NF-κB Mediated IL-6 Production by Renal Epithelial Cells Is Regulated by C-Jun NH₂-Terminal Kinase

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Tubular epithelial cells (TEC) play an important role in tubulointerstitial inflammation, a hallmark of most renal diseases, via production of cytokines and chemokines. In this study, the role of mitogen-activated protein kinases (MAPK) in regulation of the proinflammatory cytokine IL-6 in cultured human TEC in response to the leukocyte-derived factors IL-1, TNF-α, IL-17, and CD40L was investigated. IL-6 production induced by IL-1, TNF-α, and IL-17 was specifically inhibited by the c-jun NH₂-terminal kinase (JNK) inhibitor SP600125, but not by a selective inhibitor of p38 MAPK, and was moderately increased when the ERK1/2 pathway was inhibited. Also for CD40L stimulation, inhibition of JNK resulted in a pronounced inhibition of IL-6 production. Although stimulation of TEC induced activation of activator protein–1 (AP-1), the down-stream target of JNK, reporter assays demonstrated that mutation of the AP-1 binding site in the IL-6 promoter did not affect gene transcription. Furthermore, IL-1–induced transcriptional activation of the IL-6 promoter was repressed by SP600125 or by co-transfection of a dominant-negative expression plasmid of c-jun even in the absence of a functional AP-1 binding site. This suggests that IL-6 production by renal epithelial cells is regulated by JNK, via a mechanism, however, independent of the AP-1 binding site. The data rather suggest that the JNK pathway may interfere with other signaling pathways, involving NF-κB and possibly ERK.

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MAPK involved in IL-6 production by renal epithelial cells. However, we demonstrate that regulation of IL-6 gene transcription by JNK is independent of the AP-1-binding site, but rather involves interference with other signaling pathways such as NF-κB and ERK.

**Materials and Methods**

**Cell Culture**

The renal proximal tubular epithelial cell-line HK-2 was kindly provided by M.P. Ryan, University College, Dublin, Ireland (18). Cells were cultured in DMEM/HAMF12 (Bio-Whittaker, Walkersville, MD) supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), hydrocortisone (36 ng/ml), triiodothyronine (40 pg/ml), EGF (10 ng/ml, all from Sigma Chemical Co, St. Louis, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Paisley, UK). Primary human proximal TEC were isolated from kidneys not suitable for transplantation and from pretransplant biopsy samples as described previously (19).

**Western Blot Analysis**

Whole cell extracts were prepared as described previously (17). Cell extracts (10 μg) were separated with SDS-PAGE, transferred to membranes, and blocked in Tris-buffered saline/0.1% Tween-20 containing 5% nonfat dry milk. Primary antibodies used for Western blot were rabbit anti-phospho-p38 MAPK, total p38 MAPK, phospho-SAPK/JNK, total SAPK/JNK, phospho-p44/p42 MAPK, and total p44/p42 MAPK antibody (Cell Signaling Technology, Beverly, MA) diluted in Tris-buffered saline/Tween. Horseradish peroxidase-conjugated secondary antibodies were obtained from DAKO (Glostrup, Denmark). Blots were developed with an enhanced chemiluminescence substrate (Pierce, Rockford, IL) and detected with Hyperfilm ECL (Amersham Blotech). Western blots were scanned and analyzed using the Quantity One analysis program (Biorad, Hercules, CA).

**IL-6 and IL-8 Production**

Cells were stimulated for 48 h with recombinant human IL-1α, IL-17 (R&D Systems), TNF-α (Invitrogen), or irradiated CD40L-transfected L-cells (20) at a 1:1 ratio. For inhibition experiments, cells were preincubated for 2 h with PD98059, U0126, SB203580, or SP600125 (all from Calbiochem). Cytokines or L-cells were added in the continuous presence of these inhibitors. Cell viability was checked with trypan blue staining to confirm that the inhibitors were nontoxic at the concentrations used. IL-6 and IL-8 concentration in culture supernatants was measured by specific ELISA as described previously (19).

**Plasmids**

The IL-6 promoter containing plasmids p1168hu.IL6P-luc+, p50hu.IL6P-luc+, (pIL6x8B)50hu.IL6P-luc+, and the point mutants for AP-1 and NF-κB have been described previously (21). The synthetic reporter construct pAP-1-Luc contains multimerized responsive elements in front of a minimal promoter (21). The dominant-negative plasmid IκB-aDNA(71-317) (22) was generously provided by C. Scheidegurt (Max-Delbrück-Center for Molecular Medicine, Berlin, Germany). Plasmid pCMV-TAM67 (23) was kindly provided by E. Vellenga (University of Groningen, The Netherlands). Dominant-negative TRAF6 (24) was provided by Tularik (San Francisco, CA).

**Transient Transfection and Luciferase Assay**

Before transfection, cells were plated at a density of 0.5 × 10⁶ cells per well in six-well plates (Costar, Corning, NY). On the day of transfection, culture medium was switched to serum-free DMEM (Invitrogen). Plasmid pCMV-TAM67 (23) was kindly provided by E. Vellenga (University of Groningen, The Netherlands). Dominant-negative TRAF6 (24) was provided by Tularik (San Francisco, CA).

**Figure 1.** MAPK phosphorylation in response to IL-1. (A) HK-2 cells were stimulated with 5 ng/ml IL-1α for 0 to 20 min. Cell lysates were size-fractionated by SDS-PAGE and immunoblotted with antibodies to phosphorylated or total mitogen-activated protein kinase (MAPK) as indicated. (B) Densitometry analysis of the experiment described in (A). Data are representative of three independent experiments.

**Statistical Analyses**

Cytokine production is presented as mean concentration ± SEM from at least 5 independent experiments or as mean ± SD from representative experiments. Cytokine production was analyzed with a paired t test. Results were considered significant if P < 0.05.

**Results**

**MAPK Activation in Renal Epithelial Cells in Response to IL-1**

Activation of MAPK was investigated by Western blot analysis using antibodies specific for phosphorylated p38, JNK/
SAPK, and ERK1/2 (p42/p44) MAPK. Untreated HK-2 cells contained low levels of phosphorylated p38 and JNK. IL-1 stimulation induced a time-dependent increase in p38 and JNK phosphorylation (Figure 1). No difference was observed in total p38 and JNK levels, confirming equal protein loading. In contrast to p38 and JNK, ERK1/2 phosphorylation was constitutively present in untreated HK-2 cells. Stimulation with IL-1 resulted in a decrease in ERK1/2 phosphorylation without alterations in total ERK1/2 levels.

Role for MAP Kinases in IL-6 Production by Tubular Epithelial Cells

Stimulation of HK-2 cells with IL-1, IL-17, and TNF-α for 48 h resulted in increased IL-6 production of 12.5-, 6.3-, and 7.1-fold, respectively. Although we found that p38 is activated on stimulation, we did not observe an effect of SB203580 (10 μmol) on cytokine-induced IL-6 protein expression in renal epithelial cells (Figure 2A). Similarly, no inhibition of IL-6 production was found in the presence of PD98059 (50 μmol), which inhibits the activity of the MAP kinase MEK1/2, the upstream kinase of the ERK1/2 pathway (Figure 2B). Interestingly, with PD98059 we observed a consistent although nonsignificant increase in basal IL-6 production. Considering this, we investigated the effect of an alternative and more potent MEK1/2 inhibitor, U0126, on IL-6 production by TEC. In untreated cells and cytokine-treated cells, we observed a significant increase in IL-6 expression in the presence of 10 μmol U0126 (Figure 2C).

In parallel experiments, inhibition of the JNK pathway by SP600125 (10 μmol) strongly inhibited IL-6 production by all stimuli (Figure 2D). Neither of the inhibitors had an effect on cell viability as measured by trypan blue staining (data not shown).

Inhibition of IL-1–induced IL-6 production by SP600125 was dose-dependent, with 50% inhibition at 1.3 μmol (Figure 3A). We also investigated the regulation of IL-6 production in primary renal epithelial cells cultured from normal human kidney. Inhibition of IL-1–induced IL-6 production by SP600125 was dose-dependent, with a similar ID₅₀ compared with HK-2 (Figure 3B). Also in primary renal epithelial cells, IL-6 production induced by IL-17 and TNF-α was strongly inhibited by SP600125. Comparable to HK-2 cells, we observed no inhibition of IL-6 production with SB203580 in primary cells, whereas PD98059 induced a small increase in IL-6 levels (data not shown). To determine whether the effect of SP600125 was exclusive for IL-6, we measured production of IL-8 by HK-2 cells. As shown for IL-6, IL-1–induced IL-8 production was strongly inhibited (Figure 3C). No effect of PD98059 or SB203580 on IL-8 production was observed (data not shown).

Another important inducer of TEC activation is CD40L (19).

Figure 2. Effect of MAPK inhibitors on IL-6 production. HK-2 monolayers were pretreated for 2 h with selective MAPK inhibitors, SB203580 10 μmol (A), PD98059 50 μmol (B), U0126 10 μmol (C), and SP600125 10 μmol (D) before stimulation with IL-1 (5 ng/ml), IL-17 (50 ng/ml), or TNF-α (100 ng/ml) for 48 h. IL-6 production was measured in culture supernatants by specific ELISA. No effect was observed with the solvent DMSO (data not shown). Data are presented as mean ± SEM from 3 to 5 different experiments. * P < 0.05. ** P < 0.01. *** P < 0.005.
In contrast to the soluble factors, CD40L-induced IL-6 production was partially inhibited by both PD98059 and SB203580. Of the used inhibitors, SP600125 was most potent in inhibiting CD40L-induced IL-6 production (Figure 4). The increase in IL-6 production after CD40L stimulation (6.7-fold) was comparable to IL-17 and TNF-α, suggesting that the observed inhibition by PD98059 and SB203580 was not caused by a lower level of IL-6 induction. Similar results were obtained with primary renal epithelial cells (data not shown).

JNK Regulates IL-6 Promoter Activity

Two approaches were taken to confirm the specificity of SP600125. First, we determined the effect of SP600125 on MAPK phosphorylation. We focused on the stimulation with IL-1 because this cytokine was the most potent inducer of IL-6 production. IL-1–induced phosphorylation of p38 was not changed in cells that were incubated with SP600125, whereas JNK phosphorylation was completely inhibited (Figure 5A). Interestingly, the IL-1–induced decrease in ERK1/2 phosphorylation (Figure 1) was prevented by incubation with SP600125. Total MAPK levels were not altered after SP600125 treatment (Figure 5A).

One of the substrates of JNK is c-jun, an important subunit of the AP-1 transcription factor complex (25). Therefore, we transfected HK-2 cells with an AP-1 reporter construct containing multiple AP-1 binding sites in front of a luciferase reporter gene and determined the effect of SP600125. IL-1 induced a small but consistent increase in AP-1 reporter gene activity compared with untreated cells (Figure 5B). SP600125, but not PD98059 or SB203580, was able to inhibit the activity of the AP-1 construct, demonstrating the specificity of SP600125 (Figure 5C).

Knowing this, regulation of IL-6 gene expression was investigated in more detail by transient transfection of HK-2 cells with a reporter construct consisting of the wild-type IL-6 promoter in front of a luciferase gene (p1168hu.IL6P-luc) (21). In control HK-2 cells, a basal level of constitutive reporter gene activity was observed (Figure 6), consistent with the baseline IL-6 production found in supernatants. Stimulation with IL-1 resulted in increased transcriptional activation of the reporter gene of approximately two-fold, which was partially, although not significantly, inhibited by SB203580 (10 μmol) and was slightly increased by PD98059 (50 μmol). In agreement with the
cytokine data, incubation with SP600125 strongly inhibited IL-1–induced luciferase activity.

AP-1 Binding Site Is Not Required for IL-6 Gene Transcription

We have previously demonstrated that the transcription factor NF-κB is involved in IL-6 production by TEC (17). Therefore, we wanted to determine the relative contribution of AP-1 and NF-κB to transcription of the IL-6 gene with IL-6 promoter constructs in which specific binding sites were mutated (Figure 7A). As expected, mutation of the NF-κB binding site completely abrogated the ability of IL-1 to activate the reporter gene. However, although AP-1 is an important target of JNK and AP-1 reporter gene activity was induced by IL-1 stimulation, mutation of the AP-1-binding site in the IL-6 promoter did not change the ability of IL-1 to induce reporter gene activity as compared with the wild-type IL-6 promoter (Figure 7B).

Next, HK-2 cells were transfected with the IL-6 promoter construct in which the AP-1 site was mutated. The NF-κB-binding site in this construct was still functional. Accordingly, IL-1–induced reporter gene activity was strongly inhibited by the NF-κB inhibitor caffeic acid phenethyl ester (CAPE; 10 μg/ml). Surprisingly, we found that SP600125 was still able to inhibit luciferase activity to a similar extent as the wild-type IL-6 construct (63% versus 65.6% inhibition; Figure 6). Moreover, IL-1–induced reporter gene activity was inhibited by co-transfection of N-terminally truncated dominant-negative expression plasmid for c-Jun (23), the downstream target of JNK, but not by the empty vector (Figure 7C).

Cross-Talk between JNK and NF-κB Pathways

For TNF-α, it has been shown that NF-κB alone is sufficient for transcriptional activation of the IL-6 promoter (26). We found that this was also the case for IL-1, because transfection of HK-2 cells with a construct containing multiple NF-κB binding sites in front of the nonresponsive construct p50hu.IL6P-luc+ resulted in more than eight-fold induction of reporter gene activity (Figure 8A). IL-1–induced luciferase activity was completely inhibited by CAPE (Figure 8B). However, we observed an almost equal block of NF-κB reporter gene activity with SP600125, suggesting there might be an interaction of the JNK and NF-κB pathways.

To exclude a direct effect of SP600125 on NF-κB activation, we studied the effect of dominant-negative expression plasmids on constitutive and IL-1–induced activation of the NF-κB reporter construct. It is important to note that we examined the effects of these inhibitory constructs on the endogenous JNK and NF-κB pathways, rather than on transfected exogenous proteins. A degradation-resistant form of the endogenous inhibitor of NF-κB, dominant-negative IκB-α (22), served as a positive control for specific inhibition of NF-κB and was found...
to inhibit both constitutive and IL-1–induced NF-κB activation (Figure 8, C and D). Next, we used dominant-negative TRAF6, a member of the TNF receptor-associated factor (TRAF) family, which is involved in IL-1 signaling and which is known to be an upstream activator of JNK and NF-κB (27,28). Co-transfection with dominant-negative TRAF6 had little effect on constitutive NF-κB activity but strongly inhibited IL-1–induced reporter gene activity, consistent with its role in IL-1 signaling (Figure 8, C and D). More importantly, IL-1–induced NF-κB activity was blocked by co-transfection of dominant-negative c-jun, indicating that c-jun might be a point of convergence between JNK and NF-κB pathways (Figure 8D).

Discussion

Using a novel selective JNK inhibitor, SP600125 (29), we show that blockade of JNK strongly inhibits IL-6 production by renal epithelial cells that were activated with IL-1, TNF-α, or IL-17. An important downstream effector molecule of JNK is the transcription factor AP-1. However, two observations demonstrated that the AP-1 binding site of the IL-6 promoter is not required for IL-6 gene activation. First, mutation of the AP-1–responsive element in the promoter region of the IL-6 gene did not diminish IL-6 reporter gene induction. Second, transcriptional activation of the IL-6 promotors was repressed by SP600125 or by co-transfection with dominant-negative c-jun even in the absence of a functional AP-1 binding site. These data suggest that the JNK pathway regulates IL-6 gene induction via a mechanism that involves c-jun but is independent of the AP-1 binding site.

Interestingly, blockade of ERK1/2 activation with PD98059 and U0126 showed an increase in IL-6 gene expression, as observed in another study (30), suggesting that ERK1/2 may down-modulate IL-6 production. Furthermore, IL-1 stimulation of TEC decreased ERK1/2 phosphorylation, which was reversed by SP600125, suggesting that the regulation of IL-6 production by JNK might be in part mediated via inhibition of ERK1/2 phosphorylation. However, ERK1/2-inhibition by IL-1 was not complete, as shown by the additional potentiating effect of U0126 on IL-1–induced IL-6 production. Negative cross-talk between the JNK and ERK pathways has been demonstrated previously, although the functional consequences were not investigated (31). Decreased ERK phosphorylation was associated with increased tubular apoptosis in rats with persistent proteinuria (32), indicating that inhibition of ERK activation may contribute to renal failure. However, albumin-induced MCP-1 production in mouse TEC was in part mediated by ERK (33). Thus, the ERK pathway may play a dual role in renal inflammation.

In addition to JNK, our study demonstrates a central role for NF-κB in the induction of IL-6 gene expression. This might be
expected given the observation that agents that activate JNK also activate NF-κB. However, we demonstrate for the first time to our knowledge that NF-κB reporter gene activity was strongly inhibited if JNK activity was blocked via SP600125 or dominant-negative c-jun, suggesting that JNK is directly involved in gene induction by NF-κB, without need for AP-1 or other transcription factor binding sites. Cross-talk of MAP kinase pathways with the NF-κB pathway has been shown in other studies. Interaction of p38 with NF-κB is well established and has been shown to play a role in IL-6 gene expression (26,34–36) but was not observed in our study. Furthermore, it has been shown that the upstream activator of JNK, MEKK-1, directly activates IκB kinase (37). In addition, JNK has been shown to physically interact with c-rel, a protein of the NF-κB family (38). In line with another study (23), our results thus indicate that c-jun might be a novel point of convergence between JNK and NF-κB pathways. Although additional experiments have to be performed to investigate the mechanism of interaction, synergy between c-jun and NF-κB might be mediated via direct physical interaction of c-jun and the p65 subunit of NF-κB (39). Furthermore, we cannot exclude a direct effect of JNK on the phosphorylation status of NF-κB. Alternatively, recruitment of c-jun to the IL-6 promoter might be involved in chromatin modification to make the promoter accessible for NF-κB (40). Based on our observations, it will be of interest to investigate the requirement of the AP-1 binding site in this process.

The strong inhibitory effect of SP600125 on the production of IL-8 in combination with the role of NF-κB we showed previously suggests that interaction of JNK and NF-κB might also be involved in regulation of IL-8 production (17). Furthermore, JNK might be involved in cytokine and chemokine production by TEC after exposure to albumin, another important pathogenetic mediator in the progression of tubulointerstitial damage. A role for NF-κB in this process has previously been demonstrated (41,42). Another study with proximal tubular cells has demonstrated that CD40L-induced IL-8 and MCP-1 production could be blocked by inhibition of ERK1/2 and p38 (43). Although the authors show phosphorylation of JNK, the contribution of JNK to chemokine production was not addressed. We found that although all three MAP kinase pathways appeared to be involved in CD40L-mediated IL-6 production, SP600125 was the most potent inhibitor. In previous studies, cytokine production by renal epithelial cells was synergistically enhanced by a combination of CD40L with cytokines like IL-1 and IL-17 (5,6). The mechanism underlying this synergistic effect might involve the induction of multiple MAPK pathways by CD40L.

Targeting JNK as treatment in human inflammatory diseases has increasingly gained attention (44). Our study and others (45,46) demonstrate an important role for JNK in TEC activation. Given the importance of production of inflammatory mediators by renal tubular epithelial cells in renal inflammation, selective inhibition of JNK might be a powerful new tool for therapeutic intervention in the progression of renal disease. In conclusion, IL-6 gene expression by renal epithelial cells is predominantly regulated by JNK. Although AP-1 is a common mediator of cytokine gene expression and is an important
down-stream target of the JNK pathway, the AP-1-binding site is not required for activation of the IL-6 promoter. Instead, our data suggest that the JNK pathway may interfere with other signaling pathways, such as NF-κB and possibly ERK, to regulate IL-6 production.

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