Inhibition of the protein kinase MK-2 protects podocytes from nephrotic syndrome-related injury

Ruma Pengal,1 Adam J. Guess,1 Shipra Agrawal,1 Joshua Manley,1 Richard F. Ransom,1,2 Robert J. Mourey,3 Rainer Benndorf,1,2 and William E. Smoyer1,2
1Center for Clinical and Translational Research, The Research Institute at Nationwide Children’s Hospital, and 2Department of Pediatrics, The Ohio State University, Columbus, Ohio; and 3Pfizer Global Research and Development, St. Louis Laboratories, Chesterfield, Missouri

Submitted 10 November 2010; accepted in final form 24 May 2011

Inhibition of the protein kinase MK-2 protects podocytes from nephrotic syndrome-related injury. Am J Physiol Renal Physiol 301: F509–F519, 2011. First published May 25, 2011; doi:10.1152/ajprenal.00661.2010.—While mitogen-activated protein kinase (MAPK) activation has been implicated in the pathogenesis of various glomerular diseases, including nephrotic syndrome (NS), its specific role in podocyte injury is not known. We hypothesized that MK-2, a downstream substrate of p38 MAPK, mediates the adverse effects of this pathway and that inhibition of MK-2 would protect podocytes from NS-related injury. Using cultured podocytes, we analyzed 1) the roles of MK-2 and p38 MAPK in puromycin aminonucleoside (PAN)-induced podocyte injury; 2) the ability of specific MK-2 and p38 MAPK inhibitors to protect podocytes against injury; 3) the role of serum albumin, known to induce podocyte injury, in activating p38 MAPK/MK-2 signaling; and 4) the role of p38 MAPK/MK-2 signaling in the expression of Cox-2, an enzyme associated with podocyte injury. Treatment with protein kinase inhibitors specific for both MK-2 (C23, a pyrrolopyridine-type compound) or p38 MAPK (SB203580) reduced PAN-induced podocyte injury and actin cytoskeletal disruption. Both inhibitors reduced baseline podocyte p38 MAPK/MK-2 signaling, as measured by the degree of phosphorylation of HSPB1, a downstream substrate of MK-2, but exhibited disparate effects on upstream signaling. Serum albumin activated p38 MAPK/MK-2 signaling and induced Cox-2 expression, and these responses were blocked by both inhibitors. Given the critical importance of podocyte injury to both NS and other progressive glomerular diseases, these data suggest an important role for p38 MAPK/MK-2 signaling in podocyte injury and identify MK-2 inhibition as a promising potential therapeutic strategy to protect podocytes in various glomerular diseases.

NEPHROTIC SYNDROME (NS) is a common kidney disease affecting both children and adults. Although numerous treatments exist, most of these can have serious side effects. A feature shared by all forms of NS is injury to the visceral epithelial cells or podocytes that surround glomerular capillaries and comprise a key part of the kidney’s filtration barrier.

In recent years, evidence has accumulated that several mitogen-activated protein kinases (MAPK), including p38 MAPK, are involved in human renal diseases and experimental models of renal injury (22). Two of the known substrates of p38 MAPK are the protein kinases MAPK-activated protein kinase-2 (MK-2) and the related MK-3 (18, 19). p38 MAPK/MK-2 signaling is induced by various cytokines and stress stimuli including ultraviolet irradiation, heat shock, and osmotic shock, and p38 MAPK is known to be involved in cell differentiation, senescence, tumorigenesis, and apoptosis (12). It is also now well established that activation of p38 MAPK/MK-2 signaling plays a critical role in various inflammatory conditions. For example, targeted deletion of the genes encoding MK-2 (or MK-2 and MK-3) in mice provided significant protection against lipopolysaccharide-induced systemic endotoxic shock by impairing the inflammatory response (33, 48). MK-2 deletion or inhibition has also been found to be beneficial in other conditions, including cancer, atherosclerosis, and certain neurological disorders like amyotrophic lateral sclerosis (13, 14, 30, 43, 49, 51). Based on these findings, inhibition of the p38 MAPK/MK-2 signaling pathway has emerged as a novel therapeutic approach in treating several inflammatory and other disease conditions (22, 69).

One of the well-studied substrates of MK-2 is the small heat shock protein HSPB1 (synonyms: HSP27, HSP25), which is known to be involved in actin remodeling, mRNA processing, apoptosis, and other processes, partly in a phosphorylation-dependent manner (8, 18, 32). HSPB1 is phosphorylated by MK-2 at three sites in humans (Ser15, Ser78, Ser82) and at two sites in mice (Ser15, Ser86) (20, 35). In human crescentic glomerulonephritis, as well as experimental animal models of glomerulonephritis, activated p38 MAPK signaling has been observed in podocytes (46, 52, 57–59). Similarly, increased phosphorylation (activation) of p38 MAPK has been reported in various human glomerulopathies as well as in experimental rodent nephrosis models (31). In addition, in the same study reduced podocyte injury was described in vitro and in vivo using a specific p38 MAPK inhibitor. Exposure of podocytes to elevated concentrations of serum albumin (SA) has also been reported to activate p38 MAPK, and its inhibition protected the cells from SA-induced stress (68). From a clinical perspective, podocyte p38 MAPK/MK-2 pathway activation in response to increased concentrations of SA during proteinuria in NS may thus be a critical (and potentially inhabitable) mediator of cell injury and resultant outcome.

The therapeutic relevance of inhibition of p38 MAPK and of other MAPKs in various renal conditions has been summarized in a recent review (22). Since deletion of the gene for p38α MAPK results in embryonic lethality (1), targeting MK-2/MK-3, however, may be a better alternative. Indeed, in marked contrast to p38 MAPK disruption, disruption of the genes
encoding MK-2 and/or MK-3 in mice does not affect their viabili-
ty of inhibition of MK-2 and/or MK-3 (33, 48). Available p38 MAPK and MK-2 inhibitors include the pyridinyl imida-
zole SB203580 and a recently developed pyrrolopyridine sub-
stance (herein designated C23), respectively (5, 69). C23
specifically inhibited MK-2 compared with MK-3 (5). A pre-
vious study has shown that MK-3 accounted for only 10–20% of
the HSPB1-phosphorylating activity, thus indicating the
important role that MK-2 plays in signaling downstream of p38
MAPK (48). Figure 1 shows a schematic of the p38 MAPK/
MK-2/HSPB1 signaling pathway and the sites of action of both
SB203580 and C23.

In this study, we investigated the potential beneficial effects
of inhibition of MK-2 by the specific inhibitor C23 and
compared this with the protective effects of the p38 MAPK
inhibitor SB203580 in cultured podocytes injured by puromo-
cycin aminonucleoside (PAN), a commonly used toxin to mimic
NS-related podocyte injury.

MATERIALS AND METHODS

MK-2 inhibitor C23. This pyrrolopyridine compound with the
systematic name 2-[2-{2-(fluorophenyl)pyridin-4-yl]-1,5,6,7-tetra-
hydro-4H-pyrrolo[3,2-c]pyridin-4-one trifluoroacetate was provided
by Pfizer, (Chesterfield, MO). The used stock solution was 100 mM in
DMSO. C23 inhibited specifically MK-2 (IC50 = 0.126 µM) com-
pared with the related MK-3 (IC50 = 1.1 µM), and compared with a
number of other protein kinases (5).

Culture and treatment of podocytes and viability assays. The
conditionally immortalized mouse podocyte cell line JR07 was
cultivated as previously described (47). Briefly, cells were propagated at
33°C in RPMI 1640 medium in the presence of 10% fetal bovine
serum, 0.29 mg/ml glutamine, and 10 U/ml mouse γ-interferon (complete
medium) in cell culture flasks (Greiner, Monroe, NC). For
differentiation, cells were trypsinized and 12,000 cells per well were
seeded into rat tail collagen type I (BD Biosciences, Bedford, MA)-
coated six-well culture plates (Greiner) containing the same medium
without γ-interferon. Differentiation was induced by transferring the
cells to 37°C. Cells were allowed to differentiate for 10–14 days. For
podocyte injury, cells were treated with 5 or 10 µg/ml PAN (stock
solution: 10 mg/ml in water) for various time periods as specified in the
figure legends. Where indicated, cells were serum-starved over-
night or for 24 h before addition of further reagents. Podocytes were
treated with C23 (0.3, 1, 3, 10, 30, 90 µM), SB203580 (0.3, 1, 10, 20,
30 µM; stock solution: 30 mM in DMSO), bovine SA (5, 50 µg/ml in
serum-free RPMI 1640), anisomycin (5, 10, 20, 50 ng/ml; stock
solution: 50 µg/ml in 70% ethanol), or with the appropriate vehicles
alone, for various time periods as indicated. C23 and SB203580 were
added to the cells 30 min before the addition of SA or anisomycin,
unless stated otherwise. PAN, SB203580, SA, and anisomycin were
purchased from Sigma (St. Louis, MO). The efficiency of activation of
p38 MAPK signaling in podocytes by anisomycin and its inhibition by
C23 or SB203580 were verified (data not shown).

Podocyte viability was measured in 96-well plates after PAN, C23,
and SB203580 treatments, using the methylthiazolyldiphenyl-
tetrazolium bromide (MTT) viability assay method as previously described
(40). Briefly, after the treatments the culture medium was replaced with
200 µl fresh medium/well containing MTT (Sigma) at a final
concentration of 500 µg/ml, and cells were incubated for another 3 h.
Then, the MTT medium was replaced with 200 µl of solution A (200
µl DMSO + 25 µl glycine buffer containing 0.1 M glycine-NaOH, pH
10.5; 0.1 M NaCl). The absorbance in each well was measured at 570
nm on a SpectraMax M2 spectrophotometric plate reader (Molecular
Devices, Sunnyvale, CA). Cells treated for 1 h with 0.01% Triton
X-100 showed no significant viability and were used as negative
controls.

Actin staining and microscopy. For actin staining, cells were grown and
differentiated on glass coverslips in six-well plates. After treatment of
the cells, F-actin was labeled using Texas Red labeled phallolidin (Invitrogen, Carlsbad, CA) as described (47). Phase-con-
trast and fluorescence microscopy was performed on a Leica
DMI6000B inverted fluorescence microscope equipped with a TX2
cube (excitation 560/40 nm, emission 645/75 nm; Leica Microsys-
tems, Bannockburn, IL). Digital micrographs were captured using a
Retiga SRV 14-bit grayscale CCD camera (QImaging, Surrey, BC).

Electrophoretic methods and Western blotting. SDS-PAGE and
isoelectric focusing PAGE (IEF-PAGE) were performed according to
standard procedures (34, 56). For IEF-PAGE, podocytes were lysed in
100 µl of solution B (6 M urea, 2% ampholytes 3/10, 2% Triton
X-100, and 10 mM DTT). After separation, proteins were transferred onto a polyvinylidene difluoride membrane which was incubated with various primary and horseradish peroxidase-coupled secondary antibi-
odies for protein detection. Antibody binding was visualized with the
ECL chemiluminescence system (GE Healthcare Bio-Sciences, Pisc-
caway, NJ) and detected by exposure to X-ray film. Representative
blots of at least three independent experiments are shown (see see
Figs. 2, 5, 6, and 7).

The degree of phosphorylation of p38 MAPK and MK-2 was
semi-quantitatively estimated by calculating the ratio of band densi-
ties (D) of phosphorylated over non-phosphorylated forms of these
enzymes. The degree of phosphorylation of HSPB1 (expressed as RP
values) was semi-quantitatively estimated by calculating the ratio of
band densities of phosphorylated over non-phosphorylated isoforms
according to the equation: $\frac{D_{0p},D_{1p},D_{2p}}{D_{0p}}$, where RP is the
ratio of phosphorylation of HSPB1 isoforms and $D_{0p}$, $D_{1p}$, and $D_{2p}$
are band densities of the non-phosphorylated (0p), singly phosphor-
ylated (1p), and doubly phosphorylated (2p) HSPB1 isoforms, respec-
tively. All ratios of band densities and RP values are shown as
fold-changes compared with the controls, yielding relative RP values.

X-ray films were scanned using a calibrated ArtixScan M1 transillu-
mination scanner (Microtek Lab., Cerritos, CA) controlled by the
ScanWizard Pro program (version 7.042) using standard settings. The
densitometric analysis was performed using the ImageJ program
(version 1.39), which is available at http://rsb.info.nih.gov/ij/.

---

**Fig. 1.** Schematic diagram of the p38 MAPK/MK-2 signaling upstream of
HSPB1, a downstream substrate of MK-2, and the site of action of the protein
kinase inhibitors C23 and SB203580. MK-2, a downstream substrate of p38
MAPK, phosphorylates 2 sites in mouse (Ser15, Ser86) and 3 sites in human
HSPB1 (Ser15, Ser78, Ser82). While both inhibitors show strong inhibitory
effects at the level of HSPB1, they can also affect the signaling upstream of
their target kinases (cf. Fig. 6). TGF, transforming growth factor.
The following primary antibodies were used at the indicated dilutions in 3% BSA in 0.1% Tween 20 PBS: rabbit polyclonal p38 MAPK antibody (dilution 1:1,000, Cell Signaling, Danvers, MA), rabbit monoclonal phospho-Thr180/Tyr182 p38 MAPK antibody (dilution 1:1,000, Cell Signaling), rabbit polyclonal MK-2 antibody (dilution 1:1,000, Cell Signaling), rabbit polyclonal HSP25 anti-mouse HSPB1 antibody (dilution 1:5,000, Assay Designs, Ann Arbor, MI), rabbit polyclonal phospho-Ser15 HSP27 antibody (detects mouse phospho-Ser15; dilution 1:1,000, Assay Designs), rabbit polyclonal phospho-Ser82 HSP27 antibody (detects mouse phospho-Ser86; dilution 1:1,000, Cell Signaling), and mouse monoclonal anti-GAPDH antibody (dilution 1:10,000, Millipore, Billerica, MA). Secondary antibody solutions contained peroxidase-conjugated goat anti-rabbit (dilution 1:10,000) or goat anti-mouse antibodies (dilution 1:10,000, both from Jackson ImmunoResearch Laboratories, West Grove, PA).

Cox-2 expression. Differentiated podocytes were serum-starved, treated with C23, SB203580, DMSO vehicle, and/or SA as specified in the Fig. 8 legend. Total RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA). One microgram of RNA was subjected to DNase (Ambion, Austin, TX) treatment at 37°C for 30 min followed by a DNase inactivation step in the presence of 5 mM EDTA at 75°C for 10 min. cDNA was prepared from 1 μg RNA with Superscript II reverse transcriptase (Bio-Rad, Hercules, CA) at reaction conditions of 5 min at 25°C, 30 min at 42°C followed by 5 min at 55°C. Cox-2 and β-actin mRNA levels were measured by the SYBR green PCR method using an iQ5 thermal cycler (Bio-Rad). The primers used for Cox-2 amplification were 5'-CAAGACAGACATCAAGCCGAGGA (forward) and 5'-GGCCGAGTATTGTGTCTGT (reverse), and the primers used for the β-actin amplification were 5'-CTTCGTTGCGG- GTCCACACCC (forward) and 5'-CTGGGGCTCTGCACCCACAT (reverse). PCR efficiency of each primer pair was measured by plotting the efficiency curve of serial dilutions of selected cDNA samples. Fold-changes in Cox-2 mRNA levels were plotted by normalizing against β-actin as a housekeeping gene and using the calculated primer efficiency as described (45). Samples treated with SA were compared with serum-starved samples, and all values were normalized to the control to obtain fold-change mRNA levels. Each sample was analyzed in triplicate, and the experiment was repeated three times.

Statistical analysis. The statistical significance of differences between means was analyzed by Student’s t-test. Significance was assumed if *P* < 0.05.

**RESULTS**

Toxicity of MK-2 and p38 MAPK inhibitors on cultured podocytes. In preliminary experiments, the toxicity of C23 and SB203580 on differentiated podocytes was estimated by viability assays. Cells were treated with different concentrations of either inhibitor for 1, 3, and 5 days followed by viability measurements. C23 had no or a minor effect on viability at day 1 at all tested concentrations (1, 3, 10, 30, 90 μM). At days 3 and 5, it affected viability in a concentration-dependent manner, with 90 μM being most toxic. The estimated IC50 value for C23 at day 5 was ~15 μM. Similarly, SB203580 typically had no significant effect on cell viability at day 1 at all tested concentrations (0.3, 3, 10, 20, 30 μM), while it affected viability moderately at days 3 and 5, with 30 μM being most toxic. The estimated IC50 value for SB203580 at day 5 was ~250 μM. Throughout this study, inhibitor concentrations were selected that had no or only moderate (reduction to not more than ~50% viability) toxicity: 0.3, 3, and 10 μM for C23 and 0.3, 3 and 30 μM for SB203580.

Phosphorylation of HSPB1 as output measure of p38 MAPK/MK-2 signaling. Throughout this study, the degree of phosphorylation of HSPB1 was used as an output measure to determine the overall extent of activity of the p38 MAPK/MK-2 signaling cascade in response to activators such as anisomycin and to the protein kinase inhibitors (cf. Fig. 1). To validate and compare available analysis methods, podocyte extracts containing variable proportions of the HSPB1 isoforms (0p, 1p, 2p) were prepared by treating the cells for 1 h with different concentrations (0, 5, 20, 50 ng/ml) of anisomycin. These extracts were analyzed by both IEF-PAGE/Western blotting (using an antibody recognizing all isoforms of HSPB1; Fig. 2A, top) and by SDS-PAGE/Western blotting (using antibodies specific for phospho-Ser15 and phospho-Ser86 in HSPB1; Fig. 2A, bottom). IEF-PAGE demonstrated that untreated podocytes contained predominantly the 0p-isofrom (~70%), relatively less 1p-isofrom (~25%), and only traces of the 2p-isofrom (~5%). Treatment with increasing concentrations of anisomycin resulted in a gradual shift toward the 2p-isofrom. Cells treated with 50 ng/ml anisomycin contained predominantly the 2p-isofrom (~80%), relatively less 1p-isofrom (~20%), and traces (if any) of the 0p-isofrom.
The band pattern obtained by SDS-PAGE/Western blotting and using phosphorylation site-specific antibodies correlated well with the IEF-PAGE analysis (Fig. 2A, **bottom**). Phosphorylation of HSPB1 in response to anisomycin involved both known phosphorylation sites of the mouse sequence, Ser15 and Ser86. Figure 2A also demonstrates that the IEF-PAGE analysis was more informative and more sensitive than the SDS-PAGE analysis. Therefore, IEF-PAGE analysis of the HSPB1 isoforms was used in the subsequent experiments.

**Efficacy of MK-2 and p38 MAPK inhibitors.** To test the efficacy of both drugs after 3 or 5 days, C23- and SB203580-treated podocytes were stimulated with 10 or 50 ng/ml anisomycin, followed by analysis of the distribution of the HSPB1 isoforms by IEF-PAGE/Western blotting (Fig. 2B). Similar to Fig. 2A, stimulation of podocytes with anisomycin in the absence of inhibitor caused a pronounced shift toward the phosphorylated isoforms, with 50 ng/ml anisomycin being more effective than 10 ng/ml anisomycin. When cells pretreated for 3 or 5 days with various concentrations of C23 or SB203580 were stimulated with anisomycin, phosphorylation of HSPB1 was partially or completely inhibited, depending on the inhibitor and anisomycin concentrations used. When phosphorylation of HSPB1 was induced with 10 ng/ml anisomycin after 3 days, the highest concentrations of both inhibitors completely prevented this response, while after 5 days C23 (10 μM) exhibited slightly reduced efficacy compared with SB203580 (30 μM). Following the induction of HSPB1 phosphorylation with 50 ng/ml anisomycin after 3 or 5 days, both drugs were less effective in preventing this response. This was particularly evident for C23, while SB203580, at the highest tested concentration (30 μM), still completely inhibited phosphorylation of HSPB1.

In summary, both drugs, C23 and SB203580, were effective in inhibiting activation of the p38 MAPK/MK-2 pathway up to 5 days after their addition to podocytes. Although at the concentrations used, C23 was somewhat less effective than SB203580, it is also a potent inhibitor of MK-2 signaling which is consistent with its reported IC$_{50}$ (0.126 μM) (5).

**Improved viability of PAN-injured podocytes following inhibition of MK-2 or p38 MAPK.** Podocytes were injured by treatment for up to 5 days with PAN (5 or 10 μg/ml), and cell viability was measured using the MTT assay. Both PAN concentrations decreased cell viability in a concentration- and time-dependent manner, with 10 μg/ml PAN reducing viability typically to ~0–15% after 5 days (Fig. 3, open bars). To test the ability of both inhibitors to protect podocytes from PAN-induced injury, cells were coincubated with various concentrations of C23 or SB203580 and viability was measured after 3 and 5 days of treatment (Fig. 3, filled bars). At day 3 following 5 μg/ml PAN treatment, all C23 concentrations improved cell viability. Following 10 μg/ml PAN treatment at day 3, only 0.3 μM C23 improved cell viability, while higher concentrations resulted in reduced viability. At day 5 following 5 μg/ml PAN treatment, all C23 concentrations improved cell viability in a dose-dependent manner. Similar to the 3-day treatment, at day 5 following 10 μg/ml PAN treatment only 0.3 μM C23 improved cell viability. SB203580 had similar protective effects. At day 3 following 5 μg/ml PAN treatment, 30 μM SB203580 improved cell viability, while at day 3 following 10 μg/ml PAN all SB203580 concentrations improved cell viability in a dose-dependent manner. At day 5 following 5 μg/ml PAN treatment, 30 μM SB203580 improved cell viability; however, at day 5 of 10 μg/ml PAN treatment only 30 μM SB203580 improved cell viability.

These data demonstrate that inhibition of MK-2 can protect podocytes from injury by PAN. The data also confirm that inhibition of p38 MAPK can have similar protective effects in podocytes, in agreement with a prior report (31).

![Viability of puromycin aminonucleoside (PAN)-injured podocytes following treatment with C23 or SB203580.](http://ajprenal.physiology.org/)
Protection of actin cytoskeleton of PAN-injured podocytes following inhibition of MK-2 or p38 MAPK. Disruption and reorganization of the actin cytoskeleton are hallmarks of podocyte injury in human NS, animal models of NS, and PAN-treated cultured podocytes (47, 55). Untreated podocytes exhibited pronounced bundles of actin stress fibers spanning the cell body (Fig. 4). Treatment with 5 or 10 μg/ml PAN caused, in a concentration- and time-dependent manner, redistribution of the actin cytoskeleton with the stress fibers being largely replaced by subcortical rings of filamentous actin. Prolonged exposure or higher PAN concentrations also resulted in a reduced cell number with disrupted actin cytoskeleton in the surviving cells. At the concentrations and exposure times used, C23 or SB203580 alone had no major effect on podocyte morphology (control panels in Fig. 4).

Cotreatment of podocytes with PAN and increasing concentrations of C23 clearly attenuated PAN-induced actin cytoskeletal injury. This was reflected by the high number of viable cells and by the maintenance of the original cell architecture characterized by actin stress fibers. Cells cotreated with 3 or 10 μM C23 were morphologically similar to the corresponding control cells.

Similarly, increasing concentrations of SB203580 also attenuated PAN-induced actin cytoskeletal disruption. Both 3 and 30 μM SB203580 essentially restored the original morphology of the actin stress fibers.

In summary, these results demonstrate the critical role of both MK-2 and p38 MAPK in disruption and reorganization of the actin cytoskeleton in PAN-injured podocytes and that their inhibition can protect the cytoskeleton against injury.

Effects of C23 and SB203580 on p38 MAPK/MK-2 signaling. To characterize the effects of both inhibitors at the molecular level in long-term treatments (cf. Figs. 3 and 4), the extent of inhibition of the p38 MAPK/MK-2 signaling cascade was assessed by measuring the isoform distribution of the MK-2 substrate HSPB1 by IEF-PAGE/Western blotting. In untreated control cells, the ω- and 1p-isoforms of HSPB1 predominated, with little 2p-isoform being detectable (Fig. 5, A and B). This isoform pattern reflects the steady-state activity of the p38 MAPK/MK-2 signaling cascade for this experiment.

Treatment for 1, 3, or 5 days with PAN (5 or 10 μg/ml) did not result in a consistent change in HSPB1 phosphorylation (Fig. 5, PAN controls), although in some experiments a minor increase was detectable at day 1 (not shown). This long-term response of cultured podocytes to PAN was different from the short-term response to PAN (typically up to 6 h) in which we observed a moderate activation of this signaling cascade (not shown), which, however, faded in longer exposures. As a positive control, cells were stimulated for 1 h with anisomycin, resulting in a pronounced shift toward the phosphorylated HSPB1 isoforms, with the doubly phosphorylated isoform being predominant (Fig. 5B). The semiquantitative evaluation (ratio of phosphorylated over nonphosphorylated HSPB1 isoforms) yielded a strong increase in the relative RP value (~16-fold) in response to anisomycin compared with the untreated control (Fig. 5D).

Inhibition of MK-2 (with C23) and p38 MAPK (with SB203580) protected the podocyte actin cytoskeleton from PAN-induced damage.
Treatment of cells for 1 day with either inhibitor alone resulted in a concentration-dependent decrease in the phosphorylation of HSPB1, with the highest tested inhibitor concentrations being most effective (nonphosphorylated isoform predominates) (Fig. 5, A and B). The calculated relative RP values in the presence of 10 μM C23 and 30 μM SB203580 at day 1 were in the range of ~0.25–0.45 (Fig. 5, C and D). Cotreatment of cells with 5 or 10 μg/ml PAN and either inhibitor had no detectable impact on the phosphorylation pattern. After days 3 and 5 of either treatment, the decrease in HSPB1 phosphorylation resulting from either inhibitor was less pronounced (Fig. 5, A and B).

Taken together, in long-term exposures of podocytes to C23 or SB352080, both inhibitors decreased the baseline activity of p38 MAPK/MK-2 signaling.

Disparate upstream and common downstream modulation of p38 MAPK/MK-2 signaling by C23 and SB203580. To further characterize the response of podocytes to either inhibitor, their effects on the activities of p38 MAPK and MK-2, and on the phosphorylation of HSPB1 were determined in short-term experiments by SDS-PAGE and IEF-PAGE followed by Western blotting (Fig. 6). In untreated cells, the phosphorylated (activated) forms of p38 MAPK and MK-2 were readily detectable using phosphorylation site-specific antibodies (Fig. 6A). For semiquantitative evaluation, the relative ratio of phosphorylated to nonphosphorylated forms of both protein kinases (as shown in Fig. 6A) was determined by densitometry and compared with the relative RP values of HSPB1 (Fig. 6A). Surprisingly, C23 induced a strong activation of p38 MAPK, seen as increased phosphorylation of both p38 MAPK and MK-2 (Fig. 6B). This increased phosphorylation of MK-2, however, was not paralleled by an increase in its activity, as the RP value of HSPB1 actually decreased slightly, consistent with Fig. 5 and with C23 being an MK-2 inhibitor.

In contrast, treatment of podocytes with SB203580 had quite different effects (Fig. 6, A and C). Treatment with this drug resulted in a temporary decrease in phosphorylation (inactivation) of both p38 MAPK and MK-2, which, however, was fully
(p38 MAPK) or partially (MK-2) restored after 2 h. A similar inhibition of p38 MAPK by SB203580 has been observed in other experimental systems (21). Consistent with Fig. 5, in this short time period SB203580 treatment resulted only in a minor decrease in the degree of phosphorylation of HSPB1, likely reflecting the low turnover rate of these HSPB1 isoforms.

Taken together, the MK-2 and p38 MAPK inhibitors used exhibited quite disparate effects on the upstream signaling, while they exhibited a similar effect (decrease) on HSPB1 phosphorylation (cf. Fig. 5). This suggests the possibility that reduction of HSPB1 phosphorylation may have an important role in the protection of podocytes from PAN injury. This also implies that phosphorylated HSPB1 may contribute to podocyte injury in response to PAN.

**Activation of podocyte p38 MAPK/MK-2 signaling by SA.** While PAN is an experimental toxin commonly used to mimic NS-related injury, during the proteinuria associated with NS in humans, podocytes are exposed to increased concentrations of several potentially injurious serum components. Some of these are known activators of p38 MAPK in podocytes or other cells, including SA and TGF-β (16, 50, 60, 68). In short-term experiments, we thus determined whether exposure of podocytes to concentrations of SA likely to be achieved during glomerular proteinuria (i.e., 35–55 mg/ml) can activate the p38 MAPK/MK-2 signaling cascade and whether this response can be blocked by C23 or SB203580 (Fig. 7). Treatment of serum-starved podocytes with 50 mg/ml SA for 1 h activated the p38 MAPK/MK-2 cascade, resulting in increased phosphorylation of p38 MAPK (Fig. 7A) and HSPB1 (Fig. 7B), although at the level of HSPB1 this response typically was weaker than the response to anisomycin. Preincubation with C23 inhibited the response to SA at the level of HSPB1, but not at the level of p38 MAPK, which is consistent with the activating effect of C23 upstream of HSPB1 (cf. Fig. 6). Similar to C23, SB203580 inhibited SA-induced HSPB1 phosphorylation. For controls, cells were treated with C23 or SB203580 alone, resulting in a minor decrease in HSPB1 phosphorylation to below the control level observed in untreated cells. Calculation of the relative RP values obtained from the blot shown in Fig. 7B supported the above findings (Fig. 7C).

In summary, inhibition of MK-2 or p38 MAPK effectively inhibited SA-induced activation of this signaling pathway in podocytes. Given the known adverse effects of SA on podocytes (68), inhibition of the p38 MAPK/MK-2 signaling pathway at either level with substances like C23 or SB203580 may thus represent a novel therapeutic strategy to protect podocytes from SA-induced injury during glomerular proteinuria.

**Dependence of Cox-2 mRNA expression on p38 MAPK/MK-2 signaling.** Regulation of gene expression, be it through phosphorylation of transcription factors or mRNA-processing proteins, represents a known downstream activity of the p38 MAPK/MK-2 pathway (18). In addition, abnormal expression of Cox-2 has been previously shown to be critical for susceptibility of podocytes to injury, and p38 MAPK has been implicated in the regulation of expression of this gene (10, 39).

We therefore chose to determine whether Cox-2 expression in podocytes is dependent on MK-2 or p38 MAPK activity. Using quantitative RT-PCR, the data in Fig. 8A show that the amount of the baseline level of Cox-2 mRNA is significantly reduced, relative to actin, in the presence of either C23 or SB203580.

With SA activating the p38 MAPK/MK-2 pathway (cf. Fig. 7), we also determined its effects on Cox-2 expression in podocytes. Figure 8B shows that SA greatly induced Cox-2 in a concentration-dependent manner (−10- and ~30-fold increases by 5 and 50 mg/ml SA, respectively). In addition, we examined the effects of inhibition of the p38 MAPK/MK-2 pathway by C23 and SB203580 on this SA-mediated Cox-2 induction. As expected, in the presence of C23 or SB203580, the SA-mediated Cox-2 induction was significantly reduced. The relative inhibition of Cox-2 induction was more pronounced following induction with 5 mg/ml SA compared with induction with 50 mg/ml SA. This suggests that strong inducers like 50 mg/ml SA can partially override the effects of these inhibitors, similar to what we observed in podocytes treated with anisomycin (cf. Fig. 2) or in mesangial cells treated with cadmium (27).

These findings demonstrate that inhibition of both MK-2 and p38 MAPK is able to reduce the expression of at least one gene with direct relevance to podocyte injury and suggest that inhibition of MK-2 or p38 MAPK may represent a viable approach to protect podocytes from the adverse effects of Cox-2 induced by SA.
The therapeutic concepts are those focusing on protecting podocytes from NS-related injury. Among the most popular are cyclophosphamide, mycophenolate, and calcineurin inhibitors. Although most children and adults presenting with NS respond to glucocorticoids, the clinical outcome is often not satisfactory, given the high frequency of relapses, the high proportion of steroid-resistant patients, and the significant side effects of this treatment (28, 36). Alternative medications such as cyclophosphamide and calcineurin inhibitors exhibited disparate upstream effects, also supporting a critical role of HSPB1, or of another substrate of MK-2, in podocyte injury and protection. However, the exact role of phosphorylated HSPB1 in renal disease remains to be determined.

Interestingly, inhibition of HSPB1 phosphorylation was most pronounced on day 1 of treatment, but gradually faded at days 3 and 5 (cf. Fig. 5). Despite this, increased cell survival was observed in the presence of these inhibitors at days 3 and 5 (cf. Fig. 3). Similarly, short-term PAN treatment (up to 6 h) of cultured mouse podocytes has been reported to activate p38 MAPK (31), consistent with our own findings (data not shown). However, we could not detect any notable PAN-induced increases in p38 MAPK/MK-2 signaling after 1 day (cf. Fig. 5). Such a situation is not uncommon, as early phosphorylation events are known to cause late biological adverse effects of HSPB1 on cell viability, particularly in oxidative stress situations (6, 38). Renal tubular cells of HSPB1-overexpressing mice showed increased injury following ischemia-reperfusion, although this effect seemed to result from a systemic response by exacerbating renal inflammation (9). Given the well-known proapoptotic effects of p38 MAPK signaling (12), an association of phosphorylated HSPB1 with cell injury can be expected in many experimental settings and diseases including renal conditions with increased p38 MAPK signaling. The fact that decreased phosphorylation of HSPB1 was a common effect between C23 and SB203580, while both inhibitors exhibited disparate upstream effects, also supports a critical role of HSPB1, or of another substrate of MK-2, in podocyte injury and protection. However, the exact role of phosphorylated HSPB1 in renal disease remains to be determined.

In the current study, we demonstrate that inhibition of MK-2 and p38 MAPK by C23 and SB203580, respectively, was effective in protecting podocytes against NS-related injury in terms of both cell viability and integrity of the actin cytoskeleton. Downstream of MK-2, these inhibitors decreased HSPB1 phosphorylation and baseline Cox-2 mRNA expression. We also found that exposure of podocytes to normal serum concentrations of SA activated p38 MAPK/MK-2 signaling and that this response was blocked by both inhibitors. In addition, the induction of Cox-2 expression by SA was significantly reduced in the presence of these inhibitors at 5 or 50 mg/ml SA for 2 h before RNA extraction. Both C23 and SB203580 significantly reduced the induction of Cox-2 by either concentration of SA. Cox-2 mRNA was quantified by SYBR green RT-PCR, normalized to β-actin mRNA, and plotted as fold-change. Values are means ± SD of triplicate samples of 1 representative experiment of 3 independent experiments. Significant differences compared with the control values are indicated with asterisks (*), and significant differences compared with the respective SA-alone treatment are indicated by plus signs (+). SA 5 = 5 mg/ml; SA 50 = 50 mg/ml SA.

**DISCUSSION**

Involvement of p38 MAPK/MK-2 signaling in cyclooxygenase (Cox)-2 mRNA expression in podocytes. A: decrease in baseline expression of Cox-2 mRNA in podocytes upon inhibition of p38 MAPK/MK-2 signaling. Cells were left untreated (control), or were treated with 10 μM C23 or 10 μM SB203580 for 24 h before RNA extraction. B: induction of Cox-2 by SA and its inhibition by C23 or SB203580. Serum-starved cells were left untreated (control), or were pretreated with 10 μM C23 or 30 μM SB203580 for 1 h followed by treatment with 5 or 50 mg/ml SA for 2 h before RNA extraction. Both C23 and SB203580 significantly reduced the induction of Cox-2 by either concentration of SA. Cox-2 mRNA was quantified by SYBR green RT-PCR, normalized to β-actin mRNA, and plotted as fold-change. Values are means ± SD of triplicate samples of 1 representative experiment of 3 independent experiments. Significant differences compared with the control values are indicated with asterisks (*), and significant differences compared with the respective SA-alone treatment are indicated by plus signs (+). SA 5 = 5 mg/ml; SA 50 = 50 mg/ml SA.
compounds inhibit MK-2 with thiophene-type compounds (Pfizer) (3–5, 41). Some of these (63), pyrrolopyridine-type (which includes C23), and benzo-and various aminocyanopyridine-type (2), pyrimidine-type compounds (Abbott Laboratories) (7, 24), (26), benzopyranopyridine-type compounds (15), 2,4-diamin-(Pharmacia) (65), undisclosed compounds (Schering/Bayer) phen, oxazole, thiazole, triazole, and imidazole compounds pounds include a series of derivatives of pyrrole, furan, thio- rendered cell membrane permeable (37). In the last decade, consequences. Examples include the transient activation (and phosphorylation) of ERK by EGF in PC12 cells that leads eventually to cell proliferation (62), and the transient phosphorylation of the transcription factor AP-1 leading to the initiation of differentiation of human embryonic stem cells (64).

Another mechanism that could play a role in podocyte injury is the regulation of Cox-2 expression by the p38 MAPK/MK-2 pathway. p38 MAPK is known to increase Cox-2 expression through activation of transcription factors, e.g., C/EBP and CREB/ATF (29). While Cox-2 enzyme activity is important for podocyte survival, its upregulation has been reported to increase the susceptibility of podocytes to injury by PAN (10). In addition to regulating transcription, the p38 MAPK/MK-2 pathway has also been shown to regulate the stability of AU-rich element (ARE)-containing mRNAs (which includes Cox-2 mRNA) by altering the phosphorylation status of tris-tetraprolin and possibly HSPB1 (18, 54). At this time, it is unclear whether the regulation of Cox-2 expression in podocytes is achieved primarily by regulation of the synthesis or the stability of the Cox-2 mRNA. The data in Fig. 8 suggest that p38 MAPK/MK-2 is a major pathway in the regulation of both the baseline expression and SA-mediated induction of Cox-2. This finding is consistent with published data on the involvement of p38 MAPK in the regulation of Cox-2 expression in podocytes in response to the prostaglandin PGE2 (17). In summary, our findings on Cox-2 expression provide a plausible molecular explanation for the protective effects of C23 and SB203580 on podocytes.

Elevated SA is a suspected stress factor for podocytes during NS, and it is also known to activate p38 MAPK (68). Our data in Fig. 7 revealed that SA activated MK-2 downstream of p38 MAPK, as measured by increased phosphorylation of HSPB1. Although the observed response to SA was moderate, a similarly low degree of p38 MAPK activation was found to be sufficient for the effective phosphorylation of e.g., transcription factors, and can be expected to be physiologically relevant (44). Thus SA has the potential to damage podocytes through activation of p38 MAPK/MK-2/HSPB1 signaling. Consequently, substances like C23 or SB203580 may have the potential to protect podocytes from SA-induced injury.

In recent years, considerable efforts were invested in the development of MK-2 inhibitors. Initially, a specific inhibitory peptide directed to the peptide binding site of MK-2 was designed on the basis of the consensus substrate recognition motif of MK-2 (25), and a modified version of this peptide was rendered cell membrane permeable (37). In the last decade, several secondary metabolite compounds were isolated that inhibit MK-2 (23, 67). Recently developed synthetic compounds include a series of derivatives of pyrrole, furan, thiophen, oxazole, thiazole, triazole, and imidazole compounds (Pharmacia) (65), undisclosed compounds (Schering/Bayer) (26), benzopyranopyridine-type compounds (15), 2,4-diaminopyrimidine-type compounds (Abbott Laboratories) (7, 24), and various aminocyanopyridine-type (2), β-carboline-type (63), pyrrolopyridine-type (which includes C23), and benzo-thiophene-type compounds (Pfizer) (3–5, 41). Some of these compounds inhibit MK-2 with Kᵢ values in the nanomolar range. Which of these compounds eventually may lead to a clinically applicable drug is not clear at this time.

In addition to p38 MAPK/MK-2 signaling, other MAPKs including ERK1, ERK2, ERK5, or JNK also play roles in renal disease, as activation of these pathways has been demonstrated in various models of renal failure (22). Thus pharmacological inhibition of MAPK signaling (including their downstream effectors) appears to have the potential to become a promising future therapy for various acute and chronic renal diseases.

While this cell-biological study has demonstrated the potential utility of MK-2 inhibition to protect podocytes from NS-related injury, it should be noted that such in vitro studies have intrinsic limitations with respect to a therapy at the level of a whole organism. This concept should be verified in separate follow-up studies in suitable animal models of NS.

In summary, our data suggest that p38 MAPK/MK-2 signaling is involved in NS-related injury of podocytes and that inhibition of this signaling cascade is able to protect podocytes from injury. In addition, podocyte exposure to normal serum concentrations of SA (known to induce podocyte injury) activated podocyte p38 MAPK/MK-2 signaling, and this response was blocked by both inhibitors. Both inhibitors also reduced MK-2-mediated Cox-2 mRNA expression and HSPB1 phosphorylation, suggesting these processes as candidate molecular mechanisms. In light of the central role of podocyte injury in NS as well as other progressive glomerular diseases, these findings identify a critical role for p38 MAPK/MK-2 signaling in podocyte injury and suggest that MK-2 inhibition may be a promising potential therapeutic strategy to protect podocytes in a variety of glomerular disorders.

ACKNOWLEDGMENTS

We thank Pfizer, Inc. (St. Louis Laboratories, Chesterfield, MO) for kindly providing us with the MK-2 inhibitor C23.

GRANTS

This work was supported in part by National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases (NIH/NIDDK) Grants R01 DK077283 and R56 DK55602 to W. E. Smoyer, R01 DK075533 to R. F. Ransom, and NIH Grant U01 GM092655 (Principal Investigator: W. Sadee, Columbus, OH).

DISCLOSURES

R. J. Mourey owns stock in Pfizer, Inc., where he was employed. None of the other coauthors have competing financial interests.

REFERENCES

INHIBITION OF MK-2 SIGNALING IN PODOCYTES


17. F518 INHIBITION OF MK-2 SIGNALING IN PODOCYTES


19. F518 INHIBITION OF MK-2 SIGNALING IN PODOCYTES


Acknowledgments of funding agencies and/or clinical centers are acknowledged.