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Role of MAP kinase pathways in mediating IL-6 production in human primary mesangial and proximal tubular cells

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Role of MAP kinase pathways in mediating IL-6 production in human primary mesangial and proximal tubular cells.

Background. Both interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) are pleiotropic cytokines that have been implicated in the development of glomerular and tubular injury in various forms of immune-mediated renal disease, including glomerulonephritis. Although TNF-α has been shown to stimulate IL-6 production in renal cells in culture, the signaling mechanisms that regulate IL-6 production are not fully understood. The aim of this study was to examine the role of the p38 and extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathways in regulating TNF-α–mediated IL-6 production from both primary human mesangial cells (HMCs) and human proximal tubular (HPT) cells.

Methods. Primary mesangial and proximal tubular cells were prepared from nephrectomized human kidney tissue. Cells were treated for 24 hours with TNF-α in the presence and absence of the specific p38 and ERK1,2 MAPK inhibitors SB203580 and PD98059, respectively, either alone or in combination. IL-6 levels in the cell culture media were measured by enzyme-linked immunosorbent assay. MAPK activation was demonstrated by immunoblot for the active kinase (tyrosine/threonine phosphorylated) in whole cell extracts using phospho-specific antibodies. p38 MAPK activity in HPT cells was measured using an *in vitro* immunokinase assay using ATF2 as the substrate.

Results. TNF-α (0.1 to 100 ng/ml) stimulated a dose-dependent increase in IL-6 production in both renal cell types. The activation of the p38 and the ERK1,2 MAPKs occurred following TNF-α stimulation. The role of these activations in IL-6 production was confirmed by the ability of both inhibitors SB203580 (1 to 30 μM) and PD98059 (0.01 to 10 μM) to inhibit basal and TNF-α–stimulated IL-6 production in both cell types. The addition of both inhibitors in combination caused greater decreases in IL-6 production compared with either inhibitor alone. Pretreatment with SB203580 (10 μM) had no effect on basal or TNF-α–stimulated phosphorylation of p38 MAPK but completely abolished TNF-α–stimulated p38 MAPK activity. PD98059 decreased both basal and TNF-α–stimulated phosphorylation of ERK1,2.

Conclusions. This study provides evidence that both the p38 and ERK MAPK pathways are important for the regulation of the production of IL-6 from the proximal tubular and glomerular mesangial regions of the nephron. In response to TNF-α, the activation of both pathways leads to IL-6 production. These findings could aid in an understanding of the cellular mechanisms that regulate IL-6 production and could provide insights into possible pharmacological strategies in inflammatory renal disease.

Key words: interleukin-6, MAPK, inflammatory renal disease, TNF-α, cytokines, glomerulonephritis.

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Interleukin-6 (IL-6) is a pleiotropic cytokine produced by a wide variety of cell types in response to many different stimuli, including IL-1 and tumor necrosis factor-α (TNF-α) [1]. Many of its physiological and pathophysiological functions have been well characterized and include the regulation of immune and inflammatory responses, acute-phase protein production, bone metabolism, and hematopoiesis [2–4]. Within the kidney, elevated levels of both TNF-α and IL-6 have been demonstrated in both the resident and infiltrating cells in various forms of glomerulonephritis and tubulointerstitial nephritis, and indeed have been suggested to contribute to the pathogenesis or progression of the disease [5, 6]. An increased production of IL-6 from mesangial and proximal tubular epithelial cells has been shown *in vitro* in response to many diverse stimuli, including lipopolysaccharide (LPS), TNF-α, and IL-1 [7, 8]. The regulation of IL-6 production and its exact function within the inflamed kidney remain to be identified.

In other nonrenal cell types, IL-6 production has been shown to be regulated by the activation of mitogen-activated protein kinases (MAPKs) [9]. MAPKs constitute a group of important intracellular mediators of signal transduction from the cell surface to the nucleus in response to a wide variety of stimuli [10, 11]. Their activation involves dual phosphorylation of conserved threonine and tyrosine residues, allowing downstream

phosphorylation and activation of target proteins, including transcription factors that can lead to an alteration in gene expression. There are currently three well-characterized mammalian MAPK signaling cascades: (*a*) the classic p42/44 [extracellular signal-regulated kinase (ERK)] MAPK pathway, which is linked to the regulation of cell growth and differentiation; (*b*) the c-jun N-terminal kinases (JNKs) or stress-activated protein kinases (SAPKs); and (*c*) the p38-MAPKs. The latter two are involved in the cellular response to environmental stress [12, 13]. The elucidation of the biological function of the different pathways can be aided by the use of specific inhibitors. These include PD98059, an inhibitor of ERK1,2 MAPK (MEK1) activity [14], and SB203580 [15], an inhibitor of p38 MAPK activity. In renal disease, MAPKs have been shown to be activated in response to both growth factors and proinflammatory cytokines in a number of pathological conditions [16–18]. In a rat model of mesangioproliferative glomerulonephritis, the activation of the ERK MAPK pathway was proposed as a putative mechanism for the proliferative response observed in this disease [19]. *In vitro*, the activation of MAPKs has been demonstrated in mesangial cells following exposure to both IL-1 and reactive oxygen species [20].

The aims of this study were to directly compare the basal and TNF-α–stimulated IL-6 production in human primary mesangial cells (HMCs) and proximal tubular (HPT) cells. The role of MAPK cascades, particularly the p38 and ERK MAPK cascades, in mediating IL-6 production was investigated. We demonstrate that in both renal cell types, TNF-α induces IL-6 production through simultaneous activation of the p38 and ERK MAPK pathways.

METHODS

Materials

The whole cell p38 and ERK2 antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The phospho-specific p38 and phospho-specific ERK1,2 antibodies were obtained from New England BioLabs (Beverly, MA, USA). Activating transcription factor 2 (ATF2, 1–96) was from Santa Cruz. SB203580 and PD98059 were purchased from Calbiochem (La Jolla, CA, USA). For the enzyme-linked immunosorbent assay (ELISA), both the monoclonal mouse antihuman IL-6 primary antibody and the streptavidin-conjugated horseradish peroxidase (HRP) detection reagent were obtained from Genzyme (West Malling, Kent, UK), and the biotinylated polyclonal goat antihuman IL-6 secondary antibody came from R&D Systems (Abingdon, Oxon, UK). All other reageants were of the highest available purity from commercial sources.

Human proximal tubular cell culture

Human renal cortex was obtained from uninvolved sections of nephrectomized human kidneys removed because of renal cell carcinoma. Proximal tubular fragments were obtained as described previously [21, 22] using collagenase (type II; Sigma, Poole, Dorset, UK) digestion followed by percoll density gradient centrifugation at 20,000 *g* for 30 minutes at 4°C. Proximal tubular fragments were plated at a density of 5×10^4 fragments/ml. Epithelial colonies were evident after 48 hours of growth. The cells were maintained in specially formulated glucose-free 1:1 Dulbecco's modified Eagle's medium (DMEM)/HAMS F-12 (Promocell, Heidelberg, Germany) supplemented with 10% (vol/vol) fetal calf serum (FCS), 2 mM L-glutamine, 10 mM sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin. In addition, the amino acid D-valine was substituted for L-valine to inhibit the growth of fibroblasts that require L-valine for growth [23, 24]. Cells were characterized as proximal tubular in origin using morphological, immunological, and functional techniques [25, 26].

Human mesangial cell isolation

Human mesangial cells were isolated as previously described [27, 28]. In brief, glomeruli were isolated from minced renal cortex by differential sieving (mesh sizes 180, 106, and 75 μm). Glomeruli that collected on the lower two sieves were subjected to collagenase (type II; Sigma) digestion at 37°C for 20 minutes. They were then plated onto 1% gelatin-coated (Sigma) tissue culture flasks, and mesangial cells appeared as outgrowths from the digested glomeruli by day 10 and reached confluence by day 30. Cells were maintained in RPMI medium supplemented with 10% (vol/vol) FCS and penicillin-streptomycin (50 U/ml and 50 μg/ml, respectively) and were used for experiments between passages 3 and 10. The identity of the HMCs was confirmed morphologically by the display of the characteristic hillock structures in the culture and by the use of a series of cell markers [27, 28].

Experimental protocol

Confluent HMCs and HPTs were grown in either 24- or 6-multiwell tissue culture plates or 100 mm Petri dishes (Falcon, Cowley, Oxford, UK). Prior to drug treatment, cells were serum deprived for 24 hours to render them quiescent. For the determination of IL-6 production using ELISA, cells were treated for 24 hours with TNF-α (0.1 to 100 ng/ml). For MAPK inhibitor experiments, cells were pretreated for one hour with the specific MAPK inhibitors SB203580 and PD98059 either alone or in combination prior to cytokine exposure. For an analysis of MAPK activation and MAPK activity, cells were treated with 10 ng/ml TNF-α for 15 minutes at 37°C in the presence and absence of the MAPK inhibitors.

Assay for interleukin-6

The wells of a 96-well microtiter plate (NUNC Immunoplate, Hereford, UK) were coated with a monoclonal mouse antihuman IL-6 (1.25 μg/ml) capture antibody overnight at 4°C. Culture media from treated cells or IL-6 standard were then added to wells and incubated for one hour at 37°C followed by the addition of secondary antibody (25 ng/ml biotinylated polyclonal goat antihuman IL-6) also for one hour at 37°C. Following washing, detection reagent (streptavidin-conjugated HRP) was then added and incubated for 30 minutes at 37°C. Substrate solution (phosphate-citrate buffer containing 100 μg/ml 3,3′,5,5′-tetra-methylbenzidine) was added for 15 minutes at room temperature, and the reaction was halted by the addition of 1.8 M H_2SO_4. The absorbance was measured at 450 nm using a Dynatech MR5000 multiwell plate reader.

Western blot analysis

For Western blot analysis, whole cell extracts were prepared in 1 × Laemmli buffer separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels [29] and transferred to a nitrocellulose membrane using a semi-dry transfer system. The membranes were then blotted with the appropriate primary antibody: antiphospho ERK-1,2, which detects phosphorylated tyrosine 204 and threonine 202 of ERK1 and ERK2; antiphospho p38, which detects p38 when activated by dual phosphorylation at threonine 180 and tyrosine 182; or whole cell p38 and ERK2 antibodies. The primary antibodies were detected using an anti-rabbit HRP-conjugated antibody and bands visualized using enhanced chemiluminescence (Pierce, Rockford, IL, USA).

Immunoprecipitation

After treatment cells were washed three times with phosphate-buffered saline and lyzed in ice-cold Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM ethylenediaminetetraacetic acid (EDTA), 40 mM β-glycerophosphate, 200 μM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μM pepstatin, and 1% Triton X-100) for 25 minutes at 4°C. Insoluble material was removed by centrifugation at 12,000 × g for 15 minutes at 4°C. The microbicinchoninic acid assay (Pierce) was used to determine protein content using bovine serum albumin (BSA) as a standard. Cell extracts were matched for protein content and were precleared with 2 μl of preimmune serum preadsorbed to 50 μl of protein A-sepharose–coated beads (Pharmacia, Little Chalfont, Bucks, UK) for one hour at 4°C. The precleared supernatants were further incubated overnight with 13 μl of a polyclonal antibody recognizing p38 MAPK, which had been preadsorbed to protein A-sepharose for two hours. Immunocomplexes were then used to measure p38 MAPK activity.

p38 Mitogen-activated protein kinase activity assay

For measurement of p38 MAPK activity, the respective immunocomplexes were collected by centrifugation, washed four times with a washing buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EGTA, and 0.5% Triton X-100) and once with a kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 10 mM p-nitrophenylphosphate) and were resuspended in a final volume of 20 μl of kinase buffer containing 200 μg/ml GST-ATF-2, 100 μM ATP, and 10 μCi of $[[\gamma]\text{-}^{32}P]$ATP. The reaction was initiated by incubation at 30°C and was continued for 10 minutes. Thereafter, 20 μl of 2 × Laemmli sample buffer were added to terminate the reaction. Samples were then boiled for three minutes and subjected to 10% SDS-PAGE. The gels were stained in Coomassie Brilliant Blue, dried, and exposed for 24 to 48 hours to Amersham Hyperfilm MP at −70°C with intensifying screens. Kinase activity was visualized and quantitated by densitometry of the exposed autoradiographic film.

Data analysis

For analysis of the data, confidence intervals were constructed (95, 99, 99.9, and 99.99%), and an unpaired Student's t-test was used to test for statistical significance. Where appropriate, an analysis of variance followed by a Dunnett post-test was performed. Results are expressed as the mean ± SEM, and a value of $P < 0.05$ was deemed significant.

RESULTS

Cell characterization

Human proximal tubular epithelial cells formed monolayers that had the typical cobblestone appearance of epithelial cell monolayers (Fig. 1A). They stained positive for the epithelial intermediate filament protein cytokeratin but negative for the endothelial marker Factor VIII-related antigen (data not shown). The uptake of the nonmetabolizable glucose analogue ^{14}C-methyl-α-D-U-glucopyranoside was significantly reduced when sodium was replaced by N-methyl-glucamine and in the presence of the transport inhibitor 1 mM phlorizin (data not shown), indicating the presence of the proximal tubular Na/hexose cotransport system. These results would indicate that the cells are predominantly of proximal tubular origin [25, 26].

Glomerular mesangial cells appeared as a homogenous population after 30 days in culture. They displayed the typical stellate "hill and valley" morphology characteristic of mesangial cells in culture (Fig. 1B). As evi-

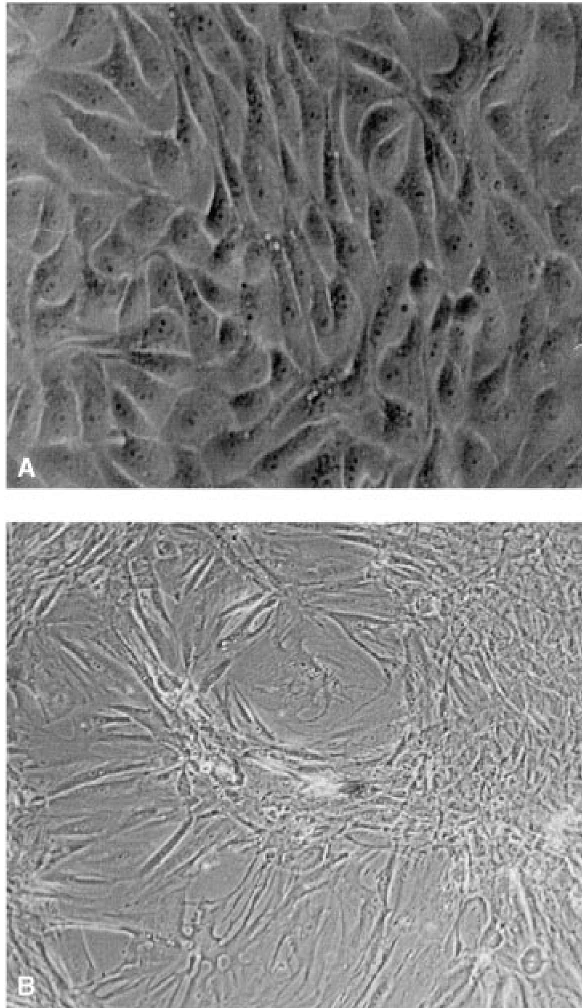

Fig. 1. Phase contrast microscopy of human proximal tubular (*A*) and human mesangial cells (*B*) in culture. Human proximal tubular epithelial cells displayed the typical epithelial cobblestone-like morphology (A). Human mesangial cells appeared as elongated cells in multilayers (B). Magnification (×450).

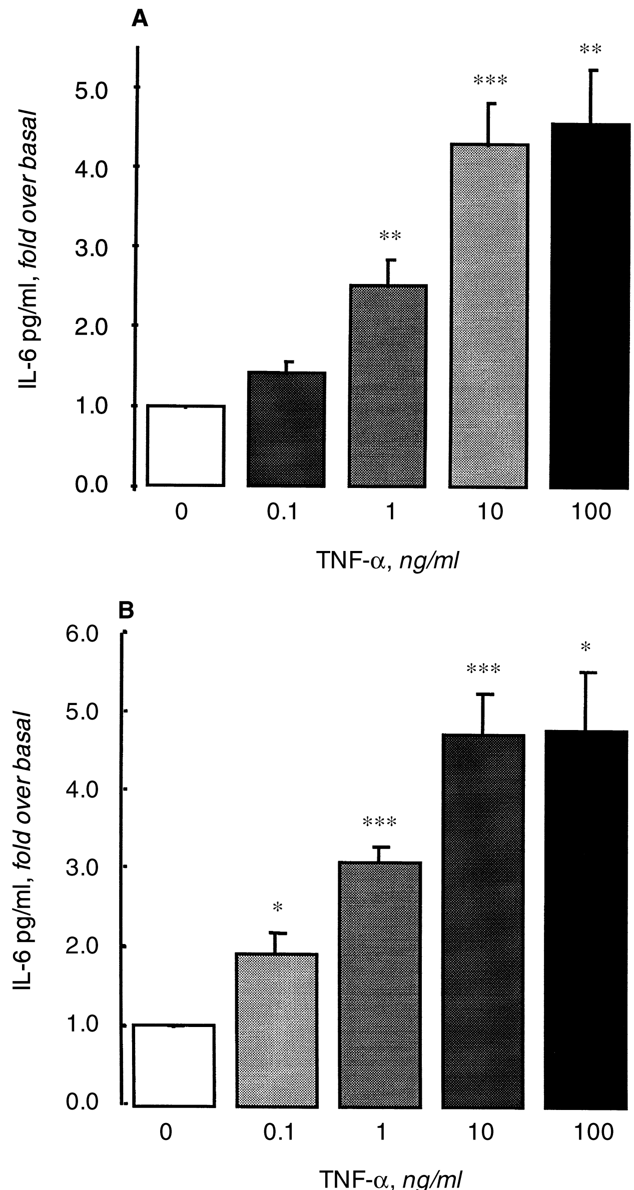

Fig. 2. Tumor necrosis factor-α (TNF-α)–induced dose-dependent increase in interleukin-6 (IL-6) production from human proximal tubular (*A*) and human mesangial (*B*) cells. Cells were grown to confluence and treated with TNF-α (0.1 to 100 ng/ml) for 24 hours. Cell culture medium was removed and assayed for IL-6 by ELISA, and levels were expressed as fold over basal concentrations (pg/ml). Each value represents the mean ± SEM of 4 to 11 experiments each performed in duplicate. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, statistically significant difference compared with control.

denced by transmission electron microscopy (data not shown), the HMCs contained bundles of microfilaments oriented parallel to the plasma membrane similar to mesangial cells *in vivo* [28]. The cells displayed positive staining for vimentin and α-smooth muscle actin and negative staining for cytokeratin, thus excluding glomerular epithelial contamination (data not shown).

Basal and TNF-α–stimulated IL-6 production from human proximal tubular and mesangial cells

Both cell types exhibited basal production of IL-6 with levels of 23.2 ± 4.7 pg/ml ($N = 10$) in proximal tubular cells and 12.9 ± 3.0 pg/ml ($N = 11$) in mesangial cells. Following 24 hours of treatment, TNF-α stimulated dose-dependent (0.1 to 100 ng/ml) increases in IL-6 production from both proximal tubular (Fig. 2A) and mesangial (Fig. 2B) cells. For example, 10 ng/ml TNF-α stimulated 4.3 ± 0.5-fold ($N = 10$) and 4.7 ± 0.5-fold ($N = 9$) over basal IL-6 levels in proximal tubular and mesangial cells, respectively. There was no further increase in IL-6 production using 100 ng/ml TNF-α. Therefore, for all subsequent experiments, cells were treated with 10 ng/ml TNF-α.

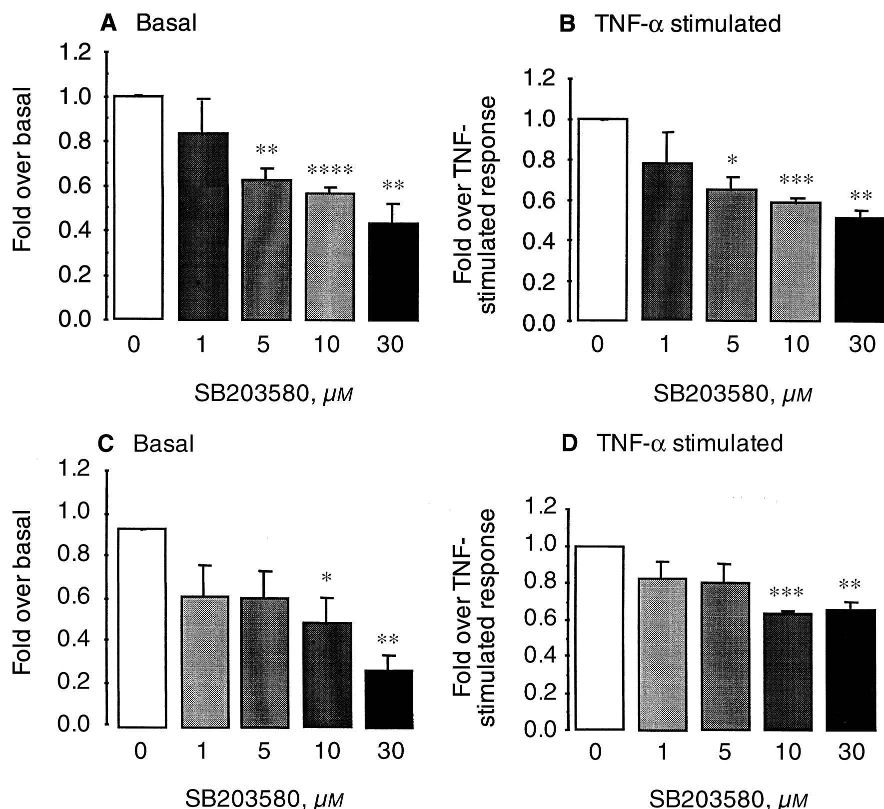

Fig. 3. Inhibition of basal (*A* and *C*) and TNF-α (*B* and *D*) stimulated IL-6 production from human proximal tubular (A and B) and human mesangial (C and D) cells by the specific p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580. Cells grown to confluence were pretreated with SB203580 (1 to 30 μM) for one hour prior to stimulation with TNF-α (10 ng/ml) for 24 hours. Cell culture medium was removed and assayed for IL-6 by ELISA, and levels in the presence of different inhibitor concentrations were expressed and compared as fold over basal or fold over TNF-α–stimulated concentrations (pg/ml). Each value represents the mean ± SEM of four experiments, each performed in duplicate. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; statistically significant difference compared with control.

Inhibition by the p38 MAPK inhibitor SB203580 of basal and TNF-α–stimulated IL-6 production

The specific p38 MAPK inhibitor SB203580 [15] was used to examine the involvement of this signaling cascade in mediating IL-6 production from mesangial and tubular cells. Cells were pretreated with SB203580 (1 to 30 μM) for one hour prior to stimulation with TNF-α (10 ng/ml) for 24 hours. SB203580 was not toxic to the cells at these concentrations, as evidenced by no losses in cellular viability (data not shown). Doses of SB203580 greater than 30 μM were, however, toxic to the cells and therefore could not be used (data not shown). SB203580 (1 to 30 μM) caused dose-dependent decreases in both basal (Fig. 3 A, C) and TNF-α–stimulated (Fig. 3 B, D) IL-6 production from both proximal tubular (Fig. 3 A, B) and mesangial cells (Fig. 3 C, D).

Basal and tumor necrosis factor-α–stimulated p38 MAPK phosphorylation (activation) was not inhibited by SB203580

The activation of p38 MAPK is normally characterized by dual phosphorylation on specific tyrosine and threonine residues. p38 MAPK phosphorylation (activation) in this study was investigated using a phospho-specific p38 MAPK antibody, which detects p38 when activated by dual phosphorylation at threonine 180 and tyrosine 182. Whole cell p38 MAPK expression was assessed to control for cytokine-induced alterations in the protein content of the kinase. No changes in whole cell p38 MAPK expression were apparent after any of the treatments used (lower panels, Fig. 4 A, B) in either cell type. TNF-α (10 ng/ml) treatment for 15 minutes caused an increase in p38 MAPK phosphorylation in both proximal tubular (upper panel, Fig. 4A) and mesangial cells (upper panel, Fig. 4B), as evidenced by increased expression of the phosphorylated form of the kinase. Pretreatment with 10 μM SB203580 had no effect on either basal or TNF-α–stimulated p38 MAPK phosphorylation in either cell types (upper panels, Fig. 4 A, B).

Inhibition of tumor necrosis factor-α–stimulated p38 MAP kinase activity by SB203580

The effect of SB203580 on the downstream kinase activity of p38 MAPK was then measured in order to establish the stage in the kinase cascade that the compounds act to inhibit IL-6 production in renal cells. p38 MAPK activity in human proximal tubular cells was measured using an *in vitro* immunokinase assay using ATF2 as the substrate for the reaction. TNF-α (10 ng/ml) induced a large increase in kinase activity in proximal tubular epithelial cells. This was completely abolished by prior treatment with 10 μM SB203580 (Fig. 5). p38

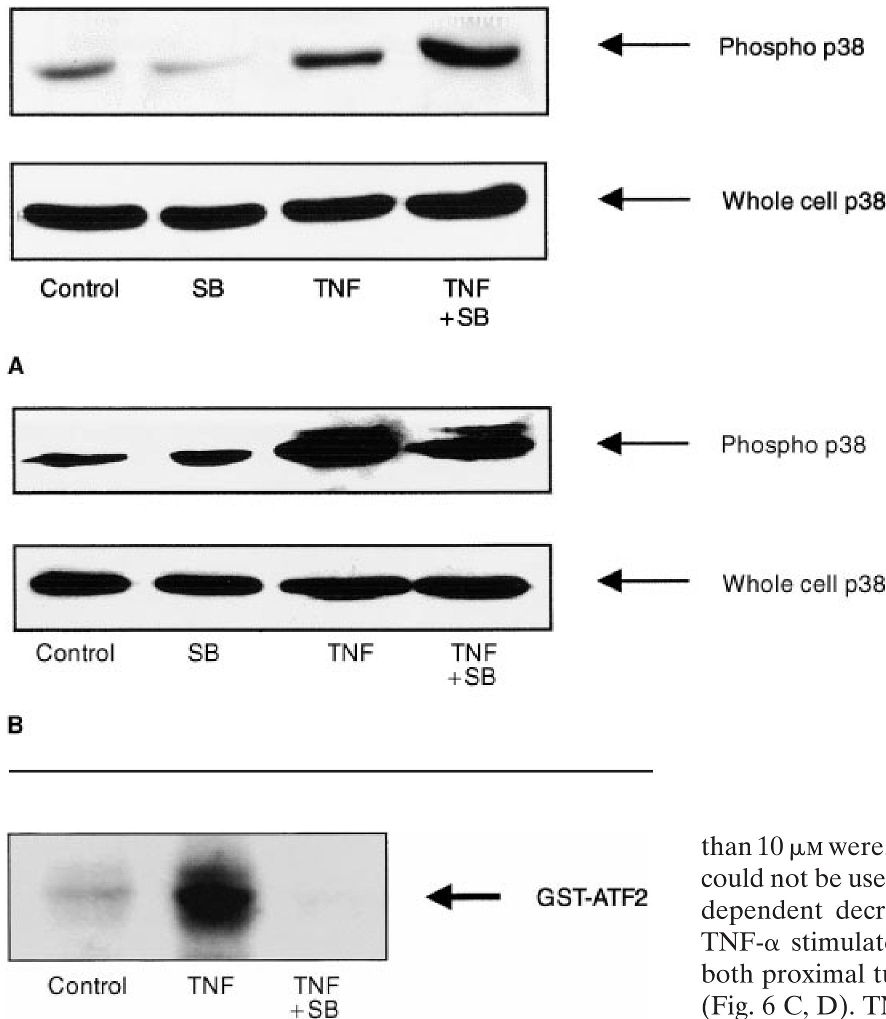

Fig. 4. TNF-α–induced stimulation of p38 MAPK phosphorylation (activation) in human proximal tubular (*A*) and human mesangial (*B*) cells, which were not affected by SB203580. Confluent cells, serum deprived for 24 hours, were pretreated with SB203580 (10 μM) for one hour followed by TNF-α (10 ng/ml) stimulation for 15 minutes. Whole cell extracts subjected to SDS-PAGE were probed using Western blotting and antibodies directed against the phosphorylated or whole cell p38 MAPK. The bands were visualized by ECL. Phosphorylated p38 MAPK is shown in the upper panels, and whole cell p38 MAPK is shown in the lower panels. Representative blots from one of three separate experiments are shown.

Fig. 5. Inhibition by SB203580 of the TNF-α–stimulated increase in p38 MAPK activity in human proximal tubular epithelial cells. Confluent cells, serum deprived for 24 hours were pretreated with SB203580 (10 μM) for one hour followed by TNF-α (10 ng/ml) stimulation for 15 minutes. p38 MAPK was immunoprecipitated from whole cell extracts, and activity was measured as the incorporation of a radiolabeled phosphate into an artificial substrate ATF2. The incorporation was visualized by autoradiography after SDS-PAGE.

MAPK activity could not be detected in mesangial cells at the level of sensitivity used in this kinase assay.

Inhibition of basal and TNF-α stimulated IL-6 production by the MEK1 inhibitor PD98059

The specific MEK1 inhibitor PD98059 [14] was used to assess the involvement of the ERK1,2 pathway in mediating IL-6 production from human renal cells. Cells were pretreated with PD98059 (0.01 to 10 μM) for one hour prior to stimulation with TNF-α (10 ng/ml) for 24 hours. PD98059 was not toxic to the cells at these concentrations, as evidenced by no losses in cellular viability (data not shown). Doses of PD98059 of greater than 10 μM were, however, toxic to the cells and therefore could not be used. PD98059 (0.01 to 10 μM) caused dose-dependent decreases in both basal (Fig. 6 A, C) and TNF-α stimulated (Fig. 6 B, D) IL-6 production from both proximal tubular (Fig. 6 A, B) and mesangial cells (Fig. 6 C, D). TNF-α–stimulated IL-6 levels were inhibited by similar amounts in both cell types (Fig. 6 B, D).

Basal and TNF-α stimulated ERK1,2 MAPK phosphorylation (activation) was inhibited by PD98059

The activation of ERK1,2 MAPK is also characterized by dual phosphorylation. The phospho-specific antibody "antiphospho ERK-1,2," which detects phosphorylated tyrosine 204 and threonine 202 of ERK1 and ERK2, was used to detect ERK phosphorylation (activation) in this system. Whole cell ERK2 expression was assessed to control for cytokine-induced alterations in the protein content of the kinase. No changes in whole cell ERK2 kinase expression were apparent after any of the treatments in either cell type used (lower panels, Fig. 7 A, B). Basal levels of ERK1,2 MAPK phosphorylation were increased after treatment with TNF-α (10 ng/ml) for 15 minutes in both proximal and mesangial cells (upper panel, Fig. 7B). The TNF-α–induced ERK1 phosphorylation appeared to be greater than that of ERK2. PD98059 (10 μM) reduced both basal and TNF-α–stimulated ERK1,2 MAPK phosphorylation in both cell types (upper panels, Fig. 7 A, B).

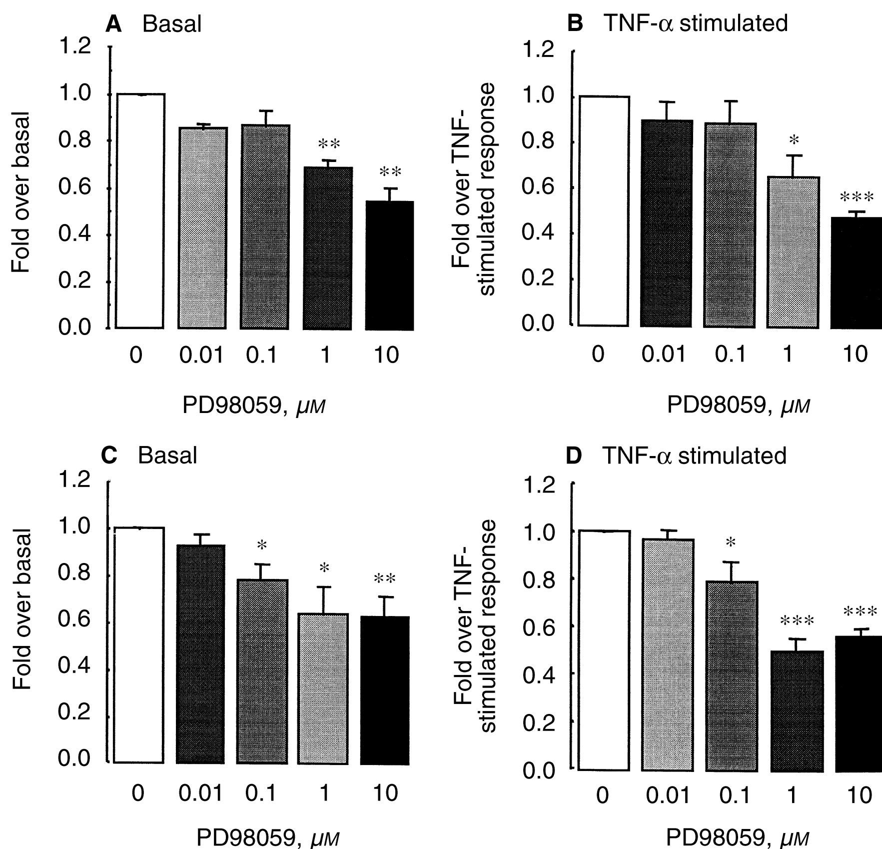

Fig. 6. Inhibition of basal (*A* and *C*) and TNF-α (*B* and *D*)–stimulated IL-6 production from human proximal tubular (A and B) and mesangial (C and D) cells by the specific MEK1 inhibitor PD98059. Cells grown to confluence were pretreated with PD98059 (0.01 to 10 μM) for one hour prior to stimulation with TNF-α (10 ng/ml) for 24 hours. Cell culture medium was removed and assayed for IL-6 by ELISA, and levels in the presence of different inhibitor concentrations were expressed and compared as fold over basal or fold over TNF-α–stimulated concentrations (pg/ml). Each value represents the mean ± SEM of four to eight experiments each performed in duplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; statistically significant difference compared to control.

Further inhibition of basal and TNF-α–stimulated IL-6 production using a combination of SB203580 and PD98059

Finally, the effects of a combination of both MAPK inhibitors on IL-6 production in mesangial and proximal tubular cells were assessed. Cells were pretreated with SB203580 (10 μM) or PD98059 (10 μM) either alone or in combination for one hour prior to stimulation with TNF-α (10 ng/ml) for 24 hours. Cotreatment of cells with both inhibitors caused significant decreases in both basal (Fig. 8 A, C), and TNF-α stimulated (Fig. 8 B, D) IL-6 production from proximal tubular (Fig. 8 A, B) and mesangial cells (Fig. 8 C, D) as compared with cells treated with the relevant inhibitor alone.

DISCUSSION

In this study, we demonstrated that primary human mesangial and proximal tubular cells produce similar amounts of basal IL-6 and respond to TNF-α with similar increases in IL-6 production. Furthermore, the TNF-α mediated IL-6 production involved the activation of the p38 and ERK MAPK pathways. These *in vitro* systems of human cells provide models to explore mechanisms of cytokine-mediated events relevant to human renal disease.

The pathophysiology of many experimental models of glomerulonephritis are consistent with the associated overproduction of cytokines in both the glomerular and tubulointerstitial regions [30–32]. Tubulointerstitial fibrosis has been proposed as a final common pathway for progressive renal injury in most renal diseases, and the level of tubulointerstitial fibrosis correlates closely with the degree of chronic renal dysfunction in these settings [33]. Tubular epithelial cells once considered passive bystanders in the disease process have since been shown to be actively involved and are indeed a rich source of cytokines, chemokines, and other inflammatory mediators [7, 34–36].

Tumor necrosis factor-α expression has been found to be increased *in vivo* in various inflammatory renal diseases and *in vitro* in both mesangial and proximal tubular cells [37, 38]. TNF-α has been reported as a potent stimulator of IL-6 production [9]. Both glomerular mesangial and tubular epithelial cells have been shown to express and secrete IL-6 [7, 8, 39]. IL-6 has been shown to be produced in mesangial cells in response to a number of stimuli, including immune complexes, IgA, and a number of proinflammatory cytokines, where it is thought to act as an autocrine growth factor stimulating mesangial cell hyperproliferation [40–42]. A recent study suggested that apoptotic monocytes are also capable of inducing

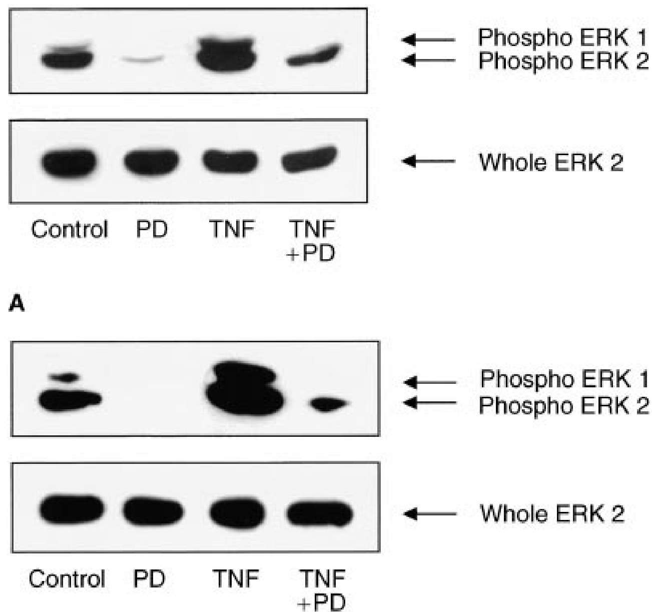

Fig. 7. TNF-α–induced stimulation of ERK1,2 phosphorylation (activation) in human proximal tubular (*A*) and mesangial (*B*) cells, which were inhibited by the specific MEK1 inhibitor PD98059. Confluent cells, serum deprived for 24 hours, were pretreated with PD98059 (10 μM) for 1 hour followed by TNF-α (10 ng/ml) stimulation for 15 minutes. Whole cell extracts subjected to SDS-PAGE were probed using Western blotting and antibodies directed against the phosphorylated form of ERK1,2 MAPK or whole cell ERK2. The bands were visualized by ECL. Phosphorylated ERK1,2 MAPK is shown in the upper panels, and whole cell ERK2 is shown in the lower panels. Representative blots from one of three separate experiments are shown.

an exaggerated mesangial IL-6 production [43]. Urinary IL-6 production has been correlated to disease progression, and it has been shown that the level of urinary IL-6 could be influenced not only by mesangial cell proliferation but also by renal tubular dysfunction [44]. However, the exact functional role of IL-6 in renal disease is controversial. In some studies, the degree of mesangial hyperproliferation, tubular atrophy, and intensity of interstitial infiltrates have been correlated to the renal expression and urine concentration of IL-6 [45, 46]. However, more recent studies have demonstrated that IL-6 may, in fact, have anti-inflammatory effects in renal disease and does not induce mesangial cell proliferation [47, 48]. Regardless of the exact functional role, IL-6 production has been tightly correlated to renal disease progression. However, the mechanisms and signaling events that regulate IL-6 production in renal cells have not been established.

To provide more insight into the roles of IL-6 and TNF-α in renal disease, it is necessary to provide models relevant to the human situation where mechanisms and signaling events can be explored. Therefore, in this study, we focused on both basal and TNF-α–stimulated IL-6 production in primary renal cells representing the mesangial and proximal tubular regions. Initially, we established that the basal production of IL-6 occurred in primary mesangial and proximal tubular cells and that the levels of IL-6 produced from both cell types were similar. Then we established that TNF-α (0.1 to 100 ng/ml) treatment for 24 hours stimulated a dose-dependent increase in IL-6 production in both glomerular mesangial and proximal tubular epithelial cells. TNF-α appeared to cause a similar level of stimulation of IL-6 production from both cell types.

Mitogen-activated protein kinases are a crucial part of cellular signal transduction machinery and play major roles in cell growth, differentiation, and transformation [10]. The use of the specific inhibitors of the p38 and ERK1,2 MAPK pathways SB203580 and PD98059, respectively, has facilitated investigations of these pathways. In these studies, the activation of both the p38 and the ERK1,2 MAPKs above basal levels occurred following TNF-α stimulation in both cell types. The biological significance of this activation was confirmed by the ability of the inhibitors SB203580 (1 to 30 μM) and PD98059 (0.01 to 10 μM) to inhibit basal and TNF-α–stimulated IL-6 production in both cell types. Pretreatment with SB203580 (10 μM) had no effect on basal or TNF-α–stimulated activation (phosphorylation) of p38 MAPK but completely abolished TNF-α–stimulated p38 MAPK activity. PD98059 decreased both basal and TNF-α–stimulated ERK1,2 phosphorylation, indicating that both inhibitors reduce IL-6 production by acting at different stages in their respective cascades. The level of inhibition observed appeared to depend on agonist (TNF-α) stimulation. We confirmed the specificity of the inhibitors by the fact that neither inhibitor had any effect on the activation of the other respective pathway (data not shown). The doses responsible for the inhibition of IL-6 production by SB203580 and PD98059 closely corresponded to those required for inhibition of p38 MAPK activity and ERK1,2 phosphorylation, respectively, confirming that MAPK activation was involved in TNF-α–induced IL-6 synthesis in renal cells. A combination of both inhibitors caused significantly greater decreases in IL-6 production as compared with either inhibitor alone, clearly indicating that both signaling pathways are involved in regulating IL-6 production in renal cells. It is interesting to note that a total abrogation of TNF-α–induced IL-6 production was not achieved even in the presence of both inhibitors, suggesting that other additional signaling pathways may be involved in the transduction of the TNF-α signal.

Although, to our knowledge, these studies are the first demonstration of the involvement of both the p38 and ERK MAPK pathways in IL-6 production from HMCs and tubular cells, other studies have been reported in nonrenal cells. In osteoblast-like MC3T3-E1 cells, PD98059 was reported to bring about a dose-dependent decrease

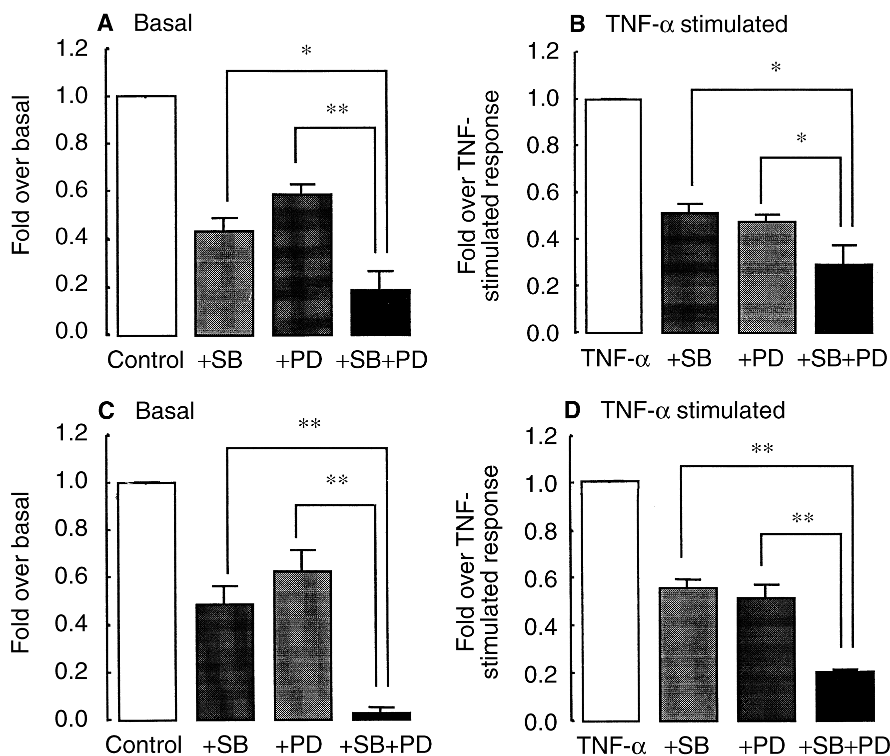

Fig. 8. Further inhibition of basal (*A* and *C*) and TNF-α (*B* and *D*)–stimulated IL-6 production from human proximal tubular (A and B) and human mesangial (C and D) cells by a combination of the MAPK inhibitors SB203580 and PD98059. Cells grown to confluence were pretreated with PD98059 (10 μM) and SB203580 (10 μM) for one hour prior to stimulation with TNF-α (10 ng/ml) for 24 hours. Cell culture medium was removed and assayed for IL-6 by ELISA, and levels in the presence of inhibitors were expressed and compared as fold over basal or fold over TNF-α–stimulated concentrations (pg/ml). Each value represents the mean ± SEM of four to eight experiments each performed in duplicate. *$P < 0.05$; **$P < 0.01$; statistically significant difference compared to each inhibitor added alone.

in sphingosine 1-phosphate–stimulated IL-6 production [49]. Sphingosine 1-phosphate is a downstream mediator of TNF-α–induced signal transduction [50]. In the MC3T3-E1 cells, PD98059 (10 μM) failed to inhibit IL-6 production by more than 50%, which is similar to that observed in mesangial and tubular cells in our studies. In HeLa and L-929 cells, a complete inhibition of TNF-α–stimulated IL-6 expression has been shown with SB203580 (10 μM) [9]. This is in contrast to our findings in mesangial and tubular cells, where only approximately 50% inhibition was found with a similar dose of SB203580. In human fibroblasts and endothelial cells, Ridley et al showed a 60 and 75% inhibition of IL-1–stimulated IL-6 production with SB203580 (10 μM) [51]. These values are more similar to the levels of inhibition observed in these studies in mesangial and proximal tubular cells. In these studies, IL-6 production in human mesangial and proximal tubular cells was inhibited by both PD98059 and SB203580, implicating the involvement of both pathways in IL-6 production. In Kupffer cells, both pathways have also been shown to mediate IL-6 production [52]. However, in Sertoli cells, IL-6 production was inhibited by SB203580 but not by PD98059, indicating that only the p38 MAPK pathway plays a role in these cells [53]. Clearly, the extent of SB203580 and PD98059 mediated the inhibition of IL-6 production is very much cell-type specific.

The MAPK pathways have also been implicated in TNF-α–mediated expression of other cytokines and chemokines. In addition to IL-6, TNF-α also induces the expression of a number of other inflammatory mediators implicated in renal disease, for example, adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) and chemokines such as IL-8 [54]. However, TNF-α appears to differentially regulate the expression of these molecules. For example, in cultured mouse sertoli cells, it has been shown that in response to TNF-α, the activation of p38 MAPK leads to IL-6 production, whereas ICAM-1 and VCAM-1 are induced by activation of the JNK pathway [53]. In addition, in vascular endothelial cells, the activation of the p38 MAPK pathway was shown to be important in IL-1–stimulated IL-6 production but was not involved in the production of IL-8 [51].

The specific roles of the p38 and ERK MAPK pathways in mediating gene expression and the level at which they mediate this expression are still relatively unresolved [55]. SB203580 has been shown to inhibit LPS-stimulated IL-1 and TNF-α expression [56] and also IL-1 induced IL-6 [57] expression at the translational level. However, the inhibition of TNF-α–induced IL-6 expression appears to be at the transcriptional level. Nuclear factor-κB (NF-κB) is the main transcriptional activator for both TNF-α and IL-1–induced IL-6 gene induction. NF-κB exists typically as a dimer between p50 and the transactivating subunit p65 [58]. A recent study used site-directed mutagenesis of the IL-6 promoter and reported a necessity for NF-κB transcriptional activity in

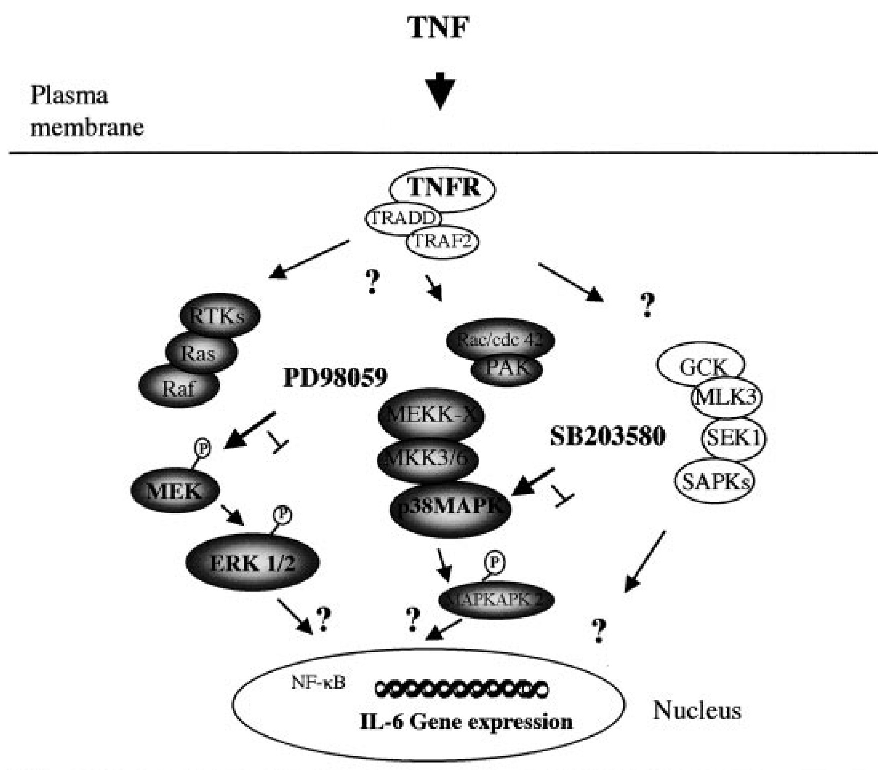

Fig. 9. Proposed hypothesis for the regulation of TNF-α–induced IL-6 production in renal cells.

mediating TNF-α–stimulated IL-6 gene transcription [59]. The MAPK inhibitors SB203580 and PD98059 were shown to have a direct repressive effect on the transactivation potential of the p65 subunit, suggesting that both the p38 and ERK MAPK pathways modulate TNF-α–induced IL-6 expression by modulating components of the transactivation machinery. A combination of both inhibitors totally abrogated the TNF-induced NF-κB transactivation potential. In this study, similar additive effects were found between both inhibitors at the level of TNF-α–induced IL-6 production. These findings suggest that a number of converging pathways and co-operative mechanisms regulate TNF-α–mediated IL-6 expression. Our findings indicate that similar mechanisms may operate in TNF-α–simulated IL-6 production in HMCs and HPT cells. A scheme outlining the possible involvement of the p38 and ERK1,2 MAPKs in IL-6 gene expression is outlined in Figure 9.

In summary, this study demonstrates that both the p38 and ERK MAPK cascades are simultaneously activated in response to TNF-α in both human mesangial and proximal tubular epithelial cells. The activation of these pathways appears to play an important role in the mediation of IL-6 production by these cells. Following TNF-α stimulation, both glomerular mesangial and tubular epithelial cells appear to utilize similar signal transduction pathways in the mediation of IL-6 production. These findings should aid in the understanding of the cellular mechanisms that regulate IL-6 production from both the glomerular and tubulointerstitial regions of the nephron.

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