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Inflammatory cytokines upregulate nephrin expression in human embryonic kidney epithelial cells and podocytes☆

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Abstract

Nephrin is an important regulator of the glomerular filtration barrier and its malfunction is associated with severe proteinuria. In this study we show that exposure of human embryonic kidney epithelial A293 cells to the proinflammatory cytokine interleukin-1β (IL-1β) causes a dose-dependent upregulation of nephrin mRNA level. Time-course analyses reveal first significant increases in nephrin mRNA levels after 4 h of stimulation. Furthermore, nephrin protein is also elevated by IL-1β treatment. Tumor necrosis factor-α (TNFα) exerted a comparable effect on nephrin mRNA and protein expression. The IL-1β-induced upregulation of nephrin expression occurs independently of nitric oxide (NO) generation, since the NO-synthase inhibitor N^G-monomethyl-L-arginine does not block the IL-1β effect. Mechanistically, we found that the IL-1β-induced response does not involve protein kinase C, protein kinase A, the classical mitogen-activated protein kinase (MAPK), the stress-activated p38-MAPK, or the NF-κB cascade, since selective inhibitors of these pathways were unable to alter the IL-1β response. Moreover, neither unselective cyclooxygenase (COX) inhibitors, like indomethacin, nor COX-2-selective inhibitors, like flosulide and NS 398, nor the anti-inflammatory glucocorticoid dexamethasone were able to alter IL-1β-induced nephrin expression. The only inhibitor that was able to block IL-1β- and TNFα-induced nephrin upregulation was rottlerin, which has been suggested to act as a selective PKCδ inhibitor. However, concerning cytokine-triggered nephrin expression, rottlerin action involved inhibition of another still to be identified protein kinase. Importantly, cytokine-induced upregulation of nephrin expression was also confirmed in primary human podocytes. In summary, these data show for the first time that inflammatory cytokines like IL-1β or TNFα can upregulate nephrin expression and this mechanistically involves a rottlerin-sensitive protein kinase.

Keywords: Nephrin; Interleukin-1β; Tumor necrosis factor α; Kidney epithelial cells; Nitric oxide; Protein kinase C isoenzymes

Proteinuria is a serious complication of many kidney diseases and the degree of proteinuria correlates well with the severity of the disease. Morphologically it is accompanied by a flattening of glomerular foot processes, which is determined by the interaction of glomerular visceral epithelial cells (podocytes) [1] with the glomerular basement membrane to form porous interconnecting slit diaphragms [1–5].

The recently identified podocyte-specific protein nephrin plays an important structural and functional role in the establishment of the kidney filtration barrier [1–5]. Deficiency of nephrin due to mutations in the *NPHS 1* gene results in the congenital nephrotic syndrome of the Finnish type (CNSF) [6,7], which is characterized by massive proteinuria and renal failure and

☆ *Abbreviations:* CNSF, congenital nephrotic syndrome of the Finnish type; COX, cyclooxygenase; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; IL-1β, interleukin-1β; iNOS, inducible NO-synthase; LNMMA, N^G-monomethyl-L-arginine; MAPK, mitogen-activated protein kinase; MAPKAPK-2, MAPK-activated protein kinase-2; MEK, MAPK kinase; NF-κB, nuclear factor kappa B; PKC, protein kinase C; PRAK, p38-regulated/activated protein kinase; TNFα, tumor necrosis factor α; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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which can only be cured by kidney transplantation. Similarly, knock-out mice generated by targeted inactivation of the nephrin gene [8] or by gene trapping [9] fail to develop foot processes and are nephrotic.

Nephrin is a 185-kDa glycosylated transmembrane protein with eight immunoglobulin-like domains and one fibronectin Ill-like domain in the extracellular part and a relatively short intracellular part. It is located in a complex with other proteins, like the *NPHS 2* gene product podocin and CD2AP [4,10,11]. Mutation of one of the complex members or a disbalance between the members is associated with foot process flattening and results in filtration barrier dysfunction. However, the detailed mechanism that is required to keep the functionality of the filtration barrier or mechanisms that are involved in barrier disruption is not understood.

In this study we have used human embryonic kidney epithelial A293 cells that have previously been shown to naturally express nephrin mRNA and protein [12], to investigate the regulation of nephrin expression under inflammatory conditions. We report for the first time that the proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα) potently upregulate nephrin mRNA and protein levels in A293 cells but also in primary cultures of human podocytes, and that this upregulation occurs in a rottlerin-sensitive manner.

Materials and methods

Chemicals. IL-1β, CGP41251, and flosulide were kindly provided by Novartis Pharma, Basel, Switzerland; TNFα was kindly provided by Knoll AG, Ludwigshafen, Germany; protein A–Sepharose 4B CL, anti-rabbit horseradish peroxidase-linked IgGs, Hyperfilms, and the enhanced chemiluminescence (ECL) reagents were purchased from Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany; all inhibitors were obtained from Merck Biosciences, Schwalbach, Germany; all cell culture nutrients were from Invitrogen/Life Technologies, Karlsruhe, Germany; two synthetic peptides (CEY EES QWT GER DTQ S and QPS GEP EDQ LPT EPPC) based on the sequence of the human nephrin (Accession No.: NM004646) were synthesized, coupled to keyhole limpet hemocyanin, and used to immunize rabbits. The antiserum was affinity purified by using a peptide-coupled Sepharose column.

Cell-culture. Human embryonic kidney epithelial cells A293 cells were cultivated as previously described [12]. Primary cultures of human podocytes were cultivated as described [13]. Before stimulations, cells were synchronized by incubation for 16 h in Dulbecco's modified Eagle's medium (DMEM) including 0.1 mg/ml of fatty acid-free bovine serum albumin.

Reverse transcriptase-PCR. Total RNA was isolated using guanidinium isothiocyanate solution. Ten micrograms of total RNA was used for reverse transcription (using a First-Strand cDNA Synthesis Kit, MBI). The following sequences were performed for PCR (Taq DNA Polymerase, recombinant, MBI): 94 °C for 45 s, 56 °C for 1 min, 72 °C for 45 s (with 35 cycles), and final extension at 72 °C for 10 min. Sequences of the primers for analysis of mRNA: human nephrin: forward, CCA ACA TCG TTT TCA CTT GG; reverse, GGG TGG TAC GAC ATC CAC AT. GAPDH: forward, AAT GCA TCC TGC ACC ACC AA; reverse, GTC ATT GAG AGC AAT GCC AGC. PCR products (length: 347 bp for nephrin and 470 bp for GAPDH) were run on a 1.0% agarose gel containing 0.5 μg/ml ethidium bromide. The identities of PCR products were confirmed by sequencing (Genetic Analyzer 310, Perkin–Elmer).

Immunoprecipitation and Western blot analysis. Confluent A293 cells in 100-mm diameter dishes were stimulated in DMEM including 0.1 mg/ml of fatty acid-free bovine serum albumin with the indicated substances. Thereafter the medium was withdrawn and the cells were washed once with ice-cold phosphate-buffered saline solution. Cells were scraped into ice-cold lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 40 mM β-glycerophosphate, 50 mM sodium fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride) and vortexed for 30 s. Samples were centrifuged for 10 min at 14,000*g* and the supernatant was taken for protein determination. Cell extracts containing 1000 μg of protein and 5% of fetal bovine serum in lysis buffer were incubated with a polyclonal anti-nephrin antiserum (at a dilution of 1:100). Thereafter, protein A–Sepharose 4B CL was added and incubated for 1 h on a rotation wheel [14]. Beads were washed three times with a low salt buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.2% Triton X-100, 2 mM EDTA, 2 mM EGTA, and 0.1% SDS) and three times with a high salt buffer (50 mM Tris/HCl, pH 7.4, 500 mM NaCl, 0.2% Triton X-100, 2 mM EDTA, 2 mM EGTA, and 0.1% SDS) and then heated for 5 min at 95 °C in Laemmli buffer. Proteins were separated on SDS–PAGE (5% acrylamide gel) and transferred to a nitrocellulose membrane for 1 h at 11 V using a semi-dry blotting apparatus. The blotting buffer used was 25 mM Tris, 190 mM glycine in 20% (v/v) methanol. After the transfer, immunostaining was performed as previously described in detail [15,16]. Antibodies were diluted in blocking buffer as indicated in the legends of the figures. Bands were detected by the ECL method as recommended by the manufacturer. [^{35}S]Metabolic labeling of A293 cells was performed as described [14].

Results

A293 cells were incubated with 1 nM of IL-1β for different time periods upto 24 h showing a time-dependent increase of nephrin mRNA steady-state levels as detected by a semi-quantitative PCR method. A maximal increase is seen at 8 h and thereafter slightly declines again but is still elevated after 24 h of stimulation (Fig. 1A). The effect of IL-1β occurs in a concentration-

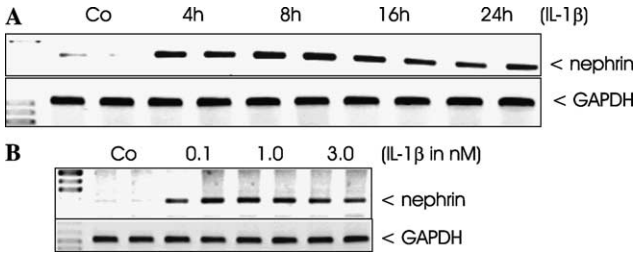

Fig. 1. IL-1β upregulates nephrin mRNA in a time- (A) and dose- (B) dependent manner in A293 cells. Confluent A293 cells were stimulated with either vehicle (Co) or 1 nM of IL-1β for the indicated time periods (A), or for 8 h with the indicated concentrations of IL-1β (B). Thereafter RNA was extracted and used for reverse transcriptase-PCR as described in Materials and methods. Samples were separated on a 1.5% agarose gel. Nephrin and GAPDH were run at a size of 349 and 470 bp, respectively. Data are representative of three independent experiments giving similar results.

dependent manner with a maximal effect at 1 nM of IL-1β (Fig. 1B). No further increase is obtained when increasing the concentration to 3 nM (Fig. 1B).

To see whether the upregulation of nephrin mRNA is followed by increased protein levels, stimulated cell lysates were taken for immunoprecipitation of nephrin with a specific anti-nephrin antibody. Samples were then subjected to Western blot analyses. As seen in Fig. 2A, nephrin, which runs as a double band at approximately 185 kDa, is constitutively expressed, but is markedly enhanced after 8 and 24 h of IL-1β stimulation. Compared to the mRNA induction, the protein increased in a delayed fashion and remained elevated at 24 h. Furthermore, increased de novo synthesis of nephrin was detected in [^{35}S]methionine/cysteine-metabolically labeled cells. As seen in Fig. 2B, unstimulated cells do not synthesize nephrin within 24 h as detected by immunoprecipitation of nephrin from ^{35}S-labeled cell lysates, suggesting a very low basal turnover. However, 24 h of IL-1β treatment leads to a marked de novo synthesis of nephrin (Fig. 2B).

In a next step, an attempt was taken to unravel the mechanism by which IL-1β induced nephrin expression. Since a previous study showed that the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) is also able to upregulate nephrin expression [12], thus suggesting an involvement of protein kinase C (PKC), we employed three inhibitors of PKC which possess varying selectivities for this class of at least 11 isoenzymes. Stimulation of cells with IL-1β in the presence of CGP41251 [17,18], which selectively inhibits the Ca^{2+}-dependent isoenzymes, or Ro 318220 [19], which inhibits all Ca^{2+}-dependent and Ca^{2+}-independent isoenzymes, does not lead to a reduction of nephrin mRNA expression (Fig. 3A). Furthermore, rottlerin was used, which was once proposed to be a selective inhibitor of PKC-δ but also of the calmodulin-dependent protein kinase III [20] but meanwhile has been proven to inhibit other protein kinases like the p38-regulated/activated protein kinase (PRAK) and the MAPK-activated protein kinase-2 (MAPKAPK-2) far more potently with IC_{50} values of 1.9 and 54 μM, respectively [21]. In contrast to the other PKC inhibitors, rottlerin dose-dependently reduced IL-1β-induced nephrin expression with a maximal effect at 10 μM (Fig. 3B).

Since gene transcription often involves either the classical mitogen-activated protein kinase (MAPK) or the stress-activated protein kinase cascades [22,23], the involvement of these cascades in nephrin expression was studied by using specific inhibitors. U0126 is a selective inhibitor of the MAPK kinase (MEK) [24] and therefore blocks the classical MAPK cascade. Surprisingly, in the presence of U0126 the IL-1β-induced increase of nephrin mRNA expression was not inhibited but rather amplified (Fig. 4A). A similar moderate amplification is found with the p38-MAPK inhibitor SB203580 [25] (Fig. 4A). Furthermore, also inhibition of the nuclear factor kappa B (NF-κB) pathway by Bay 117082 [26], which blocks the upstream IKK (inhibitor of κB protein kinase), is ineffective (Fig. 4A). In addition, cyclooxygenase (COX) inhibitors were tested because IL-1β can potently upregulate COX isoenzymes in various cell types and thereby generate prostaglandins which participate in various gene transcription events [27].

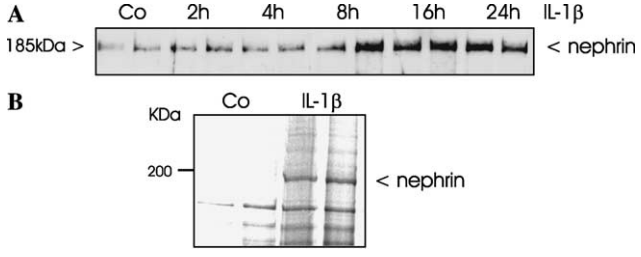

Fig. 2. IL-1β induces nephrin protein expression and stimulates de novo synthesis in [^{35}S]methionine/cysteine-labeled A293 cells. (A) Confluent A293 cells were stimulated with either vehicle (Co) or with 1 nM of IL-1β for the indicated time periods. Thereafter cell extracts were taken to immunoprecipitate nephrin with an anti-nephrin antibody at a dilution of 1:100. Samples were separated by SDS–PAGE, transferred to nitrocellulose, and subjected to Western blot analysis using the same antibody at 1:100. Bands were visualized by the ECL method. The data are representative of two independent experiments. (B) Confluent A293 cells were stimulated in methionine/cysteine-free medium with either vehicle (Co) or 1 nM of IL-1β for 24 h. During the last 4 h of stimulation, [^{35}S]methionine/[^{35}S]cysteine were added as described in Materials and methods. Thereafter cell extracts containing equal amounts of radioactivity were taken to immunoprecipitate nephrin with an anti-nephrin antibody at a dilution of 1:100. Immunocomplexes were separated by SDS–PAGE and analyzed on an Imaging system (Fuji). The data are representative of three independent experiments giving similar results.

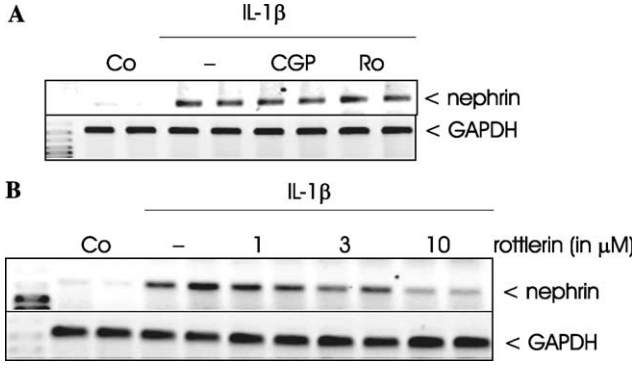

Fig. 3. Effects of various PKC inhibitors on IL-1β-induced nephrin mRNA expression in A293 cells. (A) Confluent A293 cells were stimulated for 8 h with either vehicle (Co) or IL-1β (1 nM) in the absence (–) or presence of CGP 41251 (100 nM; CGP) or Ro 31-8220 (100 nM; Ro). (B) Cells were stimulated for 8 h with either vehicle (Co) or IL-1β (1 nM) in the absence (–) or presence of indicated concentrations of rottlerin (in micromolar). Thereafter RNA was extracted and used for reverse transcriptase-PCR as described in Materials and methods. Samples were separated on a 1.5% agarose gel. Nephrin and GAPDH were run at a size of 349 and 470 bp, respectively. Data are representative of three independent experiments giving similar results.

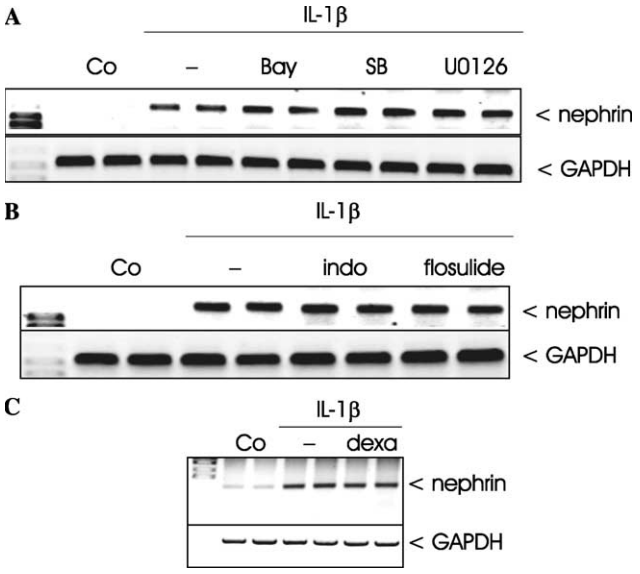

Fig. 4. Effects of diverse protein kinase and cyclooxygenase inhibitors on IL-1β-induced nephrin mRNA expression in A293 cells. (A) Confluent A293 cells were stimulated for 8 h with either vehicle (Co) or IL-1β (1 nM) in the absence (–) or presence of the IKK inhibitor Bay 117085 (100 nM; Bay), the p38-MAPK inhibitor SB 203580 (10 μM; SB) or the MEK inhibitor U0126 (20 μM; U0126). (B) Cells were stimulated for 8 h with either vehicle (Co) or IL-1β (1 nM) in the absence (–) or presence of the unselective COX inhibitor indomethacin (indo; 10 μM) or the highly selective COX-2 inhibitors flosulide (10 μM) or dexamethasone (dexa; 100 nM) (C). Thereafter RNA was extracted and used for reverse transcriptase-PCR as described in Materials and methods. Samples were separated on a 1.5% agarose gel. Nephrin and GAPDH were run at a size of 349 and 470 bp, respectively. Data are representative of 2–3 independent experiments giving similar results.

However, neither the unselective COX inhibitor indomethacin (Fig. 4B) nor the highly selective COX-2 inhibitors flosulide (Fig. 4B) and NS 398 (data not shown), nor the potent anti-inflammatory glucocorticoid dexamethasone (Fig. 4C) have any effect on nephrin expression.

Since IL-1β is also able to upregulate the inducible nitric oxide synthase (iNOS) in many renal cell types [28] and can potently affect gene expression [29,30], we further tested whether the increased nephrin expression is mediated by NO. For this, cells were stimulated with IL-1β in the presence of an NO-synthase inhibitor, N^G-monomethyl-L-arginine (LNMMA) [31], to block endogenously generated NO. However, NO-synthase inhibition does not reduce the IL-1β effect, thus suggesting that IL-1β acts independently of NO to upregulate nephrin expression (Fig. 5). This is consistent with the finding that exogenously added NO has no upregulating effect on nephrin mRNA or protein (data not shown).

Additionally, we also tested another proinflammatory cytokine, TNFα. As seen in Fig. 6, TNFα exerted a time-dependent (Fig. 6A) and concentration-dependent (Fig. 6B) induction of nephrin expression in A293 cells.

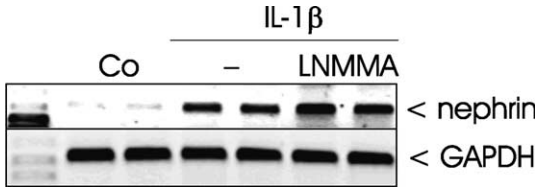

Fig. 5. Effect of nitric oxide on IL-1β-induced nephrin expression in A293 cells. Confluent A293 cells were stimulated for 24 h with IL-1β (1 nM) in the absence or presence of the NO-synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA; 3 mM). Thereafter RNA was extracted and used for reverse transcriptase-PCR as described in Materials and methods. Samples were separated on a 1.5% agarose gel. Nephrin and GAPDH were run at a size of 349 and 470 bp, respectively. Data are representative of two out of three independent experiments giving similar results.

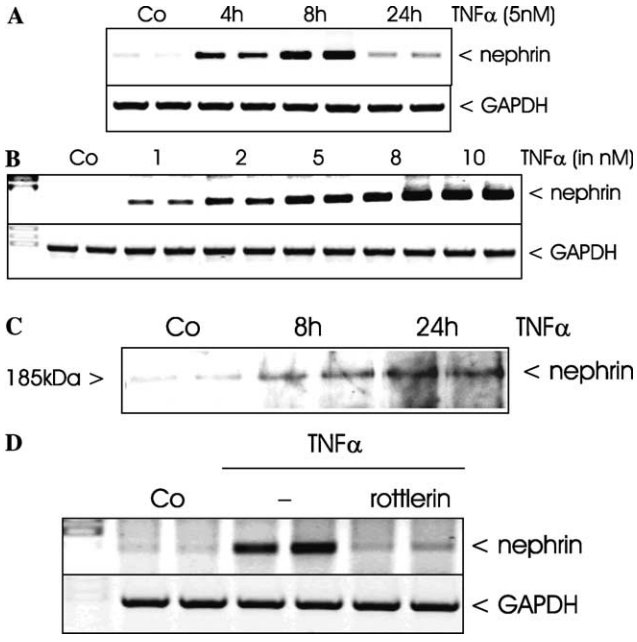

Fig. 6. Effect of TNFα on nephrin expression in A293 cells. Confluent A293 cells were stimulated for the indicated time periods with 5 nM of TNFα (A) or for 8 h with the indicated concentrations of TNFα (B), or for 8 h with TNFα (5 nM) in the presence of rottlerin (10 μM) (D). Thereafter RNA was extracted and used for reverse transcriptase-PCR. PCR products were separated on a 1.5% agarose gel. Nephrin and GAPDH were run at a size of 349 and 470 bp, respectively. (C) Cells were stimulated for 8 and 24 h with TNFα (5 nM) and protein lysates were taken for nephrin immunoprecipitation using an anti-nephrin antibody at a dilution of 1:100 as described. Immunocomplexes were subjected to SDS–PAGE to separate proteins and nephrin was analyzed by Western blot analysis. Data are representative of two independent experiments giving similar results.

In parallel, also nephrin protein levels increased after TNFα stimulation (Fig. 6C). It is worth noting that after 24 h the nephrin protein was still maximally elevated (Fig. 6C) when compared to the mRNA (Fig. 6A), suggesting that the protein is rather stable. Comparable to the IL-1β effect, also TNFα-induced nephrin expression is abolished in the presence of rottlerin (Fig. 6D).

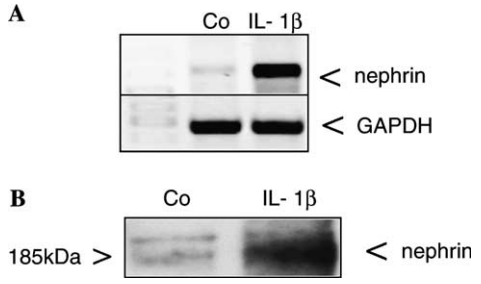

Fig. 7. Effect of IL-1β on nephrin expression in primary cultures of human podocytes. Primary human podocytes grown to confluency were stimulated for 24 h with IL-1β (1 nM). Thereafter, either RNA was extracted (A) and used for reverse transcriptase-PCR as described in Materials and methods, or protein lysates (B) were taken to immunoprecipitate nephrin with an anti-nephrin antibody at a dilution of 1:100 as described. PCR products (in A) were separated on a 1.5% agarose gel. Nephrin and GAPDH were run at a size of 349 and 470 bp, respectively. Immunocomplexes (in B) were subjected to SDS–PAGE to separate proteins and nephrin was analyzed by Western blot analysis. The data are representative of three independent experiments giving similar results.

Finally, to see whether this IL-1β-induced upregulation of nephrin also occurs in the physiologically relevant cell type, the podocyte, primary cultures of human podocytes were investigated. As seen in Fig. 7, IL-1β also causes an increase of nephrin mRNA steady-state levels (Fig. 7A) as well as protein expression (Fig. 7B) in primary podocytes.

Discussion

Since nephrin has turned out to be a key factor constituting the normal kidney filtration barrier, and deficiency or expressional downregulation [6,8,9] of nephrin is connected with dramatically increased proteinuria which, if untreated, leads to death, it is of considerable interest to understand the regulation of nephrin expression and function. A major drawback in molecular analysis of nephrin expression is the fact that it has proven very difficult to cultivate primary podocytes. For this reason, the A293 kidney epithelial cell line has been employed, which is originally derived from HEK293 cells and has previously been shown to naturally express nephrin [12].

In this study we show for the first time that the proinflammatory cytokines IL-1β and TNFα upregulate nephrin mRNA and protein expression in A293 cells and, importantly, also in primary human podocytes.

The mechanism of cytokine-induced nephrin upregulation seems to be particularly interesting because the classical MARK, the p38-MAPK as well as the NF-κB signaling cascades, which are involved in most cases of gene transcription triggered by inflammatory cytokines. In the case of MAPK and p38-MAPK, it rather seems as if these two kinases exert a negative feedback regulation and their inhibition in turn results in a further increase of nephrin expression.

The IL-1β-induced effect on nephrin expression also occurs independently of PKA and PKC activation. Previously, Wang et al. [12] reported that the phorbol ester TPA, by activating PKC, enhances nephrin expression in A293 cells. However, two potent inhibitors of PKC isoenzymes, CGP41251 and Ro 318220, which block all PKC isoenzymes except for the atypical PKC-ζ isoenzyme, as well as the PKA inhibitor H-89 (data not shown) had no effect on IL-1β-induced nephrin expression. A third PKC inhibitor employed in this study was rottlerin, which was claimed to specifically inhibit PKC-δ [20]. Rottlerin was the only substance identified so far that dose-dependently blocks IL-1β-triggered nephrin upregulation. However, a comprehensive and careful recent study [21] shed new light on the specificity of rottlerin and discarded it as a PKC-δ inhibitor because in vitro PKC-δ activity was hardly inhibited, and rather other protein kinase activities, most prominently PRAK and MAPKAPK-2, were far more susceptible to rottlerin treatment. Therefore, we have to conclude that rottlerin did not act as a PKC-δ inhibitor in our study but inhibits another yet to be defined protein kinase which is involved in nephrin upregulation.

Two additional mediators that have been excluded from triggering nephrin regulation are nitric oxide and prostaglandins. Neither an inhibitor of NO-synthases nor cyclooxygenase inhibitors affected nephrin expression. In this context, it is worth noting that human podocytes are one of the very few cell types that constitutively express COX-2 [32], and prostaglandins derived from COX-2 action would therefore represent attractive modulators of nephrin expression.

Recently, the promoters of human [33] and mouse [34] nephrin have been cloned. The existence of several putative transcription factor recognition sites has been found including those for GATA-1, GATA-2, AP4, Ets1, NFAT, AP2, C/EBP, SP1, and PPAR [33–35]. However, the functionality of these binding sites has not yet been addressed. Whether IL-1β- and TNFα-triggered signaling pathways modulate transcription factor binding to these putative binding sites is under present investigation. Although the current knowledge about transcription factors controlling nephrin gene expression is limited mainly to developmental aspects of podocytes, the modulation of nephrin expression by proinflammatory cytokines as reported here may be of utmost importance and constitute a cross-communication between immune cells and podocytes controlling podocyte functionality. The upregulation of nephrin expression initiated by IL-1β or TNF-α may have serious consequences for the stoichiometry of the proteins cooperating to form the slit diaphragm. This in turn, like nephrin downregulation or mutation, may cause a

functional destabilization of the slit diaphragm with foot process effacement and consequent proteinuria. In this context, it is worth mentioning that Aaltonen et al. [36] reported in the streptozotocin-induced diabetic nephropathy model in the rat and the non-obese diabetic mouse an upto twofold increase in nephrin mRNA expression. Further in vivo studies are essential to evaluate the relevance of our cell culture data for proteinuria in inflammatory kidney diseases.

Acknowledgments

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