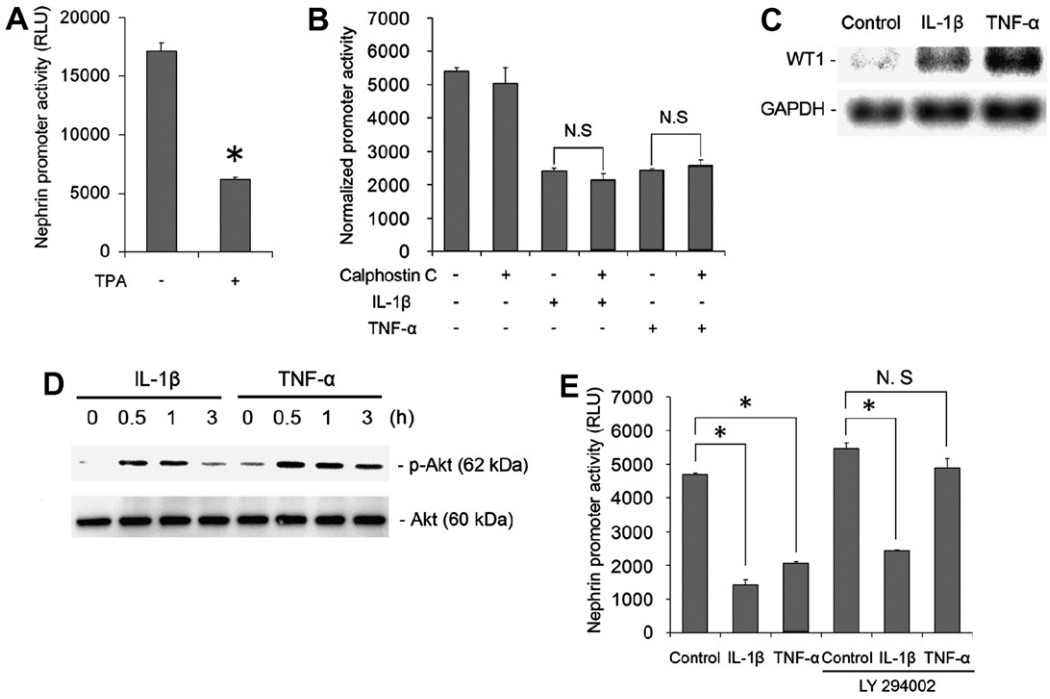

Fig. 4. Molecular mechanisms involved in transcriptional suppression of nephrin by cytokines. (A) Reporter podocytes were treated with 12-*o*-tetradecanoylphorbol-13-acetate (TPA, 50 nM) for 24 h, and culture media were subjected to SEAP assay. (B) Cells were pretreated with calphostin C (40 nM) for 30 min and subsequently exposed to IL-1β (20 ng/ml) or TNF-α (10 ng/ml). Culture media were subject to SEAP assay. (C) Reporter podocytes were stimulated with IL-1β or TNF-α for 24 h, and expression of WT1 was examined by Northern blot analysis. Expression of GAPDH is shown at the bottom as a loading control. (D) Reporter podocytes were treated with IL-1β or TNF-α for indicated time periods, and levels of phosphorylated Akt (p-Akt) and total Akt were examined by Western blot analysis. (E) Reporter podocytes were pretreated with or without LY294002 (50 μM) for 30 min, subsequently exposed to IL-1β or TNF-α, and subject to SEAP assay. Asterisks indicate statistically significant differences ($P < 0.05$). N.S, not statistically significant.

We revealed that activation of the PI3K/Akt pathway was responsible for the suppressive effect of TNF-α. Currently, downstream events involved in the suppressive effect of PI3K/Akt are unclear. The fact that substantial expression of nephrin in podocytes required ATRA (Fig. 3) raises a possibility that the PI3K/Akt pathway may affect signal transduction pathways initiated by ATRA. ATRA is an active metabolite of vitamin A and a ligand of the nuclear receptor, retinoic acid receptor (RAR). After binding of ATRA to RAR, RAR forms homodimers or heterodimers with retinoid X receptor, and the resultant complexes exert biological effects via binding to the particular *cis* element, the retinoic acid response element (RARE). Functional RAREs are present in the regulatory region of the human nephrin gene [25], and ATRA can induce expression of nephrin through activation of these regulatory elements. Recently, Srinivas et al. report that Akt inhibited retinoid-induced transactivation in lung cancer cells via phosphorylation at the Ser96 residue of the RARα's DNA-binding domain [26]. Mutation of Ser96 to alanine abrogated the suppressive effect of Akt, and overexpression of a dominant-negative mutant of Akt decreased RAR phosphorylation and increased RAR transactivation. Lefebvre et al. also reported that the PI3K/Akt pathway affected recruitment of some co-repressor to the RARβ2 promoter, leading to down-regulation of RARβ2 expression and impairment of cellular responses to ATRA [27]. These results suggest that activation of the PI3K/Akt pathway by TNF-α suppressed the function of RARs, impaired the retinoid signaling, and thereby downregulated nephrin expression. In the present study, however, we found that down-regulation of nephrin by IL-1β was independent of the PI3K/Akt pathway. Although IL-1β caused phosphorylation of Akt, inhibition of PI3K did not significantly affect the suppressive effect of this cytokine on nephrin expression. It might be due to the fact that IL-1β activated Akt to less extent and for shorter term than TNF-α, as shown in this report (Fig. 4D).

Infiltration of macrophages plays a crucial role in the development of various proteinuric glomerular diseases [12]. Activated macrophages produce inflammatory cytokines abundantly, which is involved in the development of proteinuria in glomerular diseases [28]. In anti-glomerular basement membrane antibody glomerulonephritis (a model of Goodpasture syndrome in human), activated podocytes contribute to the formation of cellular crescents where infiltrating macrophages are co-localized [29]. In this experimental model, (1) proteinuria is caused by infiltration of macrophages [30], (2) activated podocytes undergo phenotypic changes and lose expression of nephrin [29], and (3) proteinuria and crescent formation are ameliorated either by administration with IL-1 receptor antagonist or by gene knockout of TNF-α [31,32]. Based on these data together with our current findings, we propose a novel mechanism involved in the induction of proteinuria in macrophage-associated, proteinuric glomerular diseases. Under pathologic situations, infiltrating glomerular macrophages produce proinflammatory cytokines and thereby repress expression of nephrin in podocytes, leading to development of proteinuria.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.12.051.

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