Inhibition of Cyclooxygenases Reduces Complement-Induced Glomerular Epithelial Cell Injury and Proteinuria in Passive Heymann Nephritis

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ABSTRACT

In the passive Heymann nephritis (PHN) model of rat membranous nephropathy, complement induces glomerular epithelial cell injury and proteinuria, which is partially mediated by eicosanoids. Glomerular cyclooxygenase (COX)-1 and -2 are up-regulated in PHN and contribute to prostanooid generation. In the current study, we address the role of COX isoforms in proteinuria, using the nonselective COX inhibitor indomethacin and the COX-2-selective inhibitor 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone (DFU). Four groups of rats with PHN were treated twice daily, from day 7 through 14 with vehicle, 1 mg/kg DFU, 10 mg/kg DFU, or 2 mg/kg indomethacin. Vehicle-treated rats with PHN showed significant proteinuria on day 14 (163\pm 15 mg/d, n = 19), compared with normal rats (10 ± 4 mg/d, n = 3, p < 0.001). Treatment with DFU (1 or 10 mg/kg) reduced proteinuria significantly (by ~33%), compared with vehicle, but to a lesser extent than indomethacin (56% reduction). Glomerular eicosanoid generation was reduced significantly in the DFU and indomethacin groups, compared with vehicle. There were no significant differences among vehicle- or DFU-treated groups in [3H]inulin clearance, or in glomerular expression of COX-1 and -2. DFU did not affect the autologous immune response. In cultured rat glomerular epithelial cells, COX inhibition reduced complement-induced cytotoxicity, and this reduction was reversed by the thromboxane A2 analog 9,11-dideoxy-9α,11α-methanooxyprostaglandin F2α (U46619). Thus, in experimental membranous nephropathy, selective inhibition of COX-2 reduces proteinuria, without adversely affecting renal function. However, inhibition of both COX-1 and -2 is required to achieve a maximum cytoprotective and antiproteinuric effect.

The rat model of passive Heymann nephritis (PHN) closely resembles human membranous nephropathy, including exclusive subepithelial distribution of immune deposits, morphological changes of visceral glomerular epithelial cells (GECs), and severe proteinuria in the absence of any detectable cellular infiltrate or inflammatory change in the glomeruli. PHN has been used as a model to study the pathophysiology of human membranous nephropathy and its validity was recently discussed in detail in Tischer and Couser (2000). Understanding the mechanisms of GEC injury and proteinuria in PHN is likely to elucidate the pathophysiology of the human disease.

In PHN, assembly of the complement C5b-9 membrane attack complex in GEC plasma membranes leads to nonlytic GEC injury, activation of biochemical pathways, and proteinuria. The precise mechanisms of GEC injury and proteinuria have not been fully established (Cybulsky et al., 2000a). Recently, we demonstrated that C5b-9 activates cytosolic phospholipase A2 (cPLA2) and releases arachidonic acid in GECs, and that cPLA2 is activated in glomeruli of rats with PHN (Cybulsky et al., 2000b). A number of previous studies

ABBRVIATIONS: PHN, passive Heymann nephritis; GEC, glomerular epithelial cell; cPLA2, cytosolic phospholipase A2; PG, prostaglandin; TX, thromboxane; COX, cyclooxygenase; SC58236, 4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide; DFU, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone; PE, polyethylene; GFR, glomerular filtration rate; TRITC, tetramethylrhodamine; B isoiothiocyanate; EIA, enzyme immunoassay; indo, indomethacin; DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; ANOVA, analysis of variance; NS, normal human serum; SC560, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole; SC58125, 1-[4-(4-methylsulfonyl)phenyl]-3-trifluoromethyl-5-(4-fluorophenyl)pyrazole; U46619, 9,11-dideoxy-9α,11α-methanooxyprostaglandin F2α.
have demonstrated that metabolites of arachidonic acid (eicosanoids) play an important role in the pathogenesis of proteinuria in membranous nephropathy. Specifically, prostaglandin (PG) and thromboxane (TX) A2 production is enhanced in glomeruli isolated from rats with PHN, and inhibition of cyclooxygenase (COX) or TX synthase, or shifting of dienoic prostanooids to inactive metabolites using fish oil diet reduces proteinuria in certain models of membranous nephropathy (Cybulsky et al., 2000a). The effect of TXA2 on proteinuria may be through an increase in glomerular transcapillary pressure, because this parameter is elevated in rat membranous nephropathy and seems to be responsible for a portion of the enhanced urine protein excretion (Cybulsky et al., 2000a). Alternatively, eicosanoids could potentially modulate GEC injury.

COX is a key enzyme in the metabolism of arachidonic acid. COX converts arachidonic acid released from membrane phospholipids by PLA2 to PGH2, an unstable intermediate, which is further metabolized to PGE2, PGI2, PGF2 alpha, and/or TXA2. There are two isoforms of COX, namely, COX-1 and COX-2 (Otto and Smith, 1995). Both isoforms have similar primary structures and enzymatic properties. In the traditional view, COX-1 is expressed constitutively and is believed to produce prostaglandins for maintenance of normal physiology, whereas COX-2 is inducible and may produce prostaglandins/TX for inflammatory processes and mitogenesis (Otto and Smith, 1995). However, in normal adult human glomeruli, COX-2 was found to be expressed in podocytes, but COX-1 was not detected (Komhoff et al., 1997). In normal adult rat glomeruli, COX-1 and -2 either were not detected or showed low levels of expression (Harris et al., 1994; Zhang et al., 1997). In the rat, it has been demonstrated that glomerular COX-2 expression was increased in anti-glomerular basement membrane nephritis (Chamugam et al., 1995), anti-Thy1.1 nephritis (Hirose et al., 1998), renal ablation model (Wang et al., 1998), and PHN (Blume et al., 1999; Takano and Cybulsky, 2000). However, the significance of these findings has yet to be defined. Recently, COX-2-selective inhibitors became available for clinical use (e.g., celecoxib and rofecoxib) and the impact of COX-2-selective inhibitors has been studied in models of renal disease. In the rat subtotal renal ablation model, the COX-2-selective inhibitor SC58236, reduced proteinuria and glomerulosclerosis to a similar extent as enalapril, while not affecting systemic blood pressure (Wang et al., 2000). In PHN, a COX-2-selective inhibitor, flusdide, was shown to reduce proteinuria, and the reduction was equal in magnitude at low and high doses (Heise et al., 1998; Blume et al., 1999). However, glomerular expression of both COX-1 and -2 proteins was markedly inhibited in rats treated with the high dose of flusdide (Heise et al., 1998; Blume et al., 1999). Furthermore, flusdide impaired creatinine clearance (Blume et al., 1999). These findings suggest that COX-2-selective inhibitors may be beneficial in noninflammatory proteinuric glomerular injury, but the mechanisms of these beneficial effects and indeed the specificity/safety of these compounds remain in question. Thus, it would be important to define the mechanisms of the beneficial effects of COX-2 selective inhibitors in noninflammatory proteinuric glomerular injury, such as membranous nephropathy. In the current study, we address the role of COX-1 and -2 in mediating proteinuria of PHN, using nonselective COX inhibition and the COX-2-selective inhibitor 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl) phenyl-2(5'H)-furanone (DFU) (Riendeau et al., 1997). Furthermore, we address potential mechanisms of the proteinuria-reducing effect of COX inhibitors.

Materials and Methods

Materials. DFU was provided by Merck Frosst Canada (Point Claire, QC, Canada). Indomethacin, methylcellulose, and TRITC-phallolidin were from Sigma-Aldrich Canada (Oakville, ON, Canada). SC560 was from Calbiochem (San Diego, CA). Ionomycin was from Roche Diagnostics (Laval, QC, Canada). TXB2, enzyme immunoassay (EIA) kit and rabbit anti-COX-2 antiserum were from Cayman Chemical (Ann Arbor, MI). Goat anti-COX-1 antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Reagents for SDS-polyacrylamide gel electrophoresis were from Bio-Rad (Mississauga, ON, Canada). Reagents for enhanced chemiluminescence were from Amersham Biosciences, Inc. (Baie d’Urfe, QC, Canada).

Induction of PHN and Experimental Design. PHN was induced in male Sprague-Dawley rats (150–175 b.wt.; Charles River, St. Constant, QC, Canada) by intravenous injection (400 μl/rat) of sheep anti-Fx1A antiserum as described previously (Takano and Cybulsky, 2000). Preparation of anti-Fx1A antiserum was described previously (Takano and Cybulsky, 2000). With this antiserum, no significant proteinuria was observed in the heterologous phase (up to 7 days after injection), but rats developed significant proteinuria 14 days after injection. Rats were divided into four groups and were treated twice daily from day 7 through day 14 with 2% methylcellulose (vehicle), 1 mg/kg DFU (DFU1), 10 mg/kg DFU (DFU10), or 2 mg/kg indomethacin (indo). DFU was prepared in 2% methylcellulose (1 ml) and given by gavage. Indomethacin was prepared in sodium phosphate buffer (0.5 M, pH 7.4) and was given by intraperitoneal injection to minimize gastric toxicity. On day 14, 24-h urine was collected in metabolic cages and urine protein was quantified using a protein assay kit (Bio-Rad). For 3HInulin clearance study, 24-h urine was collected on day 13, and rats were allowed free access to water and chow at least for 24 h before the experiment.

Measurement of COX-1 Activity in Whole Blood. COX-1 activity in whole blood was measured as described by Warner et al. (1999) with a minor modification. In brief, at the time of sacrifice, rat blood was collected from the inferior vena cava into heparin (19 units/ml). An aliquot of 200 μl was stimulated with ionomycin (50 μM) for 30 min at 37°C and centrifuged at 1500g for 5 min at 4°C. Plasma was removed and frozen immediately. Before the measurement of TXB2, plasma proteins were precipitated by 4 vol of ice-cold methanol. After incubation on ice for 10 min, samples were centrifuged for 10 min at 4°C. Supernatants were diluted 100 times with buffer and TXB2 concentration was quantified using TXB2 EIA kit (Cyamich Chemical).
each rat received the narcotic analgesic buprenorphine (Temgesic, 0.01 mg/kg i.p.; Reckitt and Colman Pharmaceuticals Inc., Wayne, NJ). Anesthesia was induced by 4% isoflurane in inspired gas (30% O_2, 70% air). After induction, the anesthetic concentration was reduced to ~2%. The animal was transferred to a servo-controlled, heated table to maintain body temperature at 37°C, intubated, and ventilated by a small animal respirator (RSP 1002; Kent Scientific Corp., Litchfield, CT). Cannulas were placed in the femoral artery (PE-90 with narrowed tip) and vein (PE-50). A constant infusion of normal saline, containing 2% charcoal-washed bovine serum albumin, delivered 1% of body weight per hour. This infusion was begun upon placement of the venous cannula and continued throughout the experiment. A PE-90 bladder cannula was placed through a small, suprapubic incision.

After 1-h equilibration, two consecutive 30-min clearances were acquired with 100-μl arterial blood samples immediately before and after each urine collection. Urine volume was measured gravimetrically. Glomerular filtration rate (GFR) and fractional excretions were determined from standard formulas, and urine and plasma Na^+ and K^+ were measured by flame photometry (Cupples and Sonnenschein, 1987).

**Immunoblotting.** Immunoblotting was performed as described previously (Takano and Cybulsky, 2000). In brief, rat glomeruli were lysed and sonicated in buffer containing 62.5 mM Tris, 2% SDS, 10% glycerol, and 0.01% bromophenol blue, pH 6.8. After centrifugation at 14,000 g, supernatants were collected and protein content was quantified by a modified Lowry method (protein DC assay; Bio-Rad). Equal amounts of protein were separated by 8% SDS-polyacrylamide gel electrophoresis under reducing conditions. Proteins were then electrophoretically transferred to a nitrocellulose membrane, blocked with 5% dry milk, and incubated with goat anti-COX-1 antiserum or rabbit anti-COX-2 antiserum. After three washes, membranes were incubated with respective secondary antibodies conjugated with horseradish peroxidase, and the signal was visualized with enhanced chemiluminescence. Protein content was quantified using scanning densitometry (NIH Image software).

**Enzyme-Linked Immunosorbent Assay for Measurement of Rat Anti-Sheep IgG Titer.** Enzyme-linked immunosorbent assay was performed according to a standard protocol (Horbeck, 1997). Flat-bottom microtiter plates were coated with sheep IgG prepared at 1 μg/ml (100 μl/well). After washing and blocking, serially diluted serum samples from rats with PHN, or normal-control rat serum (16-fold dilutions), were added and incubated for 1 h at 37°C. After washing, mouse anti-rat IgG conjugated with alkaline phosphatase (Sigma-Aldrich Canada) (1:3000 dilution) was added and the mixture was incubated further at 37°C until color developed. The titer was determined as the highest dilution of PHN serum that differed significantly from negative serum. The level of positivity was designated arbitrarily as the mean value of absorbance of the diluted negative-control serum augmented by two standard deviations.

**GEC Culture, Transfection, and Stimulation by Complement.** Rat GEC culture, characterization, and stable transfection were described previously (Takano and Cybulsky, 2000). To generate a subclone of GECs that stably overexpresses cPLA2 and COX-2, GEC-cPLA2 (a subclone of GECs that stably overexpresses cPLA2; Cybulsky et al., 1995) was stably transfected with rat COX-2 cDNA subcloned into the mammalian expression vector pcDNA3.1/zeo (Invitrogen, Burlington, ON, Canada), using zeomycin (Invitrogen) selection. Incubation of GECs with complement was detailed previously (Takano and Cybulsky, 2000). Briefly, GECs were incubated with rabbit anti-GEC antiserum [5% (v/v) in measurement buffer] for 40 min at 22°C. Antibody-sensitized GECs were exposed to normal human serum (in measurement buffer) for 40 min at 37°C to assemble C5b-9. Heat-inactivated human serum (56°C for 30 min) was used as control. Complement lysis was determined by measuring release of lactate dehydrogenase (LDH), similarly to the method described previously (Quigg et al., 1988). Specific release of LDH was calculated as described previously (Quigg et al., 1988).

**Quantification of P-actin was performed as described by Goecckerl and Wysolmerski (1995) with minor modifications, using cells in 24-well plates. All procedures were performed at room temperature. After stimulation, cells were washed with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde and 4% sucrose in PBS for 10 min. After incubation in NH_4Cl (50 mM) for 5 min, cells were permeabilized with 0.5% Triton X-100 for 10 min and blocked with 3% bovine serum albumin in PBS for 30 min. Cells were incubated with TRITC-phalloidin (0.1 μg/ml) for 60 min. After three washes with PBS, TRITC-phalloidin was extracted with 2 ml of methanol for 30 min with agitation, and cells were further washed with an additional 1 ml of methanol. Fluorescence of the pooled methanol extracts was quantified in a fluorometer (542-nm excitation/563-nm emission). To confirm that there were similar numbers of viable cells in each well, cells were further washed three times with PBS and were incubated with a nucleic acid-binding fluorescent dye, Toto-3 (100 nM; Molecular Probes, Eugene, OR). Cells were resuspended with a rubber policeman and cell suspensions were transferred to test tubes and sonicated. Fluorescence of the samples was quantified in a fluorometer (642-nm excitation/660-nm emission) and results of TRITC-phalloidin were normalized to Toto-3 values.

**Statistics.** Data are presented as mean ± S.E.M. The t statistic was used to determine significant differences between two groups. One-way analysis of variance (ANOVA) was used to determine significant differences among groups. Where significant differences were found, individual comparisons were made between groups using the t statistic, and adjusting the critical value according to the Bonferroni method. Two-way ANOVA was used to determine significant differences among groups containing multiple subsets of measurements.

**Results**

**COX Inhibition Reduces Proteinuria in PHN.** The anti-Fx1A antiserum used in the current study did not cause significant proteinuria up to day 7, but induced significant proteinuria on day 14 (Fig. 1). To determine whether a COX-2-selective inhibitor reduces proteinuria in PHN, we chose to initiate the treatment before proteinuria was established.
Thus, drugs were administered from day 7 through 14 and urine protein was quantified on day 14. Two doses of DFU, previously shown to inhibit carrageenan-induced paw edema in rats by ~50% (1 mg/kg) or ~100% (10 mg/kg), were selected (Riendeau et al., 1997). A twice-daily regimen was chosen because of the short half-life of DFU (~5 h; Riendeau et al., 1997; Nicoll-Griffith et al., 1999). DFU, given at two doses (1 and 10 mg/kg/day), reduced proteinuria by ~33%, compared with vehicle (Fig. 1). The nonselective COX inhibitor indomethacin also reduced proteinuria, consistent with a previous report (Cybulsky et al., 2000a). The inhibitory effect of indomethacin was significantly greater than the effect of DFU (Fig. 1).

To verify the COX-2 selectivity of DFU, we evaluated COX-1 activity of platelets by measuring TXB2 (stable metabolite of TXA2) in ionicin-stimulated whole blood from rats with PHN (Warner et al., 1999). The amounts of TXB2 generation in PHN rats treated with DFU1 (546 ± 57 pg/ml, n = 4) or DFU10 (267 ± 61 pg/ml, n = 5) were not significantly different from rats treated with vehicle (398 ± 72 pg/ml, n = 7), but TXB2 generation in indomethacin-treated rats was inhibited significantly (115 ± 25 pg/ml, n = 3, p < 0.05 versus vehicle). These results confirm the selectivity of DFU toward COX-2.

**COX Inhibition Does Not Affect the GFR in PHN.** Because COX metabolites can influence renal hemodynamics, DFU or indomethacin may have reduced proteinuria by reducing the GFR. To evaluate this possibility, we studied the impact of DFU and indomethacin on [3H]inulin clearance according to a standard protocol (see Materials and Methods). Neither DFU nor indomethacin affected the GFR (Table 1).

It has been reported that COX products modulate tubular handling of electrolytes (Rossat et al., 1999). Thus, tubular handling of sodium and potassium was also evaluated. In rats with PHN, treated with DFU or indomethacin, there were trends toward lower fractional excretion of sodium and potassium, and a higher plasma potassium concentration (Table 1). These differences did not reach statistical significance possibly because of the small number of measurements.

**DFU and Indomethacin Inhibit Glomerular Eicosanoid Generation in PHN.** To address the mechanisms of the proteinuria-reducing effect of DFU in PHN, we investigated whether reduction of proteinuria by DFU was associated with inhibition of glomerular eicosanoid generation. It has been established that glomeruli isolated from rats with PHN generate more TXA2 and PGE2 than glomeruli from normal rats (Weise et al., 1993; Nagao et al., 1996; Takano and Cybulsky, 2000). Glomeruli from DFU-treated rats with PHN generated 42 to 63% less TXA2 and 31 to 52% less PGE2, compared with vehicle-treated rats (Fig. 2, A and B). The effect of DFU reached statistical significance at the higher dose. Indomethacin inhibited glomerular TXA2 and PGE2 generation by 63 and 70%, respectively (Fig. 2A). We also studied urinary eicosanoid excretion, which mainly reflects eicosanoid generation in the renal medulla. Urinary TXB2 and PGE2 excretion was inhibited by DFU by 10 to 37% and 36 to 37%, respectively (Fig. 2B). Similar to glomerular eicosanoid blockade, the effect of DFU was statistically significant at the higher dose. Indomethacin inhibited urinary TXB2 and PGE2 excretion by 58 and 69%, respectively (Fig. 2B).

**DFU and Indomethacin Do Not Decrease Glomerular COX-1 and -2 Protein Expression in PHN.** A previous report by Blume et al. (1999) showed that a COX-2-selective inhibitor, flosulide, decreased glomerular expression of COX-1 and -2 protein in PHN, which may have accounted for decreased eicosanoid generation in glomeruli. To determine whether DFU affected the expression of COX isoforms, we studied glomerular expression of COX-1 and -2 proteins by immunoblotting. Previously, we showed that both COX-1 and -2 proteins are up-regulated in glomeruli of rats with PHN, compared with control rats (Takano and Cybulsky, 2000). The induced COX-1 protein expression levels were not affected by treatment with DFU nor indomethacin (Fig. 3). The induced COX-2 protein levels were not affected by DFU, but were augmented significantly by indomethacin (Fig. 3). Together, the results indicate that reduction of proteinuria by DFU or indomethacin is associated with inhibition of glomerular eicosanoid generation and that inhibition of eicosanoid generation by these two drugs occurs via inhibition of COX catalytic activity, and not via down-regulation of COX protein expression.

**COX-1 and COX-2 Both Contribute to Glomerular TXA2 Generation in PHN.** In previous studies, COX-1 protein expression had not been clearly demonstrated in GECs in normal rats, but we have shown that COX-1 and COX-2 are up-regulated in glomeruli of rats with PHN (Fig. 3; Takano and Cybulsky, 2000). Because C5b-9-mediated injury in PHN is localized to GECs (Tischer and Couser, 2000), it is reasonable to conclude that in PHN, COX isoforms are actually up-regulated in GECs. To provide further evidence for a functional role of COX-1 and -2 in GECs, we further characterized COX activities in glomeruli isolated from normal rats and from rats with PHN. Glomeruli were incubated in vitro with DFU or the COX-1-selective inhibitor SC560 (Smith et al., 1998), and production of TXB2 was measured. Glomeruli from rats with PHN generated more TXB2, compared with normal rats (Fig. 4). The increase in TXB2 generation in PHN (1.4 ng/ml; Fig. 4, PHN versus normal untreated) is attributed to complement-mediated TXA2 generation in GECs.
the complement-stimulated increase in TXB₂ (1.4 ng/ml), 58% (0.8 ng/ml) was inhibited by DFU (Fig. 4), indicating that the remaining 42% (0.6 ng/ml) was generated by COX-1. In normal glomeruli, DFU did not affect TXB₂ production significantly (Fig. 4). SC560 markedly decreased glomerular TXA₂ generation both in normal rats and rats with PHN (Fig. 4). This result confirms that glomeruli contain abundant COX-1 activity, although in normal glomeruli, it is not possible to attribute the COX-1 activity solely to GECs.

**Rat Anti-Sheep IgG Titer Is Not Affected by DFU.**
COX-2 is known to be expressed in lymphocytes, and under certain conditions may contribute to immune responses (Iniguez et al., 1999; Phipps et al., 2000). Thus, another possible mechanism for the proteinuria-reducing effect of DFU is via modulation of the immune response. To test whether DFU affected the autologous immune response of rats to sheep anti-Fx1A antibody IgG, rat anti-sheep IgG titers were quantified in plasma collected at the time of sacrifice (day 14). The anti-sheep IgG titer was 172 (H11006) 50 in the vehicle-treated group (n = 9) and 150 (H11006) 30 in the DFU10 group (n = 10; p = not significant). The 24-h urinary protein excretion of the same set of rats was 203 (H11006) 20 mg/day in the vehicle-treated group and 141 (H11006) 21 mg/day for the DFU10 group (p < 0.05). These results indicate that the proteinuria-reducing effect of DFU is not likely to be due to impairment of the autologous immune response to anti-Fx1A antibody IgG.

**Prostanoid Production Modulates Complement-Dependent GEC Injury.**
The above-mentioned results demonstrate that inhibition of COX reduces proteinuria in PHN. To determine whether the decrease in proteinuria may have been associated with a reduction in complement-dependent GEC injury, we tested the effect of COX inhibition on complement-mediated cytotoxicity in cultured rat GECs. In these experiments, we used GECs that stably overexpress cPLA₂ (GEC-cPLA₂), to amplify complement-dependent release of arachidonic acid and production of prostanoids (Takano and Cybulsky, 2000). GECs were pretreated with indomethacin, DFU, or the COX-1-selective inhibitor SC560 (Smith et al., 1998). After incubation with anti-GEC antibody, GECs were incubated for 3 h with serially increasing concentrations of complement (normal serum) that induced minimal to moderate cell lysis within this time interval. The 3-h time point was chosen because previous studies showed that sublytic complement increases COX-2 expression within 3 h (Takano and Cybulsky, 2000). Cell lysis (LDH release) was determined after completion of incubations. Indomethacin (10 μM) (IC₅₀ of 0.2 and 0.7 μM for COX-1 and COX-2, respectively) (Tegeder et al., 2001) reduced the complement-induced re-
lease of LDH significantly (Fig. 5A). LDH release tended to be lower in the presence of 10 μM DFU (IC50 of >50 and 0.04 μM for COX-1 and COX-2, respectively) (Riendeau et al., 1997), although the change did not reach statistical significance (Fig. 5A). Similar to indomethacin, 10 μM SC560 (IC50 of 0.009 and 6.3 μM for COX-1 and COX-2, respectively) (Smith et al., 1998) reduced the complement-induced release of LDH significantly (Fig. 5B). At the 10 μM concentration, SC560 blocked COX-1, but may have also blocked COX-2 to some extent. In the presence of 1.0 μM SC560, a concentration at which SC560 is less likely to block COX-2, complement-induced release of LDH tended to be reduced, but the change was not statistically significant (data not shown). Together, these results indicate that complement-mediated production of eicosanoids exacerbates complement-induced cytotoxicity. This effect seemed to be mediated mainly via COX-1, but inhibition of both COX isoforms may be required to achieve maximum cytoprotection.

It was reported that a high concentration of indomethacin may inhibit the activity of cPLA2 (Chang et al., 1987). Activated cPLA2 could perturb the phospholipid composition of the plasma membrane and thus could contribute to cytotoxicity. To verify whether the cytoprotective effect of indomethacin was via inhibition of cPLA2 activity, we used an in vitro PLA2 assay (Cybulsky et al., 1995) to study the effect of indomethacin on cPLA2 activity in GECs. Pretreatment of GECs with 10 μM indomethacin did not affect the PLA2 activity in GEC extracts, and PLA2 activity was not affected by the addition of 10 μM indomethacin directly to the in vitro assay (data not shown). Therefore, the cytoprotective effect of indomethacin was most likely mediated by inhibition of COX activities, rather than cPLA2 activity.

We have previously shown that complement stimulates generation of PGE2 and TXA2 in GECs (Takano and Cybulsky, 2000). To determine which of these eicosanoids contributes to complement-mediated cytotoxicity, we assessed whether exogenous PGE2 or TXA2 could reverse the cytoprotective effect of SC560. After pretreatment with 10 μM SC560, and incubation with anti-GEC antiserum and normal serum, complement-mediated cytotoxicity was reduced significantly, compared with vehicle-treated cells (Fig. 6), consistent with the results in Fig. 5. Addition of the TXA2 analog U46619 (10 μM) to the normal serum abrogated the cytoprotective effect of SC560, whereas addition of PGE2 did not
have any effect (Fig. 6). These results suggest that complement-induced generation of TXA$_2$ (but not PGE$_2$) enhances complement-mediated cytotoxicity.

Inhibition of COX-1 seemed to be more cytoprotective than inhibition of COX-2 (Fig. 5), and cytotoxicity seemed to be dependent mainly on TXA$_2$ (Fig. 6). These results raise the possibility that in GECs, COX-1 is preferentially coupled to TXA$_2$ generation, whereas COX-2 is preferentially coupled to generation of PGE$_2$. To test this hypothesis, we compared the profile of complement-mediated PGE$_2$ and TXA$_2$ generation in GECs that stably overexpress cPLA$_2$ (GEC-cPLA$_2$) with GECs that stably overexpress cPLA$_2$ plus COX-2 (GEC-cPLA$_2$-COX-2). Under basal conditions (and after 40-min incubation with complement), both cell lines express COX-1, whereas COX-2 is expressed only in GEC-cPLA$_2$-COX-2 (Fig. 7A). After incubation of GEC-cPLA$_2$ with complement (normal human serum, NS) for 40 min, release of TXB$_2$ and PGE$_2$ into the medium was 617 ± 65 and 25 ± 3 pg/0.1 ml, respectively, indicating that COX-1 generates quantitatively more TXA$_2$ than PGE$_2$. Incubation of GEC-cPLA$_2$-COX-2 with complement increased PGE$_2$ generation by 3.8 ± 0.4-fold, compared with GEC-cPLA$_2$, whereas TXB$_2$ generation increased by 1.8 ± 0.1-fold, which was significantly less than the change in PGE$_2$ ($p < 0.005$, $n = 6$; Fig. 7B). Furthermore, 10 µM DFU inhibited the complement-stimulated PGE$_2$ generation in GEC-cPLA$_2$-COX-2 by 45 ± 4%, whereas TXB$_2$ generation was inhibited by only 19 ± 3% ($p < 0.01$, $n = 3$). Taken together, although both COX-1 and COX-2 contribute to PGE$_2$ and TXA$_2$ generation, there seems to be preferential coupling of COX-1 with TXA$_2$, whereas COX-2 couples with PGE$_2$.

**COX Inhibitors Do Not Affect Complement-Mediated Actin Depolymerization.** It was reported previously that complement causes disruption of the actin cytoskeleton in cultured rat GECs (Topham et al., 1999). We hypothesized that COX inhibitors may protect GECs from complement-mediated injury by preventing the disruption of the actin cytoskeleton. GECs that overexpress cPLA$_2$ were incubated with complement, with or without COX inhibitors (10 µM indomethacin, 10 µM DFU, 10 µM SC560) for 3 h, and the amount of F-actin was quantified using TRITC-labeled phalloidin (see Materials and Methods). Complement decreased the amount of F-actin by ~40%, consistent with previous results (Topham et al., 1999). However, COX inhibitors did not affect the complement-induced reduction in the amount
of F-actin (Fig. 8). Thus, the cytoprotective effect of COX inhibitors does not seem to be mediated by the stabilization of F-actin.

Discussion

In the present study, we used the COX-2-selective inhibitor DFU and the nonselective inhibitor indomethacin to address the role of COX-1 and COX-2 in the pathogenesis of proteinuria in PHN. Proteinuria was reduced significantly by DFU at high and low doses, but to a lesser extent than indomethacin (Fig. 1). We established that DFU was COX-2-selective in vivo. Reduction of proteinuria by DFU or indomethacin was accompanied by reduced eicosanoid generation in glomeruli (Fig. 2), which was due to inhibition of COX enzymatic activity, and not decreased expression of COX proteins (Fig. 3). GFR and rat anti-sheep IgG titer were not affected by DFU, indicating that the proteinuria-reducing effect of DFU was most likely not mediated by a reduction in the GFR nor impairment in the autologous immune response (Table 1; see Results). The modulatory effect of eicosanoids on proteinuria in PHN is, therefore, dependent on both COX-1 and COX-2 activities. Furthermore, we provide evidence that stimulated glomerular TXA2 production in PHN is mediated by both COX isoforms (Fig. 4).

The results of this study confirm previous work from our group (Cybulsky et al., 2000a) and that of others (Weise et al., 1993; Nagao et al., 1996) that production of eicosanoids exacerbates proteinuria in PHN, and extend these previous findings by showing that the regulatory effect of eicosanoids depends on both COX-1 and COX-2 activities (Figs. 2–4). In PHN, proteinuria is due to structural alterations in the glomerular capillary wall, including C5b-9-mediated GEC in-
jury, but is also, in part, due to an increase in glomerular transcapillary pressure (Cybulsky et al., 2000a). Because GECs are the site of injury in PHN (Tischer and Couser, 2000), these cells are also the most likely source of TXA2. The mediator(s) of altered glomerular hemodynamics and proteinuria in PHN has not been fully defined, and vasoconstrictor prostanoids may potentially enhance proteinuria by increasing the glomerular transcapillary pressure difference. Other studies have demonstrated that COX inhibition can either induce or prevent cell death, depending on the cell type (Marx, 2001). By analogy, the present study suggests that eicosanoids production may enhance proteinuria by directly exacerbating complement-mediated GEC injury. Using a GEC culture model of complement cytotoxicity, we demonstrated that COX inhibition reduced complement lysis (Fig. 5) and that the cytoprotective effect of COX inhibition was abrogated by exogenous TXA2 (Fig. 6). Furthermore, the enhancement of complement lysis by production of eicosanoids was most likely due to TXA2 generation via COX-1 (Marx, 2001). By analogy, the present study suggests that eicosanoids (e.g., TXA2) may directly exacerbate complement-induced GEC injury in vivo, and thus enhance the amount of urine protein excretion. The role of eicosanoids in GEC injury is further supported by a recent study, which showed that glomerular transcapillary albumin flux, induced by addition of focal segmental glomerulosclerosis serum to isolated glomeruli in vitro, was attenuated by indomethacin (McCarthy and Sharma, 2002). Selective inhibition of COX-2 reduced proteinuria in PHN (Fig. 1), but seemed to have a minor effect in protecting cultured GECs from complement lysis (Fig. 5). These results do not necessarily rule out a role for complement-mediated induction of COX-2 and COX-2 products in directly exacerbating GEC injury in vivo, but they allude to the involvement of a separate mechanism for the enhancement of proteinuria via COX-2, perhaps through a glomerular hemodynamic effect.

The precise mechanism for the action of eicosanoids in GEC injury requires further study. For example, TXA2 may act in an autocrine or paracrine manner, because receptors for TXA2 (and other prostanoids) are expressed on GECs (Bek et al., 1999). It has been reported that eicosanoids may modulate the actin cytoskeleton (Lozano et al., 1996; Yang et al., 1998; Pierce et al., 1999). In view of recent discoveries that structural proteins of GEC, including nephrin, podocin, and α-actinin-4, are essential for the morphological integrity of GEC and glomerular barrier function, and that these molecules seem to exert their functions via interaction with the actin cytoskeleton (Kerjaschki, 2001), it was reasonable to hypothesize that eicosanoids may modulate GEC function and capillary wall integrity by perturbing the actin cytoskeleton. However, we were unable to demonstrate a direct relationship between COX products and actin polymerization. Yuan et al. (2002) recently reported that nephrin is dissociated from the actin cytoskeleton in glomeruli of rats with PHN. Possibly, COX products may modulate interactions of podocyte slit diaphragm-related molecules with the actin cytoskeleton. Further studies are required to determine whether the cytotoxic effects of eicosanoids in complement-treated GECs are associated with cytoskeletal alterations.

Because COX-2-selective inhibitors are now available for use in clinical practice and are better tolerated and less likely to induce gastrointestinal complications, compared with nonselective COX inhibitors, their potential role in the treatment of renal diseases has received increasing attention. Schneider and Stahl (1998) showed that the COX-2-selective inhibitors meloxicam and SC58125 augmented glomerular chemokine expression and monocyte infiltration in glomerulonephritis models, suggesting that COX-2 products may be acting as anti-inflammatory mediators in these models. In contrast, Wang et al. (2000) showed that in the noninflammatory rat subtotal renal ablation model, a COX-2-selective inhibitor, SC58236, reduced proteinuria and glomerulosclerosis to a similar extent as enalapril, while not affecting systemic blood pressure. Our results indicate that in membranous nephropathy, selective inhibition of the catalytic activity of COX-2 may reduce proteinuria, without adversely affecting renal function. However, inhibition of both COX-1 and -2 may be required to achieve a maximum cytoprotective and antiproteinuric effect.

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References


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