p38 MAP kinase mediates mechanically induced COX-2 and PG EP₄ receptor expression in podocytes: implications for the actin cytoskeleton

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PODOCYTES ARE HIGHLY differentiated epithelial cells that form the final barrier to protein in the glomerular capillary bed. Normally, the podocyte foot processes that extend from each cell body counteract the pulsatile distensive forces exerted on the capillary walls within each glomerular tuft. However, pathophysiological conditions that increase intraglomerular capillary pressure (Pgc), such as hypertension or reduced nephron mass, expose these cells to cycles of abnormal mechanical load. The podocytes are thought to respond to such forces by undergoing aberrant morphological changes, hyper-trophy, or detachment from the glomerular basement membrane. This damage compromises the filtration barrier, thereby giving rise to proteinuria, a key predictor of progressive renal failure (3, 6, 18, 19). The pressure-induced intracellular responses underlying these deleterious events are poorly understood and are only now being characterized.

Experimental models of elevated Pgc in vivo revealed increased expression of the inducible isoform of cyclooxygenase (COX-2) in podocytes. For example, in rats with subtotal renal ablation, glomerular COX-2 expression was upregulated in mesangial cells and podocytes concomitant with increased Pgc (37). Such increased COX-2 expression might therefore translate into elevated intraglomerular prostaglandin (PG) levels capable of acting in an auto- and/or paracrine manner. PGs may compromise the effectiveness of the permselectivity barrier by inducing remodeling of the actin cytoskeleton within the podocyte foot processes through a mechanism similar to that conferred by PG-stimulated vasodilatation (28).

We recently demonstrated COX-2 expression and PGE₂ production in a conditionally immortalized mouse podocyte cell line (23). PGE₂ is the major renal COX metabolite and can interact with at least four G protein-coupled E-prostanoid (EP) receptor subtypes designated EP₁, EP₂, EP₃, and EP₄. Bek and co-workers (4) demonstrated the expression and signaling characteristics for both EP₁ and EP₄ receptors in this podocyte cell line. Whether podocyte expression of COX isoforms and EP receptor subtypes is subject to mechanical stress has not been investigated.

The increase in distension forces acting on podocytes as a result of increased Pgc can be mimicked in vitro by subjecting cells grown on a flexible substratum to cyclical strain. Recent studies employing this type of model showed that podocytes respond to mechanical stimulation by reducing proliferation rates, reorganizing their actin cytoskeleton, and enhancing gene expression (10, 11, 30). In vitro mechanical stimulation therefore represents a useful experimental approach for investigating the interplay between mechanical stress, the expression of COX-2 and other components of this eicosanoid pathway, and their effects on the actin cytoskeleton.

The purpose of the present study was to characterize mechanically regulated changes in expression of COX isoforms and EP receptor subtypes, as well as mechanically dependent changes in cytoskeletal dynamics driven by PGE₂ signaling. We show stretch-induced upregulation of COX-2 and EP₄ receptor expression, dependent on p38 MAPK signaling. Also,

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we demonstrate that increased EP4 receptor expression renders the actin cytoskeleton susceptible to disruption by PGE2. Our data support that, under conditions of increased PGcs, increased PGE2 signaling via the EP4 receptor may perturb podocyte cytoskeletal dynamics thereby undermining the integrity of the filtration barrier.

**MATERIALS AND METHODS**

**Cell culture and mechanical stimulation.** Culture of conditionally immortalized mouse podocytes, kindly provided by Dr. P. Mundel, was carried out as described in detail previously (25). Briefly, cells were grown on type I collagen-coated plastic tissue culture dishes in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Podocytes were routinely propagated in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Podocytes were maintained at 37°C, under conditions of increased PGcs, increased PGE2 signaling via the EP4 receptor may perturb podocyte cytoskeletal dynamics thereby undermining the integrity of the filtration barrier.

**Western immunoblotting.** Differentiated podocytes seeded on BioFlex plates, serum-starved overnight, and cyclically stretched to 5% elongation at 0.5 Hz for 10 min, 6 h, or 24 h, as well as time-matched, nonstretched controls, were harvested by rinsing twice with 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.5 mM PMSF) with a protease inhibitor cocktail (pem-

**Fig. 1.** Stretch increases cyclooxygenase-2 (COX-2) and E-prostanoid (EP4) receptor mRNA expression. Differentiated podocytes cultured on flexible silicone membranes were serum-starved overnight and then mechanically stimulated for 2 h with +5% equibiaxial surface elongation applied cyclically at 0.5 Hz with a sinusoidal waveform. Total RNA was extracted and analyzed by real-time RT-PCR using specific TaqMan primer/probe sets as indicated. Values have been normalized to GAPDH content and are presented as fold of respective nonstretched controls. *P < 0.01 vs. respective nonstretched control (n = 7).
RNA was extracted and analyzed by real-time RT-PCR using specific primer/probe sets for either COX-2 or EP4 receptor. Differentiated podocytes cultured on flexible membranes were serum-starved overnight and then cyclically stretched 5% for 1 to 24 h. Total expression was normalized to GAPDH content and are presented as fold of respective non-stretched controls.

Reactions were terminated with the addition of statin A, bestatin, leupeptin, and aprotinin; Sigma) and phosphatase standard. Samples containing 50 μg of total cellular protein were diluted with 2× Laemmli buffer, electrophoresed on 8% resolving gels, and electrotransferred to nitrocellulose membranes (Amer sham Pharmacia Biotech, Baie d’Urfe, QC). Membranes were probed with rabbit antibodies directed against either COX-1 or COX-2 (1:250 dilution; Cayman Chemical, Ann Arbor, MI), actin (1:1,000 dilution; Sigma), or a rabbit antibody that recognizes the phosphorylated (activated) form of p38 MAP kinase (1:500 dilution of anti-active p38; Promega). After incubation with an appropriate horseradish peroxidase-conjugated secondary antibody (1:5,000 to 1:40,000), blots were incubated in chemiluminescent substrate (Pierce, Rockford, IL) and exposed to blue light-sensitive film. Densitometric analyses of scanned blots were carried out using Kodak 1D version 3.5 software.

cAMP assay. Differentiated podocytes seeded on BioFlex plates and serum-starved overnight were incubated for 30 min with RPMI-1640 containing 20 μM indomethacin before being cyclically stretched to 5% elongation at 0.5 Hz for 6 h or incubated 6 h without stretch. Cells were then incubated for 30 min with HBSS + 0.05% BSA containing 0.5 mM isobutylmethylxanthine (IBMX; Sigma) to inhibit phosphodiesterase activity, with or without other agents as specified. Incubations were next carried out for 10 min in HBSS + 0.05% BSA containing 0.5 mM IBMX with or without 1 μM PGE2 (Cayman Chemical). Reactions were terminated with the addition of ice-cold 10% (vol/vol) trichloroacetic acid and after 30 min at 4°C each sample was washed four times with 4 vol of water-saturated diethyl ether and brought to pH 7.4 with Tris base. The cAMP content was determined with a radioimmunoassay kit (Research Diagnostics, Los Angeles, CA). Values were normalized to total cellular protein per well as determined by Bradford protein assay with BSA as a standard.

Immunofluorescence of the actin cytoskeleton. Differentiated podocytes seeded on BioFlex plates and serum-starved overnight were incubated for 30 min with RPMI-1640 containing 20 μM indomethacin before being cyclically stretched to 5% elongation at 0.5 Hz for 6 h or being incubated for 6 h without stretch. Cells were then treated with 1 μM PGE2 for 90 min, fixed for 30 min in 4% paraformaldehyde in PBS, and were then rinsed with PBS, incubated in 50 mM NH4Cl in PBS for 15 min, and permeabilized by incubation with 0.2% Triton X-100 in PBS for 15 min at 25°C. Cells were rinsed with PBS and blocked with 2% BSA/0.1% Triton X-100/PBS for 15 min, followed by incubation with 0.5 μg/ml FITC-phalloidin (Sigma) for 1 h. The wells were then washed with 0.1% Triton X-100/PBS, and the silicone membranes were excised from the plates and mounted on slides using Vectashield medium (Vector Laboratories, Burlingame, CA) for analysis by fluorescence microscopy at an excitation wavelength of 492 nm and an emission of 520 nm using a Zeiss Axioskop 2 fluorescence microscope. Images were captured with a Zeiss AxioCam and the number of cells displaying robust actin filaments vs. those with a diffuse pattern was counted in a blinded manner.

RESULTS

Stretch increases COX-2 and EP4 receptor mRNA expression. To mimic the pressure cycles experienced by podocytes in vivo, a conditionally immortalized mouse podocyte cell line was cultured on flexible collagen I-coated silicone membranes and subjected to 5% biaxial mechanical strain at 0.5 Hz for 2 h, and the mRNA expression of EP receptor subtypes, COX-1, and COX-2 was determined by real-time RT-PCR. The degree of stretch was chosen to approximate pressures experienced by podocytes in vivo (29, 32). Stretch increased both COX-2 (3.3 ± 0.6-fold of nonstretched control, P < 0.01) and EP4
Cyclical stretch (Fig. 2, before approaching baseline levels at 24 h of continuous response to 8% stretch (data not shown). Stretch for 2 h, and maximal induction was observed in significantly increased with as little as 1% cyclical levels were significantly increased with as little as 1% cyclical stretch for 2 h, and maximal induction was observed in response to 8% stretch (data not shown).

**Time course of stretch-induced COX-2 and EP4 receptor upregulation.** Stretch-induced upregulation of COX-2 and EP4 receptor mRNA levels was found to peak at 2 h of continuous cyclical 5% stretch (P < 0.001 vs. nonstretched control), before approaching baseline levels at 24 h of continuous cyclical stretch (Fig. 2, A and B). COX-2 and EP4 mRNA levels were significantly increased with as little as 1% cyclical stretch for 2 h, and maximal induction was observed in response to 8% stretch (data not shown).

**Induction of COX-2 and EP4 expression by stretch is blocked by actinomycin D.** The observed increases in COX-2 and EP4 receptor mRNA levels may derive from changes in regulation at the transcriptional or posttranscriptional level. To determine whether inhibition of transcription would block the stretch-induced expression of COX-2 and/or EP4 receptor mRNA, podocytes were incubated with actinomycin D for 6 h. Treatment with actinomycin D completely abolished the stretch-induced increase in both COX-2 and EP4 receptor mRNA expression (Fig. 3).

**Stretch increases COX-2 protein expression.** To determine whether increased COX-2 mRNA levels were followed by elevated protein expression, podocytes were stretched for 6 h and the cell lysates were assayed by Western blot analysis for immunoreactive COX. Stretch increased the expression of COX-2 protein following 6 h of stretch, whereas COX-1 protein expression was unchanged by stretch (Fig. 4). Interestingly, mechanical stretch elicited a minor increase in both arachidonic acid and PGE2 levels (i.e., <25% above nonstretched controls, data not shown), implying that other factors in addition to stretch might be required to realize the potential of COX-2 expression. Alternatively, these modest effects might be attributable to the culture conditions employed.

**Stretch increases PGE2-mediated cAMP synthesis.** To determine whether increased EP4 receptor mRNA levels were followed by elevations in functional receptor expression,
PGE₂-induced cAMP production was assayed following stretch. The EP₄ receptor subtype is coupled to adenyl cyclase through a stimulatory G protein (13). Cells cyclically stretched 5% for 6 h, followed by incubation with 1 μM PGE₂ for 10 min, yielded threefold more intracellular cAMP compared with nonstretched cells treated with PGE₂ (P < 0.001; Fig. 5).

Induction of COX-2 and EP₄ expression by stretch is blocked by p38 MAP kinase inhibition. The p38, ERK, and JNK families of MAP kinases are activated by mechanical stress in various cell types, including mesangial cells (14, 15, 27), rat glomerular epithelial cells (unpublished data), and mouse podocytes (29). To determine whether these pathways are required for stretch-induced COX-2 and EP₄ receptor
expression in podocytes, COX-2 mRNA and protein, as well as EP<sub>4</sub> receptor mRNA and PGE<sub>2</sub>-induced cAMP production, were assessed in cells cyclically stretched in the presence or absence of either p38 inhibitor (SB-202190; 25 μM), MEK inhibitor (PD-98059; 25 μM), or JNK inhibitor (SP-600125; 25 μM). The p38 inhibitor significantly reduced the stretch-induced COX-2 and EP<sub>4</sub> receptor mRNA expression (P < 0.01 vs. stretched vehicle control; Fig. 6A) and similarly reduced stretch-induced COX-2 protein levels (P < 0.05 vs. stretched vehicle control; Fig. 6B) and PGE<sub>2</sub>-stimulated cAMP production (P < 0.01 vs. stretched vehicle control; Fig. 6C). Inhibitors of the ERK or JNK pathways had no statistically significant effects, although PD-98059 did partly reduce EP<sub>4</sub> receptor mRNA and COX-2 protein induction following stretch. Consistent with these findings, podocytes that were stretched for 10 min exhibited increased phosphorylation of p38 MAPK, as detected by an antibody that specifically recognizes the phosphorylated/activated form of the enzyme (Fig. 6D) (31). Phosphorylation could be prevented by the p38 MAPK inhibitor (SB-202190) but not by the JNK inhibitor (SP-600125). A slight reduction in phospho-p38 levels was observed in cells treated with the MEK inhibitor (PD-98059; 25 μM), suggesting that this compound might exert subtle, yet non-specific, effects on p38 MAPK activation.

**PGE<sub>2</sub> induces actin stress fiber dissociation following mechanical stress.** It has been suggested that vasoactive eicosanoids such as PGE<sub>2</sub> can alter the morphology of glomerular podocytes, thereby modifying the periswiectivity barrier (8, 20, 21). Such effects likely involve modification of the podocyte actin cytoskeleton. To determine whether increased EP<sub>4</sub> receptor expression and signaling can influence actin cytoskeletal dynamics, murine podocytes were cyclically stretched for 6 h and then stimulated with PGE<sub>2</sub> (1 μM, 90 min). Actin stress fibers were visualized in fixed/permeabilized cells using FITC-conjugated phalloidin under fluorescence microscopy. The elaborate network of actin stress fibers characteristic of this cell line remained intact following mechanical stimulation, or in response to PGE<sub>2</sub> in non-stretched cells (Fig. 7A). However, PGE<sub>2</sub> stimulation of stretched cells resulted in a dramatic loss of actin stress fiber organization, as exhibited by a diffuse pattern of fluorescence (Fig. 7A) in >70% of stretched podocytes (Fig. 7B).

**DISCUSSION**

By employing an in vitro model of cell stretch, the present study investigated the interplay among mechanical stress, COX and EP receptor expression, and the actin cytoskeleton in podocytes. In vivo, the podocyte foot processes are subjected to continuous cycles of mechanical strain as these epithelial cells counteract the distensive forces acting on the glomerular wall. However, under conditions of glomerular hypertension, an increased P<sub>sc</sub> coupled with exposure to vasoactive hormones, such as locally produced prostaglandins, may challenge the ability of the foot processes to withstand mechanical stress, thereby resulting in proteinuria (26). Support for this notion comes from the finding that both mesangial cell and podocyte COX-2 expression are upregulated following increases in P<sub>sc</sub> brought about by subtotal renal ablation in rats (37) and that the associated proteinuria can be prevented by inhibiting COX-2 (36). Our present data are consistent with these observations and show that podocytes cultured in vitro respond to mechanical stress by enhancing the expression of both COX-2 and the EP<sub>4</sub> receptor but not that of COX-1, EP<sub>1</sub>, EP<sub>2</sub>, or EP<sub>3</sub>, thereby suggesting that the EP<sub>4</sub> receptor is a key mediator for COX-2-derived PGE<sub>2</sub> in the glomerulus under conditions where P<sub>sc</sub> is elevated. On the other hand, the transient nature of this upregulation (Fig. 2) and the small increase in PGE<sub>2</sub> synthesis in cultured cells suggest that other factors encountered in vivo may be required to sustain the expression of COX-2 and/or the EP<sub>4</sub> receptor following changes in mechanical load.

Mechanical forces applied to podocytes can have significant impact on gene expression (11) and can reduce proliferation (30). Such effects suggest that numerous signaling cascades are activated in podocytes by mechanical stress. We investigated the signal transduction pathway by which stretch elevates EP<sub>4</sub> and COX-2 expression. Our findings implicate p38 MAP kinase activation in mediating the levels of these gene products. The p38 MAP kinase pathway regulates COX-2 expression in a number of cell types in response to a variety of stimuli. For example, Faour et al. (12) showed that IL-1β-stimulated COX-2 expression is p38 MAP kinase dependent in fibroblasts, whereas Cheng et al. (7) demonstrated that extracellular Cl<sup>-</sup> can increase COX-2 expression in a p38-dependent manner in rabbit cortical thick ascending limb cells. Mechanical stretch upregulates COX-2 expression in mesangial cells via protein kinase C, although a role for p38 MAP kinase was not ruled out in those studies (1). Our results are the first to link mechanical stress with COX-2 expression via the p38 MAP kinase signaling cascade.

Although a variety of factors have been shown to regulate COX-2 levels, little is known regarding those affecting EP<sub>4</sub> receptor expression. Our data are the first to implicate p38 MAP kinase activation in EP<sub>4</sub> receptor expression initiated by mechanical stress. The fact that both COX-2 and EP<sub>4</sub> share similar time courses and require p38 activation suggests the involvement of concurrent regulatory mechanisms. Actinomycin D completely abrogated stretch-induced COX-2 and EP<sub>4</sub> receptor mRNA increases, thereby suggesting that control of expression is exerted at a transcriptional level rather than posttranscriptionally (i.e., increased mRNA stability or translational efficiency). Future experiments will elucidate the specific gene promoter elements subject to mechanical stretch. An initial examination of the COX-2 and EP<sub>4</sub> promoter regions (2) reveals the presence of several shared putative cis-acting elements, including NF-IL6 (C/EBP<sub>α</sub>) (35), c-Jun through v-Src (39–41), C/EBPδ (16), and NF-κB (42).

The elaborate morphology of the podocyte is upheld by a cytoskeletal framework comprising a number of components including actin. The actin network in each podocyte is dynamic, and remodeling has been shown to occur in response to mechanical stimulation. Studies by Endlich et al. (10) show that podocytes cultured in vitro rearrange their actin cytoskeleton into radial stress fibers in response to chronic application of mechanical stress. Our results indicate that actin stress fiber organization observed in stretched cells is disrupted by PGE<sub>2</sub>. This EP<sub>4</sub> receptor-mediated effect on the podocyte cytoskeleton might hinder adaptations to mechanical stress and is consistent with a growing body of evidence suggesting that podocyte cell morphology...
and actin assembly are influenced by cAMP (9). Early studies of rat glomerular epithelial cells grown on plastic demonstrated that cAMP mimetics cause rearrangement of the actin cytoskeleton into a distinct stellate pattern (33). Our findings are therefore highly relevant because podocyte foot processes are known to contain high concentrations of actin, and several reports documented an abnormal distribution and disaggregation of podocyte actin microfilaments during the development of foot process effacement in vivo (17, 22, 38). Upregulation of EP4 receptor signaling in podocytes may therefore play a critical role in pressure-induced morphological changes associated with proteinuria. However, we must temper such conclusions because the progression of podocyte foot process lesions in some diseases can yield an enrichment of F-actin density. For example, a rat model of focal and segmental glomerulosclerosis is characterized by effacement and proteinuria that correlate with increased foot process F-actin content (34). Similarly,

Fig. 7. PGE2-induced actin stress fiber dissociation following stretch. Differentiated podocytes cultured on flexible membranes were serum-starved overnight and then cyclically stretched 5% for 6 h. Cells were then preincubated for 30 min with 20 μM indomethacin and subsequently incubated with vehicle or 1 μM PGE2 for 90 min. Cells were fixed and processed for fluorescence microscopy using FITC-conjugated phalloidin (0.5 μg/ml). A: magnification ×400. B: 100 cells were analyzed under fluorescence microscopy for each condition to determine the number of cells lacking organized actin stress fibers. *P < 0.001 vs. nonstretched + PGE2 (n = 3).
the role of PGE2 in modulating podocyte structure/function is also likely to be subject to the prevailing disease etiology, as some studies showed that oxidative stress-mediated podocyte damage can be attenuated by this eicosanoid through its induction of Stra13 (5). In light of these observations, future studies should be aimed at more clearly defining the interactions between cytoskeleton and PGE2/EP receptors in vivo, specifically in disease contexts involving Pgc changes, while taking into account other signaling influences.

In summary, our data demonstrate that both EP3 receptor and COX-2 expression in mouse podocytes are upregulated by mechanical stimulation. Enhanced PGE2 signaling may be a facilitating event for morphological changes and might directly influence podocyte function under pathophysiological conditions that promote PGE2 synthesis.

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REFERENCES


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