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Cyclooxygenase-2, prostaglandin E$_2$, and prostanoid receptor EP2 in fluid flow shear stress-mediated injury in the solitary kidney

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A NORMAL GLOMERULAR FILTRATION rate (GFR) is important for maintaining homeostasis. Adaptive changes in glomerular hemodynamics start even before metabolic and clinical signs of chronic kidney disease (CKD) begin to manifest. In children born with congenital anomalies of the kidney and urinary tract (CAKUT) including a solitary kidney, the decrease in functional nephron mass leads to adaptive hyperfiltration. Hyperfiltration involves increased renal blood flow, glomerular capillary pressure (P$_{gc}$), single-nephron GFR (SNFGR), filtration fraction, and decreased hydraulic conductivity associated with glomerular hypertrophy (4, 5). A considerable number of children born with a solitary kidney develop albuminuria during adolescence and progress to renal end-stage disease (ESRD) as young adults (34, 51–53). Investigating the interplay of mechanical forces on podocytes within the glomerulus may provide a better understanding of hyperfiltration-mediated kidney injury.

Podocytes are exposed to mechanical forces such as tensile stress as a result of capillary wall stretch and fluid flow shear stress (FFSS) due to the flow of the ultrafiltrate (11, 17, 40, 41). Glomerular capillary pressure creates tensile stress on the capillary wall, which leads to stretching of the podocyte foot processes that tightly cover the capillary. The tensile stress (or stretch) is exerted over the basolateral aspect of the podocyte from the stress in the vascular compartment that is studied in vitro using a biaxial elongation (or substrate stretch) model (11). The flow of glomerular ultrafiltrate creates shear stress on the surface of the podocyte and causes cellular deformation. FFSS, largely exerted over the exposed outer aspect of major processes and the soma of podocytes, is studied in vitro using a flow chamber (17, 41). Differences between the effects of these forces are not well understood, but FFSS appears to mediate greater change in cell morphology compared with stretch (27).

Previously, we showed that the calculated FFSS over podocytes is increased 1.5–2.0-fold in unilateral nephrectomy animal models (40). FFSS applied to podocytes in vitro resulted in an altered actin cytoskeleton, upregulation of cyclooxygenase (COX)-2, and increased secretion of PGE$_2$ (41). PGE$_2$ is known to modulate glomerular hemodynamics and permeability (26, 37). We hypothesized that a hyperfiltration-induced...
increase in FFSS over podocytes increases PGE2 synthesis, which alters the filtration barrier, leading to albuminuria, kidney injury, and progression of CKD. To investigate the role of COX-2 and PGE2 in hyperfiltration-mediated kidney injury, we studied the effect of FFSS on 1) cultured podocytes in vitro, 2) isolated decapsulated rat glomeruli, and 3) sv129 mice following unilateral nephrectomy. This report highlights the significance of the COX2-PGE2-EP2 axis in podocytes exposed to FFSS. FFSS-mediated changes could be the mechanistic basis for albuminuria and progression of CKD in hyperfiltration-mediated kidney injury. We believe this COX-2-PGE2-EP2 axis is a potential target for developing interventions to prevent CKD progression in CAKUT.

MATERIALS AND METHODS

Animals. Studies involving rats and mice were carried out using protocols approved by the Institutional Animal Care and Use Committee (IACUC), Safety Subcommittee, and the R&D Committee at the Veterans Affairs Medical Center (Kansas City, MO). Male Sprague-Dawley rats (7–8 wk old) from Harlan (Madison, WI) and male sv129 mice (13–14 wk) from Charles River (Indianapolis, IN) were obtained. Animals were maintained at Association for Assessment and Accreditation of Laboratory Animal Care-approved facilities with unrestricted access to food and water under 12:12-h light-dark cycles.

Cell culture. Conditionally immortalized mouse podocyte line (kind gift from Peter Mundel) with thermosensitive tsA58 mutant T antigen was used in these studies. Podocytes were propagated in RPMI 1640 with l-glutamine supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Invitrogen, Carlsbad, CA) under permissive conditions (33°C with 10 U/ml of γ-interferon, Cell Sciences, Norwood, MA). To induce differentiation, cells were transferred to nonpermissive conditions (37°C without γ-interferon). Three glass slides (25 × 75 × 1 mm, Fischer Scientific, Pittsburgh, PA) were maintained in one culture dish containing 12 ml of culture medium. Differentiated podocytes were used for FFSS experiments on day 14. We evaluated the podocyte actin cytoskeleton with phalloidin tagged to Alexa Fluor 568 and cell morphology by crystal violet stain as described earlier by us (41).

FFSS application to podocytes. FFSS was applied to differentiated podocytes using a FlexCell Streamer Gold apparatus (Flexcell, Hillsborough, NC). The apparatus was sterilized with 300 ml of 70% ethanol for 30 min and checked for leaks. This was followed by two wash steps with 300–400 ml of sterilized PBS for 5 min each. PBS was then replaced by 350 ml of media. The flow chamber was moved to a sterile hood by using sterilized forceps. One glass slide with podocytes was placed in each of the slots of the flow device chamber. All six slots were filled to ensure consistent flow. The chamber was then placed in the incubator at 37°C with 5% CO2. The computer was programmed to apply FFSS at 2 dynes/cm2 (or 75 ml/min) for 2 h at 37°C with 5% CO2. In another set of experiments, podocytes were incubated with indomethacin (2.5 μM, 1 h) before application of FFSS. Following FFSS treatment, podocytes on slides were returned to the original medium for recovery up to 24 h in the incubator at 37°C under a 5% CO2 humidified atmosphere. In a set of experiments a three-way stop-cock was attached to collect 1.5 ml aliquots of the medium at 0, 30, and 120 min during application of FFSS. Control podocytes were grown on glass slides and placed in the same hood and incubator as experimental cells but were not exposed to FFSS. We evaluated the suitability of untreated “no-flow” control as a surrogate for very low FFSS under physiological conditions. Untreated cells were compared with podocytes exposed to low FFSS at 0.2 dynes/cm2 for 2 h using the flow cell we developed to apply FFSS to glomeruli (see below). Samples obtained before FFSS and post-FFSS treatment were termed pre-FFSS, post-2hr and post-24hr, respectively. Culture media were collected from control podocytes at the same time points. Pre-FFSS and post-FFSS (2 h/24 h) cells were harvested for analysis.

Enzyme immunoassay to determine changes in secreted and intracellular PGE2. PGE2 in media and the cell lysate was measured using a PGE2 EIA kit (514010, Cayman Chemical, Ann Arbor, MI) following the manufacturer’s instructions.
Quantitative real-time PCR to determine gene expression of cyclooxygenase enzymes and prostanoid receptors in podocytes. Podocyte total RNA was extracted using the Micro-Midi Total RNA Purification System (Invitrogen) and analyzed for quality and quantity by absorbance at 260 and 280 nm using a DNA/RNA calculator (Pharmacia Biotech/GE Healthcare, Uppsala, Sweden). The OD_{260}/OD_{280} absorbance ratio was 1.8–2.0, indicating clean RNA preparations. One microgram of total RNA was reverse transcribed using the SuperScript III First Strand Synthesis System (Invitrogen). Quantitative real-time PCR (RT-qPCR) was performed with SYBR Supermix (Bio-Rad, Hercules, CA) using a Bio-Rad iCycler (Bio-Rad) with specific sets of primers for each of the COX enzymes, EP receptors, and β-actin. β-Actin was used as the housekeeping gene. Samples from five separate experiments were analyzed by RT-qPCR for each experimental condition, i.e., FFSS with or without indomethacin. The sequences (5'-3') of primers used for RT-qPCR are shown in Table 5. PCR products were sequenced to further confirm the COX enzymes, EP receptors, and β-actin at the KUMC Core Facilities (Kansas City, KS).

Western blotting to determine protein expression of COX enzymes and prostanoid receptors in podocytes. Podocytes were lysed with RIPA buffer containing protease and phosphatase inhibitors. Total protein was determined using a bicinchoninic acid protein assay kit (BCA1, Sigma-Aldrich, St. Louis, MO). Western blotting was performed as described previously (42). Briefly, proteins were denatured in sample buffer containing β-mercaptoethanol at 94°C for 5 min. Ten micrograms of total protein was electrophoresed on a 10% Bis-Tris gel. Proteins were transferred to a polyvinylidene membrane, washed with PBST (0.1% Tween 20) and blocked using 5% nonfat milk. A rabbit polyclonal COX-1 antibody (sc-7950, Santa Cruz Biotechnology, Dallas, TX) at 1:200 dilution, COX-2 antibody (ab6665, Abcam, Cambridge, MA) at 1:2,000 dilution, EP2 antibody (sc-20675, Santa Cruz) at 1:500 dilution, and EP4 antibody (ab2446, Abcam) at 1:2,000 dilution were used. After washing with PBST, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody to each primary antibody. Chemiluminescence (ECL, GE Healthcare Biosciences, Piscataway, NJ) reagent was used for detection on X-ray film. Developed X-ray films were imaged and analyzed using FluorChem using built-in AlphaEaseFC software (Alpha Innotech, San Leandro, CA). Samples from three to five replicates for each experimental treatment (FFSS with or without indomethacin) were analyzed.

FFSS treatment of isolated rat glomeruli. Glomeruli were isolated as described below and placed on a stainless steel in a flow chamber designed and constructed in our laboratory. The flow chamber is constructed of a two-part metal shell to house a flow cell with fixed dimensions. The plexiglas base and top are lined with rubber gaskets to seal the glomeruli in a compartment with defined dimensions (Fig. 1). The thickness of the transparent plastic spacer determines the height of the fluid column. FFSS (τ, in dynes/cm²) was calculated as τ = 6ηQ/(w * h²), where η is viscosity, Q is rate of fluid flow, and w and h are width and height of the fluid column, respectively. FFSS was applied at 0.3 dyne/cm² for 60 or 120 min, and again following a recovery for 120 min at 37°C. In separate experiments, glomeruli were pretreated with 2.5 μmol/l indomethacin before FFSS treatment. Untreated glomeruli maintained in parallel without FFSS treatment were used as a control at time T₀ and T_{final}. Glomeruli from four rats were used in separate experiments (20 glomeruli/group from n = 4 rats with 5 glomeruli/rat). Glomeruli from these treatment and control
groups were used to determine effect of FFSS on albumin permeability ($P_{\text{alb}}$) in vitro using the assay described.

In vitro glomerular $P_{\text{alb}}$ assay following FFSS. Control and FFSS-treated rat glomeruli were used to study the changes in glomerular filtration barrier characteristics using an in vitro assay established in our laboratory. Details of the assay to measure volume of glomerular capillaries to an oncotic gradient generated by defined concentrations of albumin have been described previously (35, 38). Briefly, kidneys were removed from anesthetized male Sprague-Dawley rats (200–250 g) and decapsulated. The renal cortex was cut into fine fragments and consecutively passed through 80- and 120-mesh stainless screens. Glomeruli were recovered from atop the 200-mesh screen. Glomeruli were isolated at room temperature in a physiological buffer solution (pH 7.4). BSA (5 g/dl) was included in the medium as an oncotic agent (isolation/ incubation buffer). Oncotic gradient was induced by changing BSA concentration in the bath medium to 1%. Oncotic pressure was measured using a membrane colloid osmometer (model 4100, Wescor, Logan, UT).

Following experimental treatments, glomeruli were incubated in 5% BSA medium for 15 min at 37°C, transferred to glass coverslips coated with poly-l-lysine, and observed using video microscopy. Measurem ents of glomerular volume were made before and 1 min after the initial incubation medium containing 5 gm/dl BSA was replaced by medium containing 1 g/dl BSA. Change of the medium produces an oncotic gradient across the glomerular capillary wall (5 g/dl BSA in the lumen vs. 1 g/dl BSA in the bathing medium) and results in net fluid influx and an increase in glomerular volume. Glomerular volume was calculated from the average of four diameters of the video image and the change in volume ($\Delta V$) of each glomerulus in response to the oncotic gradient: $\Delta V = (V_{\text{final}} - V_{\text{initial}})/V_{\text{initial}} \times 100\%$. The increase in glomerular volume ($\Delta V$) is directly related to the oncotic gradient ($\Delta I$) applied across the capillary wall. We use this principle to calculate the reflection coefficient ($\sigma_{\text{alb}}$) using the ratio of $\Delta V$ of experimental to $\Delta V$ of control glomeruli in response to identical oncotic gradients: $\sigma_{\text{alb}} = \Delta V_{\text{experimental}}/\Delta V_{\text{control}}$. Convective $P_{\text{alb}}$ is defined as (1 – $\sigma_{\text{alb}}$) and describes the movement of albumin consequent to water flow on a unitless scale of 0 to 1. When $\sigma_{\text{alb}}$ is zero, albumin moves at the same rate as water and $P_{\text{alb}}$ is 1.0. When $\sigma_{\text{alb}}$ is 1.0, albumin cannot cross the membrane with water and $P_{\text{alb}}$ is zero.

Unilateral nephrectomy in sv129 mice. Five 13- to 14-wk-old sv129 mice underwent surgical removal of the right kidney. Another set of five 13- to 14-wk-old sv129 mice underwent a sham operation for experimental control. Four weeks later, the left kidney was harvested from all animals for analysis. The day before harvesting of kidneys, urine was collected for urine albumin and creatinine measurement. Urine albumin and creatinine were measured at the Mouse Metabolic Phenotyping Center, Vanderbilt University Medical Center (Nashville, TN). Kidneys were fixed in 10% formalin, embedded in paraffin, sectioned at 3–5 μm, and stained using Jones silver stain. Images were obtained using an Olympus BX60 (Hamburg, Germany) for light microscopy, and morphological measurements were made using computerized image-analysis software (Analysis) as described earlier (40, 41).

Immunohistochemistry to determine expression of COX enzymes and prostanoid receptors in sv129 mouse kidneys. Immunohistochemistry for COX and EP proteins was performed as described earlier (42). Primary antibodies used included a rabbit polyclonal COX-1 antibody (sc-7950, Santa Cruz Biotechnology, Dallas, TX) at 1:100 dilution, COX-2 antibody (ab6665, Abcam) at 1:150 dilution, EP2 antibody (sc-20675, Santa Cruz Biotechnology) at 1:25 dilution, and EP4 antibody (ab93486, Abcam) at 1:100 dilution. Tissue sections were mounted in 9:1 (glycerol:PBS) + 5% N-propyl gallate, and photomicrographed as described. We performed image analysis using the National Institutes of Health Image J software suite by quantifying the mean integrated density (average grey value × area) of 3,3-diaminobenzidine (DAB) staining within the glomerulus for COX and EP proteins to obtain the net protein expression/glomerulus. Endothelial cells are localized on the intraluminal aspect of the glomerular capillary, while podocytes surround and cover the extraluminal aspect of the capillary wall. We also performed additional semiquantitative analysis using a scale of 0–4. Each glomerulus examined was assigned a score based upon the percentage of total podocytes that showed positive staining (above background to brown-black): 0 (no staining), 1 (<10% of podocytes), 2 (10–25% of podocytes), 3 (25–50% of podocytes), or 4 (>50% of podocytes staining).

RESULTS

FFSS increases gene expression of COX-2, but not COX-1, in podocytes. Figure 2 (top) shows results of RT-PCR (RT-qPCR) quantification of COX-1 and COX-2 gene expression at 2 (post-2hr) or 24 h (post-24hr) after FFSS with or without pretreatment with indomethacin. COX-2 gene fold-expression increased significantly at post-2hr (15.7 ± 3.9, P < 0.001) and returned to near control levels by post-24hr (2.7 ± 1.6, P = 0.51). Indomethacin markedly attenuated the effect of FFSS, and the fold-increase in COX-2 gene expression was only 4.1 ± 2.7, P = 0.06 at post-2hr that returned to the control level (1.5 ± 0.8, P = 0.89) at post-24hr. In contrast, FFSS did not alter COX-1 expression (Fig. 2, top).

FFSS increases protein expression of COX-2, but not COX-1, in podocytes. Figure 3 shows results of Western blotting using antibodies to detect COX-1 and COX-2 proteins at post-2hr and post-24hr after FFSS with or without pretreatment with indomethacin. As with gene expression, on density analysis protein expression of COX-2/β-actin compared with control (2.67 ± 1.37) was increase at post-2hr (4.56 ± 0.55, P = 0.035) and post-24hr (4.06 ± 1.16, P = 0.10) and is shown as a fold-change in Fig. 3. Indomethacin attenuated the
Fig. 4. FFSS increases secreted and intracellular PGE₂. Cultured podocytes were exposed to FFSS at 2 dynes/cm² for 2 h with and without pretreatment with indomethacin. The ELISA method was used to determine levels of PGE₂ in samples from 5–6 replicate experiments. Values are means ± SD. Left: PGE₂ levels (pg/ml) were elevated in aliquots of the medium collected at 30 and 120 min (P = 0.04) during FFSS application. The FFSS-induced increase in the levels of secreted PGE₂ was attenuated by indomethacin. Right: intracellular PGE₂ was normalized using DNA in each sample and expressed as PGE₂/DNA (pg/µg). FFSS caused an increase in the PGE₂/DNA ratio at post-2hr (P = 0.02 vs. control) and post-24hr (P = NS vs. control) following FFSS. Pretreatment with indomethacin caused a decrease in intracellular PGE₂ at post-2hr (P = 0.001 vs. control) and post-24hr (P = 0.001 vs. control). *P < 0.05.

Increase in COX-2. COX-1 protein expression did not change following FFSS with or without indomethacin.

FFSS stimulates PGE₂ synthesis in podocytes. Figure 4 shows the enzyme immunoassay results on the levels of PGE₂ in the medium at the onset (0 min), during FFSS (30 min), and at the end of FFSS (120 min) and the effect of FFSS on podocytes pretreated with indomethacin. PGE₂ was not detectable in the medium before applying FFSS (<15 pg/ml). FFSS caused an increase in PGE₂ levels in the medium by 30.6 ± 13.6, 120 min (179.0 ± 149.1, P = 0.04). However, FFSS application for 30 or 120 min following pretreatment with indomethacin attenuated the increase in PGE₂ at 30 (33.6 ± 11.4, P = 0.03) and 120 min (17.4 ± 17.5, P = 0.81) (Fig. 4, left).

Figure 4 also shows that FFSS caused an approximately threefold increase in intracellular PGE₂. Pre-FFSS levels were 0.57 ± 0.16 pg PGE₂/µg DNA and post-2hr (1.56 ± 0.71 pg PGE₂/µg DNA, P = 0.02). Intracellular PGE₂ levels returned close to baseline by post-24hr FFSS (0.81 ± 0.11 pg PGE₂/µg DNA, P = 0.69). In contrast, pretreatment of podocytes with indomethacin caused a significant (P = 0.001) decrease in intracellular PGE₂ (control 0.53 ± 0.11, post-2hr 0.30 ± 0.07 and post-24hr 0.18 ± 0.03 pg PGE₂/µg DNA) (Fig. 4, right).

Table 1 summarizes the pattern of PGE₂ secretion up to 24 h following FFSS. PGE₂ levels were determined using ELISA in aliquots of medium collected at 2 and 24 h following FFSS treatment. FFSS caused a significant increase in PGE₂ concentration at post-2hr and post-24hr compared with control (Table 1). Pretreatment with indomethacin followed by FFSS blocked the increase in PGE₂ at both time points (Table 1).

FFSS increases gene expression of prostanoid receptor EP₂ but not EP₄. PGE₂ mediates its effect through four prostanoid receptors, EP₁–EP₄. Podocytes express EP₁, EP₂, and EP₄ but not EP₃ (42). Figure 2 (bottom) shows results of RT-qPCR measuring the gene expression of EP₂ and EP₄ at 2 (post-2hr) and 24 h (post-24hr) following FFSS with or without indomethacin pretreatment. FFSS significantly increased the gene expression of the EP₂ receptor by post-2hr (2.7 ± 1.4, P = 0.02) but not at post-2hr (1.7 ± 0.4, P = 0.4). However, indomethacin did not alter EP₂ gene expression significantly (post-2hr 0.5 ± 0.7, P = 0.55; post-24hr 1.4 ± 0.9, P = 0.73). In contrast, as shown in Fig. 2 (bottom), FFSS did not affect EP₄ expression (post-2hr was 0.9 ± 0.2, P = 0.86; post-24hr 0.9 ± 0.3, P = 0.76). Interestingly, indomethacin caused a significant increase in EP₄ gene expression at post-2hr 1.8 ± 0.4 (P = 0.007) but not at post-24hr 0.8 ± 0.2 (P = 0.53) following FFSS (Fig. 2, Lower panel).

FFSS increases protein expression of prostanoid receptor EP₂ but not EP₄. Figure 5 shows the effect of FFSS on podocyte EP₂ and EP₄ protein expression at post-2hr or
post-24hr using Western blotting. FFSS caused a gradual change in the (EP2/β-actin) density that increased from (0.29 ± 0.12, P = 0.72) at post-2hr to (0.50 ± 0.13, P = 0.06) by post-24hr compared with control (0.25 ± 0.16) and is shown as a fold-change in Fig. 5. Indomethacin attenuated the effect of FFSS on EP2 protein expression. However, FFSS, with or without indomethacin, did not alter the expression of EP4 protein.

Thus an FFSS stimulus to cultured podocytes increased the synthesis and secretion of PGE2 into the extracellular milieu and upregulated the expression of the EP2 receptor, but not of EP4. An ~30% increase in secreted PGE2 by post-2hr and only an ~10% increase between 2 and 24 h post-FFSS suggest a parallel rise in PGE2 secretion and COX-2 expression (Table 1 and Fig. 3). Indomethacin effectively attenuated the changes induced by FFSS.

Control podocytes with no flow vs. low FFSS at 0.2 dynes/cm. Podocytes did not show a qualitative change in cell morphology or the actin cytoskeleton at post-2hr and post-24hr treated with low FFSS at 0.2 dynes/cm² for 2 h compared with the no-flow control (n = 3). In contrast, we have shown that FFSS at 2 dynes/cm² for 2 h causes significant changes in cell morphology and the actin cytoskeleton (41). FFSS at 0.2 dynes/cm² on podocytes did not increase the secreted PGE2. PGE2 levels in the medium at 30 and 120 min during FFSS were below the detection limit. Intracellular PGE2 at post-2hr FFSS at 0.2 dynes/cm² (0.79 ± 0.20 pg/µg DNA) did not increase compared with the no-flow control (0.71 ± 0.14, P = 0.56). In contrast, both secreted and intracellular PGE2 levels were increased after application of FFSS at 2 dynes/cm² for 2 h (Fig. 4, Table 1). Thus the no-flow control used in our studies and low FFSS at 0.2 dynes/cm² approximating physiological conditions did not alter cellular structure or PGE2 production.

Ex vivo application of FFSS to isolated decapsulated glomeruli results in increased P alb. Previously, we showed that the PGE2-induced increase in P alb was blocked by indomethacin (26). Following the observation that application of FFSS increases PGE2 in podocytes (Fig. 4), we determined the effects of FFSS on P alb in isolated decapsulated rat glomeruli.

Table 2 and Fig. 6 summarize the effect of FFSS on P alb with or without indomethacin. In the first set of experiments, the effect of the duration of FFSS on the glomerular filtration barrier was determined. Results show that FFSS application for 60 or 120 min resulted in increased P alb (P < 0.001). There was no change in P alb in untreated control (T0) and time-matched (Tfinal) controls (Table 2). In the second set of experiments, results showed persistently elevated P alb at 2 h after (post-2hr) FFSS (Fig. 6, Table 2). Figure 6 shows that pretreatment with indomethacin blocked the effect of FFSS on P alb. In the third and fourth sets of experiments, the effect of FFSS for 120 min on glomeruli pretreated with indomethacin and after an additional 120 min (post-2hr) of recovery following FFSS was studied. The FFSS-induced increase in P alb was significantly blocked by indomethacin. The untreated control (T0) and time-matched controls (Tfinal) showed no change in P alb (Fig. 6).

Unilateral nephrectomy in sv129 mice is associated with glomerular hypertrophy and increased expression of COX-2 and prostanooid receptor EP2.

Male sv129 mice were uninephrectomized by removing the right kidney to induce hyperfiltration in the remnant solitary kidney. The left kidney was harvested 4 wk later. Table 3 shows that uninephrectomy resulted in significant glomerular hypertrophy by 4 wk as indicated by an increase in glomerular area and volume compared with the left kidney from the sham-operated mice (control). Urine albumin excretion was significantly increased in nephrectomized mice (246.8 ± 141.9 mg/g creatinine) compared with sham-operated mice (25.1 ± 11.4 mg/g creatinine, P = 0.001). The mean integrated density (average grey value × area) performed using Image J software for DAB staining for COX and EP proteins provides the net protein expression/glomerulus. Immunohistochemistry showed
increased mean integrated density for podocyte expression of COX-2 but not COX-1 and prostanoid receptor EP2 but not EP4 (Fig. 7 and Table 4). We also performed a semiquantitative analysis using a 0–4 scale. The mean ± SD on the 0–4 scale for COX-2 staining in the glomeruli for sham and nephrectomy sv129 mice was 1.06 ± 0.40 and 2.45 ± 0.40, respectively (P = <0.001), and for COX-1 was 1.29 ± 0.21 and 1.28 ± 0.10, respectively (P = 0.91). The mean ± SD integrated density for EP2 staining in the glomeruli for sham and nephrectomy sv129 mice was 0.96 ± 0.34 and 2.45 ± 0.31, respectively (P = <0.001) and for EP4 was 3.22 ± 0.34 (sham) vs. 2.83 ± 0.34 (nephrectomy) P = 0.053. Thus hyperfiltration resulting from renal mass ablation leads to upregulation of COX-2 and EP2 expression in remnant kidneys of uninephrectomized mice.

**DISCUSSION**

CAKUT including a solitary kidney is the most common cause of childhood CKD progressing to ESRD. One in 500–1,000 children born with a solitary kidney maintain their total GFR through increased SNGFR and develop adaptive hyperfiltration (34, 51–53). Continued hyperfiltration results in glomerulosclerosis, albuminuria/proteinuria, and progressive azotemia (1, 31, 34, 51–53). The absence of the traditional risk factors of hypertension and proteinuria in early childhood supports the concept that persistent hyperfiltration contributes to kidney injury (34, 52). Additionally, hyperfiltration is an important risk factor in the progression of CKD in adults with diabetes, obesity, and other kidney diseases (1, 8, 20, 31, 50). We believe that hyperfiltration would alter the homeostasis of biomechanical forces that regulates the rheological dynamics within the glomerulus and maintains the filtration barrier.

Glomerular anatomy positions podocytes in Bowmam’s space, thus exposing these large cells with extensive foot processes to biomechanical forces. Increased intracapillary pressure causes tensile stress on foot processes covering the outer aspect of capillaries, and bulk flow of the filtrate causes FFSS on podocytes (10, 11, 17, 25, 40, 41). A direct measurement of FFSS may be extremely difficult, if not impossible. Mathematical models are heavily relied upon to explore complicated biological problems. We found that the calculated FFSS over podocytes increases 1.5–2-fold in animal models of unilateral nephrectomy (40). We also showed that increased SNGFR, not filtration fraction, is the basis of increased FFSS over podocytes in a solitary kidney. We postulated that hyperfiltration impacts podocyte homeostasis mainly through increased FFSS.

Arachidonic acid metabolites are important mediators of hemodynamic regulation in the kidney. PGE2 is a major product of kidney arachidonic acid metabolism by cyclooxygenases COX-1 and COX-2 (14). As shown in Figs. 2 and 2, COX-2 expression increased as a result of FFSS applied to podocytes, but pretreatment of cells with indomethacin blocked the increase in PGE2 levels and COX-2 expression (Fig. 4 and Table 1). Upregulation of PGE2 synthesis appears to be a specific response to FFSS as neither Martinez et al. (25) nor we (abstract EPAS2008:3803.1) (40a) detected increased PGE2 in podocytes subjected to experimental stretch. A similar increase in COX-2 expression and PGE2 synthesis in renal collecting duct cells was recently demonstrated by Rohatgi and colleagues (15, 23). We did not see a significant change in PGE2 synthesis in control podocytes with no flow and FFSS at 0.2 dynes/cm2 in contrast to increased PGE2 synthesis and secretion seen with FFSS at 2 dynes/cm2. Similarly, it has been shown that an increase in PGE2 is seen after a threshold of

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**Table 3. Unilateral nephrectomy results in increased glomerular size**

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<th>Sham Mice</th>
<th>Uninephrectomy Mice</th>
<th>P Value</th>
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<tr>
<td>Mean G0, μm</td>
<td>65.6 ± 2.8</td>
<td>72.2 ± 5.0</td>
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<tr>
<td>Maximum G0, μm</td>
<td>78.4 ± 4.2</td>
<td>82.3 ± 5.2</td>
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<td>Glomerular area, μm2</td>
<td>3.0 ± 0.3 x 10³</td>
<td>4.4 ± 0.6 x 10³</td>
<td>0.006</td>
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<tr>
<td>Glomerular volume, μm3</td>
<td>297.5 ± 31.1 x 10³</td>
<td>393.4 ± 74.3 x 10³</td>
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Values are means ± SD; n = 7/group. A solitary kidney was harvested at 4 wk after unilateral nephrectomy of male sv129 mice. Sham-operated mice were used as a control. Morphometric data of measured mean and maximum glomerular diameter (G0) are shown along with calculated glomerular area and glomerular volume.
Fig. 7. Unilateral nephrectomy of sv129 mice results in increased glomerular COX-2 and EP2 expression. Hyperfiltration was induced in male sv129 mice by removing the right kidney. The left kidney was harvested 4 wk later. Renal cortical slices were processed for immunohistochemistry using specific antibodies. COX-1, COX-2, EP2, and EP4 expression in podocytes was determined using light microscopy and image analysis. In each glomerulus, a few representative podocytes are marked that show intense immunostaining for the COX and EP proteins. The difference in intensity is shown in the adjoining box plot. Images were observed and analyzed in a masked manner. 

Top 2 rows: light microscopy images of glomerular COX-1 and COX-2 (left) with corresponding box plots (right). Increased FFSS after unilateral nephrectomy resulted in increased COX-2 expression ($P < 0.001$).

Bottom 2 rows: light microscopy images of glomerular EP2 and EP4 (left) with corresponding box plots (right). Increased FFSS after unilateral nephrectomy resulted in increased EP2 expression ($P = 0.039$). 

*Outlier. **P < 0.05.
shear stress is reached in renal collecting duct cells (3, 24). These observations also validate the use of cells with no flow as an adequate although not perfect control for in vitro experiments. A continuous treatment with low FFSS is not feasible at the present time due to methodological limitations. Results from in vivo studies also support these findings. For example, in the classic 5/6 nephrectomy rat model, hyperfiltration-mediated injury results in increased PGE2 synthesis and COX-2 expression (30, 43, 49). Since the 5/6 nephrectomy model is confounded by uremia, we preferred the unilateral nephrectomy model to induce hyperfiltration. The unilateral nephrectomy resulted in increased glomerular COX-2 expression in sv129 mice (Fig. 7). Additionally, several studies have shown that chronic inhibition of COX attenuates the progression of kidney injury in animal models and human disease (45–48). NSAIDs that attenuate PGE2 synthesis, also decreased proteinuria. In contrast, sulindac, which does not influence PGE2 synthesis, showed no effect on protein excretion. Thus PGE2 appears to significantly modulate glomerular filtration barrier permselectivity. These results corroborate and complement our findings, suggesting a role for COX-2 and PGE2 in the mechanism of FFSS-induced injury leading to albuminuria/proteinuria.

PGE2 interacts with G protein-coupled receptors (GPCR), of which four (EP1-EP4) have been characterized and cloned (2, 29). EP2, when present, is a low-abundance receptor and conditions associated with progressive chronic kidney disease (CKD) or FSGS in conditions such as congenital anomalies of the kidney and urinary tract (CAKUT), a solitary kidney, obesity, and diabetes. Right: summary of current working hypothesis that FFSS alters podocyte structure and the actin cytoskeleton via the COX-2-PGE2 axis and its downstream signaling in pathophysiological conditions associated with hyperfiltration.

Table 4. Unilateral nephrectomy results in increased glomerular COX-2 and EP2 protein expression

<table>
<thead>
<tr>
<th>Target</th>
<th>Sham Mice</th>
<th>Uninephrectomy Mice</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-1</td>
<td>810.7 ± 90.1 × 10^3</td>
<td>638.7 ± 136.3 × 10^3</td>
<td>0.07</td>
</tr>
<tr>
<td>COX-2</td>
<td>498.5 ± 171.4 × 10^3</td>
<td>1,174.5 ± 136.3 × 10^3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EP2</td>
<td>274.5 ± 95.9 × 10^3</td>
<td>431.0 ± 46.6 × 10^3</td>
<td>0.039</td>
</tr>
<tr>
<td>EP4</td>
<td>381.1 ± 266.8 × 10^3</td>
<td>464.9 ± 133.7 × 10^3</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Values are means ± SD for cyclooxygenase (COX)-1, COX-2, EP2, and EP4 staining in 20 consecutive glomeruli in the kidney sections from nephrectomized mice and sham-treated control mice. Solitary kidney was harvested at 4 wk after unilateral nephrectomy of male sv129 mice. Immunohistochemical analysis of COX-1, COX-2, EP2, and EP4 proteins for mean integrated density by Image J analysis is shown. COX-2 protein and EP2 protein were increased following unilateral nephrectomy.

Table 5. Primer sequences used for qRT-PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>PCR Product Size</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-1</td>
<td>70 bp</td>
<td>Forward 5′-GCTCTTTCCAGGACCTCACA-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5′-TGGATGTAGCAGCTACAACTG-3′</td>
</tr>
<tr>
<td>COX-2</td>
<td>75 bp</td>
<td>Forward 5′-GATGCTTCTCCAGGAGCTTG-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5′-GGATTGGAACAGCAAGGATT-3′</td>
</tr>
<tr>
<td>EP2</td>
<td>73 bp</td>
<td>Forward 5′-GCGTGAGGTCGGACATTTTC-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5′-ACGAGAGGTCGAAAGACAG-3′</td>
</tr>
<tr>
<td>EP4</td>
<td>92 bp</td>
<td>Forward 5′-CTAAAGGCAACGGTGAAAG-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5′-ACCAGAGGATCACAGGGACA-3′</td>
</tr>
</tbody>
</table>
protein expression of only EP2 and not EP4 in podocytes. Also, unilateral nephrectomy resulted in the upregulation of glomerular EP2 but not EP4 in sv129 mice (Fig. 7). We have also shown that EP2 is upregulated in mice with low nephron number. Mice with the gene for oligosyndactylism (Os/+ ) are used as a model to study the effect of congenital deficiency in nephron number. The Os/+ mice are born with ~50% fewer nephrons and have increased SNGFR compared with their wild-type controls (42). Thus in vivo and in vivo studies using podocytes suggest a role for the EP2 receptor in hyperfiltration-induced injury.

EP2 and EP4 are membrane-localized GPCRs that share 30% homology and activate adenylate cyclase. However, these receptors differ with regard to their response to stimuli, nature of ligands, and regulatory mechanisms. For example, resting peritoneal macrophages C3H/HeN express only EP4. While stimulation of these cells by LPS alone induces EP2 and downregulates EP4 expression (18, 19), LPS with indomethacin upregulates EP4. Additionally, EP2 and EP4 respond differently to various ligands. For example, in Chinese hamster ovary (CHO) cells, PGE2 causes only short-term desensitization of EP4 without affecting EP2, and 15-keto-PGE2 causes an immediate reduction in EP4 activity and a gradual but greater loss of EP2 activity (32). Thus the increase in EP4 gene expression by indomethacin observed in our experiments (Fig. 2) is intriguing and unexplained but not surprising.

Studies on EP receptors in osteocytes serve as a very supportive parallel example for our observations (6, 7, 16, 21). Osteocytes located within the bone canaliculi form a network of cytoplasmic processes and respond to tensile and shear stress (27). Both osteocytes and podocytes are terminally differentiated cells that possess elaborate cytoplasmic extensions and respond to FFSS (6, 7, 16, 21). A head-to-head comparison of shear and tensile forces in osteocytes shows that FFSS induces greater cellular deformity (27) and upregulates COX-2 (6, 7, 16, 21). Furthermore, FFSS upregulates EP2 in osteocytes without altering EP4 expression (6, 7, 16, 21). These studies have been useful in comprehending the role of biomechanical forces in diverse cell types.

A comparison of mechanical forces shows that tensile stress (1) causes formation of actin-rich centers and radial stress fibers, 2) upregulates COX-2 without increasing PGE2 synthesis, and 3) upregulates EP4 but not EP2 in podocyte cultures (12, 13, 25, 44). In contrast, FFSS (1) disrupts actin stress fibers with the formation of a cortical actin ring, 2) upregulates COX-2 with increased PGE2 levels, and 3) upregulates EP2 but not EP4 in cultured podocytes (17, 41, 42). Based on these considerations, we have developed a model (Fig. 8; see the legend) to study the role of FFSS in hyperfiltration-mediated injury in the progression of CKD. Although in vitro studies can dissect the subtle differences between FFSS and stretch, these forces are likely to exert their influence concurrently in vivo. Future studies using specific receptor agonists/antagonists, an idealized control, and animal models will further strengthen our observations and delineate the cellular signaling events.

In summary, similarities in the effects of FFSS on podocytes, osteocytes, and recently reported results on renal collecting duct cells suggest a conserved cellular response involving the COX2-PGE2-EP2 axis that warrants additional investigation for its role in hyperfiltration-mediated kidney injury. We believe the COX2-PGE2-EP2 axis is a potential target for developing new interventions to prevent CKD progression.

REFERENCES


