Complement C5b-9 induces cyclooxygenase-2 gene transcription in glomerular epithelial cells

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Background: Complement C5b-9 induces cyclooxygenase-2 (COX-2) gene transcription in glomerular epithelial cells (GEC). COX-2 is constitutively expressed in certain cell types and its regulation is cell or stimulus specific. We have previously demonstrated that sublytic injury by C5b-9 of GEC leads to assembly of terminal components, exposure of complement membrane attack complex into the lipid bilayer of the plasma membrane, and activation of the complement cascade near a cell surface. We have also shown that complement C5b-9 stimulates COX-2 promoter activity in GEC (1). To further investigate the COX-2 promoter activation induced by C5b-9, we examined the roles of upstream signaling pathways in the regulation of COX-2 expression.

Results: Our results indicate that nuclear factor-kB (NF-kB) and c-Jun NH2-terminal kinase (JNK) pathways contribute independently to complement-induced COX-2 expression.

Discussion: The complement system plays an important role in mediating inflammation, cytolysis, and phagocytosis. Activation of the complement cascade near a cell surface leads to assembly of terminal components, exposure of hydrophobic domains, and insertion of the C5b-9 membrane attack complex into the lipid bilayer of the plasma membrane. Nucleated cells require multiple pathways for lysis, but, at lower doses, C5b-9 induces sublytic injury and various metabolic effects. These may include activation of phospholipases and protein kinases and induction of certain genes, e.g., growth factors, nuclear factor (NF)-kB, and c-Jun (5, 6, 16, 22, 30, 34–36, 38, 41). An example of C5b-9-mediated injury in vivo is passive Heymann nephritis (PHN) in the rat, a well-established experimental model of human membranous nephropathy. In PHN, C5b-9 induces nonlytic injury of glomerular visceral epithelial cells (GEC) in association with altered GEC morphology and proteinuria (3, 43). A number of studies have demonstrated that COX-derived metabolites of arachidonic acid (eicosanoids) play an important role in the pathogenesis of proteinuria in membranous nephropathy. Specifically, PG and thromboxane production is enhanced in glomeruli isolated from rats with PHN, and inhibition of enzymes involved in prostanooid synthesis may lead to amelioration of proteinuria (4, 33, 59). Fish oil diet also decreased proteinuria in PHN by

Cyclooxygenase (COX) plays a key role in the metabolism of arachidonic acid to the important inflammatory mediators, prostaglandins (PGs) and thromboxane A2 (47). Two isoforms of COX, namely COX-1 and COX-2, have been characterized so far. Although both isoforms have similar structures, enzymatic properties, and intracellular distribution, their modes of regulation are distinct. COX-1 is expressed constitutively in most mammalian cells, whereas COX-2 is not expressed in most tissues under normal physiological conditions but is induced in certain cell types in response to various stimuli (47). The regulation of COX-2 gene expression and the role of COX-2 in pathophysiology have received considerable attention in recent years. COX-2 is usually induced as an immediate early gene by mitogenic or inflammatory stimuli, as well as by stimuli that act via G protein- and protein kinase C (PKC)-mediated pathways (18). More recently, mitogen-activated protein (MAP) kinase (MAPK) signaling cascades have also been implicated in the regulation of COX-2 (15, 53). There are at least three MAPK pathways: 1) the extracellular signal-regulated kinase (ERK)-1/2 (p44/p42) pathway, typically activated by growth factors; 2) the c-Jun NH2-terminal kinase (JNK), or stress-activated protein kinase pathway; and 3) the p38 pathway, activated by diverse stimuli, including stress (45). All three MAPK pathways may contribute to COX-2 gene regulation (13, 53, 54); however, involvement of the pathways appears to be cell or stimulus specific, and there appears to be cross talk among the MAPK pathways, as well as with other signaling pathways, including PKC (12, 14, 28, 54).

The complement system plays an important role in mediating inflammation, cytolysis, and phagocytosis. Activation of the complement cascade near a cell surface leads to assembly of terminal components, exposure of hydrophobic domains, and insertion of the C5b-9 membrane attack complex into the lipid bilayer of the plasma membrane (21, 30, 34). Nucleated cells require multiple pathways for lysis, but, at lower doses, C5b-9 induces sublytic injury and various metabolic effects. These may include activation of phospholipases and protein kinases and induction of certain genes, e.g., growth factors, nuclear factor (NF)-kB, and c-Jun (5, 6, 16, 22, 30, 34–36, 38, 41). An example of C5b-9-mediated injury in vivo is passive Heymann nephritis (PHN) in the rat, a well-established experimental model of human membranous nephropathy. In PHN, C5b-9 induces nonlytic injury of glomerular visceral epithelial cells (GEC) in association with altered GEC morphology and proteinuria (3, 43). A number of studies have demonstrated that COX-derived metabolites of arachidonic acid (eicosanoids) play an important role in the pathogenesis of proteinuria in membranous nephropathy. Specifically, PG and thromboxane production is enhanced in glomeruli isolated from rats with PHN, and inhibition of enzymes involved in prostanooid synthesis may lead to amelioration of proteinuria (4, 33, 59). Fish oil diet also decreased proteinuria in PHN by
shifting production of certain endogenous glomerular eicosanoids away from dienoic prostanoids to inactive metabolites (52). We have recently reported that the activity of cytosolic phospholipase A₂ (cPLA₂), the key enzyme that provides substrate for eicosanoid synthesis, is increased in glomeruli of rats with PHN (7).

Previously, we have employed well-differentiated rat GEC in culture to characterize biochemical changes induced by sublytic C5b-9, which include arachidonic acid release and metabolism. We have shown that sublytic C5b-9 activates cPLA₂ (5, 6, 38). This activation is dependent on a rise in intracellular Ca^{2+} concentration and occurs secondarily to the activation of PKC. Although C5b-9 also activated ERK2, cPLA₂ activation occurred independently of ERK2. Free arachidonic acid released by cPLA₂ was further converted to arachidonic acid metabolites (52). We have recently reported that the activity of cPLA₂-selective inhibitors provides an opportunity for shifting production of certain endogenous glomerular eicosanoids away from dienoic prostanoids to inactive metabolites (52). We have also reported that COX-2 and -1 are upregulated in glomeruli of rats with PHN, compared with control rats. Thus, the current study addresses complement-mediated eicosanoid generation in GEC. We have also reported that COX-1 and -2 are upregulated in glomeruli of rats with PHN, compared with control rats, and that both isoforms contribute to eicosanoid generation in PHN (49). The recent development of COX-2-selective inhibitors provides an opportunity for blockade of COX-2 activity without significant alteration of the effects of COX-1. However, this approach requires a better understanding of the roles of the COX isoforms in kidney physiology as well as in pathological conditions, such as glomerulonephritis. Because several studies support a role for COX isoforms in the pathogenesis of complement-mediated glomerular injury in vivo, it is important to understand the mechanisms of C5b-9-induced expression of COX-2. The present study addresses complement-mediated COX-2 regulation in cultured rat GEC. We demonstrate that COX-2 expression is regulated at the transcriptional level and is mediated via PKC and JNK pathways.

MATERIALS AND METHODS

Materials. Tissue culture media and T4 nucleotide kinase were purchased from Gibco BRL (Burlington, ON). NuSerum was from Collaborative Research (Bedford, MA). C8-deficient human serum (CSD), purified human C8, PGE₂, phorbol 12-myristate 13-acetate (PMA), and pyrrolidinedithiocarbamate (PDTC) were from Sigma (St. Louis, MO). Protease inhibitor cocktail and FuGENE 6 were from Roche Diagnostics (Laval, QC). The gel shift assay system, luciferase assay system, and chloramphenicol acetyltransferase (CAT) enzyme assay system were from Promega (Madison, WI). [γ-³²P]-ATP (3,000 Ci/mmol), [α-³²P]-dCTP (3,000 Ci/mmol), and [α-³²P]-dCTP (3,000 Ci/mmol) were from New England Nuclear (Boston, MA). PD-98059 and JNK assay kit were from New England Biolabs (Mississauga, ON). SC-68376 and MG-132 were from Calbiochem (La Jolla, CA). Rabbit anti-COX-2 antiserum was from Cayman Chemical (Ann Arbor, MI). This antiserum was raised against a synthetic peptide corresponding to amino acids 584–598 of murine COX-2. It recognizes COX-2 as a 72- to 74-kDa protein by immunoblotting and does not cross-react with COX-1. Rabbit anti-MAP/ERK kinase antisera were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-NF-κB p65 subunit antibody were from Santa Cruz Biotechnology (Santa Cruz, CA).

GEC culture. Activation, complement, and induction of PHN. Culture and characterization of rat GEC were described previously (5, 49). A subclone of GEC, which grows on plastic substratum in serum-replete K1 medium, was used in this study. Rabbit antiserum to GEC (5) was used to activate complement on GEC membranes. Briefly, GEC were incubated with antiserum (5% vol/vol) for 40 min at 22°C. GEC were then incubated with normal human serum (NS; 2.5–5.0% vol/vol) or heat-inactivated (decomplemented) human serum (HS; 56°C, 30 min, 2.5–5.0% vol/vol) in controls for the indicated times at 37°C. In some experiments, antibody-sensitized GEC were incubated with C8D (5.0% vol/vol) reconstituted with or without purified human C8 (80 μg/ml undiluted serum). We have generally used heterologous complement to facilitate studies with complement-deficient sera and to minimize possible signaling via complement-regulatory proteins; however, in previous studies, results of several experiments involving arachidonic acid metabolism were confirmed with homologous (rat) complement (5). Sublethal concentrations of complement (≤5% NS) were established previously (5). Previous studies have shown that in GEC, complement is not activated in the absence of antibody (5). PHN was induced in male Sprague-Dawley rats (150–175 g body wt; Charles River, St. Constant, QC) by intravenous injection (400 μg/rat) of sheep anti-Fx1A antiserum as described previously (49). Preparation of anti-Fx1A antiserum was described previously (42). Rats developed significant proteinuria 14 days after injection (~250 mg/day; normal rats excrete <10 mg protein/day). At the 14-day time point, rats were killed, and glomeruli were isolated by differential sieving as described previously (49).

Assay of JNK activity. Confluent GEC in 6-cm culture plates were incubated with complement as described above for 20–40 min. Cells were washed with ice-cold homogenization buffer (50 mM HEPES, 0.25 mM sucrose, 1 mM EDTA, 1 mM EGTA, 20 μM leupeptin, 20 μM pepstatin, and 0.1 M phenylmethylsulfonyl fluoride (PMSF), pH 7.40) and lysed with 100 μl of cold lysis buffer [0.1% Triton X-100, 25 mM HEPES, 20 mM β-glycerophosphate, pH 7.7, 150 mM NaCl, 2 mM MgCl₂, 1 mM EGTA, 0.5 mM 1,4-dithiothreitol (DTT), 20 μM leupeptin, 20 μM pepstatin, and 0.2 mM PMSF, and 1 mM Na₃VO₄]. After centrifugation (10,000 g, 10 min), protein concentration was quantified and adjusted to 4 mg/ml. Fifty microliters of cell lysate (200 μg) were incubated with glutathione S-transferase (GST)-c-Jun-agarose (100 μl, 10% suspension) for 2 h at 4°C. After four washes, pellets were resuspended in 40 μl of kinase buffer (50 mM β-glycerophosphate, 5 mM DTT, 20 mM MgCl₂, 20 mM β-glycerophosphate, pH 7.8, and 20 μl of activated MEKK1) for 10 min at 30°C. After centrifugation (10,000 g, 1 min), the supernatant was assayed.
phate, pH 7.6, 10 mM MgCl₂, 1 mM Na₂VO₄, 20 μM ATP, and 5 μCi [γ-32P]ATP) and incubated for 20 min at 30°C. The kinase reaction was terminated with 20 μl of 3× Laemmlli reducing buffer. Samples were boiled and subjected to SDS-PAGE and autoradiography. In some experiments, JNK activity was measured by use of the JNK assay kit. Samples were processed in an analogous manner, and GST-c-Jun phosphorylated at Ser⁶³ was detected by immunoblotting with anti-phospho-c-Jun antibody. Equal loading was verified by immunoblotting with anti-c-Jun antibody. Signals were quantified by use of scanning densitometry (NIH Image software).

Transfection of GEC and reporter assays. Different transfection methods and reporter constructs were used to achieve optimal transfection efficiency and protein expression of specific plasmids. Confluent GEC were harvested, washed with ice-cold serum-free culture medium, and resuspended in ice-cold serum-free medium (2.4 × 10⁶ cells/ml). Cell suspensions (0.5 ml) were placed in electroporation cuvettes (0.4 cm) and mixed with indicated amounts of DNA by pipetting. Cuvettes were placed on ice and electroporation was performed with the use of Gene-Pulser II (Bio-Rad) at a voltage of 200 V and a capacitance of 975 μF. The time constant was typically 32–35 ms. Cuvettes were placed on ice for 10 min before cells were transferred to 35-mm plates (cells from 1 cuvette were divided into 8 plates). Cells were incubated with antibody and complement 2–3 days after electroporation. At the end of 6 h, cells were washed with PBS and harvested in 0.4 ml of reporter lysis buffer (Promega). After the freezing and thawing, cell lysates were centrifuged at 14,000 g for 5 min, and 25 μl of supernatant were mixed with 100 μl of luciferase reagent (Promega). Luciferase activity was measured in a luminometer.

In some experiments, GEC were transfected with plasmid DNA using FuGENE 6 transfection reagent according to the manufacturer’s instructions. For gene reporter assays, GEC were passed into 35-mm plates at 3–10⁶ cells/ml. On the following day, plasmids were mixed with FuGENE 6 at a ratio of 1:6 (micrograms of plasmid to microliters of FuGENE 6 in 100 μl of serum-free medium) and incubated with 50,000–100,000 counts/min (cpm) of 32P-labeled NF-kB probe for 20 min at room temperature in binding buffer [20% glycerol, 50 mM HEPES, pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM EDTA]. Nonidet P-40 was added to a final concentration of 0.5%, and nuclei were incubated for 10 min on ice. After being vortexed for 10 s, nuclei were sedimented by centrifugation for 10 min at 1,500 g. The nuclear pellets were extracted with 100 μl of high-salt extraction buffer (20 mM HEPES, pH 7.4, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 20% glycerol, and 0.5 mM DTT) for 10 min on ice with frequent agitation. The nuclear debris was discarded by centrifugation at 12,000 g for 10 min, and the nuclear extracts were collected and stored at −85°C.

Electrophoretic gel mobility shift assay. An electrophoretic gel mobility shift assay (EMSA) was performed with the use of the gel shift assay system (Promega) according to the manufacturer’s instructions. Double-stranded oligonucleotide for the human NF-κB consensus binding site (5'-AGTGAGGGGACTTTCCAGG-3') was radiolabeled by use of T4 polynucleotide kinase and [γ-32P]ATP and was purified by centrifugation through G-25 Sephadex spin columns. Ten micrograms of nuclear protein were incubated with 50,000–200,000 counts/min (cpm) of 32P-labeled NF-κB probe for 20 min at room temperature in binding buffer [20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris·HCl, pH 7.5, and 0.25 mg/ml poly(dI-dC)]. Samples were loaded onto 7% polyacrylamide gels with Tris-borate buffer (pH 8.3) at 200 V for 3 h. The gel was dried under vacuum and exposed to X-ray film for 24 h. For supershift assays, anti-NF-κB p65 subunit antibody was incubated with the reaction mixture for 10 min before addition of radiolabeled NF-κB probe.

Statistics. Data are presented as means ± SE. The t-statistic was used to determine significant differences between two groups. One-way 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 by adjusting the critical value according to the Bonferroni method.

RESULTS

Complement-induced COX-2 upregulation is partially dependent on PKC. Previously, we demonstrated that sublytic C5b-9 activates PKC and ERK and up-
regulates COX-2, but not COX-1, expression in cultured rat GEC (49). In keeping with these results, when GEC were stimulated with antibody and complement (NS), COX-2 mRNA and protein were upregulated compared with control (HIS; Fig. 1A). C8D, which forms C5b-7 and is biologically inactive in GEC (49), did not cause significant COX-2 mRNA upregulation, whereas C8D reconstituted with purified C8 clearly upregulated COX-2 mRNA, indicating that formation of C5b-9 is required for COX-2 upregulation (Fig. 1A).

Next, we addressed the role of PKC and ERK in complement-induced COX-2 expression. Prolonged incubation of GEC with PMA (2 µg/ml for 18 h) downregulates PKC activity by 100% (6). In PKC-depleted cells, complement-induced COX-2 mRNA expression was inhibited by 45%, indicating that mRNA upregulation is, at least in part, mediated by PKC (Fig. 1, B and C). In contrast, PD-98059, a specific inhibitor of MAP/ERK kinase (MEK)-1 and -2 and thus of the ERK pathway, did not affect COX-2 upregulation at a concentration that inhibited complement-stimulated ERK2 activity by >90% (6). Thus the ERK pathway, although activated by complement, does not appear to have a major role in complement-induced COX-2 upregulation.

**Complement activates JNK in GEC.** PKC appeared to mediate complement-induced COX-2 expression in GEC, but downregulation of PKC did not completely inhibit COX-2 upregulation. This result suggested involvement of additional mediators, which may include JNK. We therefore examined whether complement stimulates JNK activity in GEC. Incubation of GEC with antibody and complement (20–40 min) increased JNK activity 2.3- to 3.3-fold compared with control (HIS; Fig. 2A). To verify that C5b-9 assembly was actually required for JNK activation, antibody-treated GEC were incubated with C8D, with or without reconstitution with purified C8. C8D alone did not activate JNK compared with buffer. However, when C8D was reconstituted with purified C8, JNK activation was evident, indicating that formation of C5b-9 was required. It should also be noted that complement-induced JNK activation is unaffected by PKC depletion (data not shown).

It is important to verify that complement-induced JNK activation in cultured GEC is relevant to GEC injury in vivo, i.e., in the PHN model, where C5b-9 induces visceral GEC injury and proteinuria (49). JNK activity was studied in glomeruli isolated from rats with PHN on day 14, when the rats showed significant proteinuria. JNK activity in glomerular lysates was approximately four times higher in rats with PHN compared with control rats (Fig. 2B). Thus the JNK pathway is activated by C5b-9 both in cultured GEC and in vivo.

**JNK mediates complement-induced COX-2 expression in GEC.** Chemical inhibitors of the JNK pathway are currently not available. Thus we utilized a kinase-inactive form of JNK1, JNK1(apf) (8), and a COX-2 promoter-reporter construct to evaluate the contribution of the JNK pathway to complement-induced COX-2 upregulation. After transient transfection of GEC with the rat COX-2 promoter-luciferase construct, rCOX2–1.9-luc, a high basal luciferase activity was noted compared with cells transfected with vector (20–200 times baseline). This high basal luciferase activity does not reflect basal COX-2 mRNA expression in unstimulated GEC, which is trivial (49), and most likely is due to pronounced stability of the luciferase mRNA compared with the relatively unstable COX-2 mRNA. After GEC transiently transfected with rCOX2–1.9-luc were stimulated with complement, pro
moter activity (as reflected by luciferase activity) increased by \( \sim 82\% \) (Fig. 3). In consideration of the high basal activity of this promoter, the complement-induced increase is actually substantial. The COX-2 promoter was also activated significantly by PMA (42\%) (Fig. 3A). Although promoter activation by PMA was relatively small in magnitude, incubation of GEC with PMA also induced expression of COX-2 mRNA and protein (Fig. 3B), implying that the effect of PMA on COX-2 induction is biologically significant. When rCOX2–1.9-luc was transiently co-transfected with JNK1(apf), the complement-induced promoter activation was markedly attenuated, indicating that JNK(apf) most likely acted as a dominant inhibitor of endogenous JNK and prevented activation of the COX-2 promoter (Fig. 3A). JNK(apf) did not affect the COX-2 promoter activation by PMA. These results strongly suggest that the JNK pathway is an important mediator of complement-induced COX-2 upregulation. We also attempted to establish a subclone of GEC that stably expresses JNK1(apf) to demonstrate directly a blunted COX-2 response of such clone to complement. However, we were not able to obtain a clone that had a high expression level of JNK1(apf), because dominant-negative JNK may have impaired GEC proliferation.

To further confirm the role of the JNK pathway in COX-2 upregulation, we transfected GEC with a constitutively active MEKK1 (pFC-MEKK), a kinase upstream of JNK, and evaluated COX-2 promoter activity. Cotransfection of pFC-MEKK with rCOX2–2.7-CAT increased COX-2 promoter activity 2.2- to 2.4-fold compared with control (vector; Fig. 4, A and B).
keeping with the results in Fig. 3, the COX-2 promoter in vector-transfected cells was activated by 6 h of treatment with PMA (~40%; Fig. 4A). The effects of MEKK1 and PMA were additive, because stimulation of pFC-MEKK-transfected cells with PMA further activated the COX-2 promoter (Fig. 4A). PKC depletion did not inhibit MEKK1-induced COX-2 promoter activity, indicating that the effect of MEKK1 on the COX-2 promoter is not dependent on PKC (Fig. 4A). These results indicate that MEKK most likely induces COX-2 expression via JNK activation and that PKC acts in concert with the JNK pathway. PKC- and JNK-mediated COX-2 upregulation are independent of each other, and these two pathways likely act in an additive manner.

Although MEKK primarily activates the JNK pathway, it has been reported that MEKK, when overexpressed, may also activate the ERK or p38 MAPK pathways (25). To verify the potential contribution of the ERK and p38 pathways to MEKK-induced COX-2 promoter activation, we utilized specific inhibitors of these two pathways. Neither PD-98059 (an inhibitor of the ERK pathway) nor SC-68376 (an inhibitor of the p38 pathway) inhibited MEKK-induced COX-2 promoter activity (Fig. 4B). Furthermore, PD-98059 and SC-68376 did not inhibit complement-mediated COX-2 mRNA upregulation in GEC (Figs. 1 and 4C). It has also been reported that MEKK1 can activate the NF-κB pathway via activation of the IkB kinase complex (27). To rule out the potential contribution of the NF-κB pathway in MEKK-induced COX-2 promoter activity, we utilized a mouse COX-2 promoter in which the NF-κB binding site was mutated (pGC815NF-κBM). When the wild-type promoter pGC815 was co-transfected with pFC-MEKK, COX-2 promoter activity was increased 1.6 ± 0.2-fold (N = 5 experiments conducted in duplicate). Promoter activity of pGC815NF-κBM was increased to a similar extent by pFC-MEKK (1.8 ± 0.4-fold, N = 5 experiments conducted in duplicate), indicating that NF-κB is not involved in MEKK-induced COX-2 promoter activity. Taken together, these results indicate that activation of the JNK pathway by MEKK leads to promoter activation of the COX-2 gene in GEC.

To verify that MEKK-induced COX-2 promoter activity is biologically significant, i.e., associated with COX-2 protein expression, we transiently transfected GEC with pFC-MEKK and evaluated the expression of COX-2 protein by immunoblotting. COX-2 protein was clearly induced when the constitutively active MEKK was overexpressed (Fig. 4D).

![Fig. 4. MEK kinase (MEKK)-1 induces COX-2 expression via the JNK pathway. A: GEC in 35-mm plates were transiently transfected with COX-2 promoter coupled to chloramphenicol acetyltransferase (CAT), rCOX2-2.6-CAT (0.5 μg), plus constitutively active MEKK (pFC-MEKK) or vector (50 ng), using FuGENE 6. Left: after 24 h, cells were incubated with PMA (250 ng/ml) or vehicle (0.1% DMSO) for 6 h, and CAT activity in cell lysates was quantified. Right: to deplete PKC, cells were incubated with 2 μg/ml of PMA for ~18 h before transfection. Cells were transfected as above, and CAT activity in cell lysates was quantified after 24 h. *P < 0.002 vs. vector+DMSO; N = 3–6 experiments conducted in duplicate. B: vehicle (DMSO), PD98059 (50 μM), or SC68373 (10 μM) was added to the medium immediately after transfection. CAT activity in cell lysates was quantified after 24 h. *P < 0.05 and **P < 0.01 vs. vector; each bar represents 4 values. C: GEC were stimulated with complement (NS) for 100 min in the presence of vehicle (DMSO), PD98059 (50 μM), or SC68373 (10 μM). Total RNA was analyzed for COX-2 mRNA expression by Northern hybridization. Equal loading was confirmed by ethidium bromide staining of the gel (not shown). D: GEC plated in 6-cm plates were transfected with the indicated amounts of pFC-MEKK, using FuGENE 6. After 24 h, nuclear fractions and postnuclear supernatants were analyzed for expression of COX-2 and MEKK1 protein, respectively, by immunoblotting.](http://ajprenal.physiology.org/.../F846.pdf)
Complement activates nuclear factor (NF-κB). A: antibody-sensitized GEC were incubated with complement (NS) or HIS for 1 h. Nuclear extracts were analyzed by electrophoretic gel mobility shift assay (EMSA) to evaluate nuclear translocation of NF-κB. B: nuclear extracts from complement-stimulated GEC were subjected to EMSA with or without preincubation with rabbit anti-p65 antibody or nonimmune rabbit serum (NRS). The top 2 bands (labeled NF-κB) were supershifted by the antibody. The bottom band was not supershifted by the antibody, indicating that this band is nonspecific. (The spot in the lane farthest right is nonspecific.) C: GEC (1.2 × 10⁶ cells) were transfected with NF-κB binding sequence coupled to luciferase (NF-κB-luc; 15 μg) by electroporation and were plated into 35-mm wells. After 48 h, cells were incubated with antibody and complement (NS) or PMA (250 ng/ml) for 6 h. Luciferase activity was measured in cell lysates. *P < 0.05, **P < 0.005, and ***P < 0.002 vs. HIS; N = 6 experiments conducted in duplicate.

Complement-induced COX-2 upregulation is not mediated by NF-κB. Another pathway that has been reported to regulate COX-2 is NF-κB. To monitor activation of NF-κB, GEC were incubated with antibody and complement, and translocation of NF-κB from the cytosol to the nucleus was evaluated by EMSA, using 32P-labeled NF-κB-binding oligonucleotide as probe. After stimulation with complement, at least three distinct bands were observed by EMSA (Fig. 5A). The two top bands were supershifted by preincubation with the anti-NF-κB p65 antibody, indicating that these bands are specific to NF-κB. The bottom band was not supershifted by this antibody, suggesting that it is nonspecific (Fig. 5B).

To determine whether complement can activate gene transcription via activation of NF-κB, GEC were transfected with the luciferase gene driven by the NF-κB binding sequence (NF-κB-luc), and the cells were incubated with antibody and complement for 6 h. GEC stimulated with 2.5% NS showed an ∼2.2-fold increase in luciferase activity compared with control (HIS). NS (5% vol/vol) caused an ∼3.3-fold increase, whereas PMA, a known stimulator of NF-κB, caused an ∼8.4-fold increase compared with control (Fig. 5C). Thus complement activates gene transcription via NF-κB, although to a lesser extent than PMA.

To address the role of NF-κB in complement-mediated COX-2 expression, we utilized two inhibitors of the NF-κB pathway that are known to act via different mechanisms. PDTC is an antioxidant known to inhibit the NF-κB pathway in various systems (1). MG-132 is a proteasome inhibitor that inhibits the NF-κB pathway by inhibiting degradation of the inhibitory protein IκB (37). Using EMSA, we confirmed that these two compounds inhibited complement-mediated nuclear translocation of NF-κB (Fig. 6A). However, when GEC were incubated with antibody and complement (100 min), neither PDTC (100 μM) nor MG-132 (20 μM) inhibited complement-induced COX-2 mRNA expression (NS+PDTC: 140 ± 16% of NS alone, 3 experiments; NS+MG-132: 150 ± 15% of NS alone, 3 experiments; mRNA levels were quantified by densitometry).

Fig. 5. Complement activates nuclear factor (NF-κB). A: antibody-sensitized GEC were incubated with complement (NS) or HIS for 1 h. Nuclear extracts were analyzed by electrophoretic gel mobility shift assay (EMSA) to evaluate nuclear translocation of NF-κB. B: nuclear extracts from complement-stimulated GEC were subjected to EMSA with or without preincubation with rabbit anti-p65 antibody or nonimmune rabbit serum (NRS). The top 2 bands (labeled NF-κB) were supershifted by the antibody. The bottom band was not supershifted by the antibody, indicating that this band is nonspecific. (The spot in the lane farthest right is nonspecific.) C: GEC (1.2 × 10⁶ cells) were transfected with NF-κB binding sequence coupled to luciferase (NF-κB-luc; 15 μg) by electroporation and were plated into 35-mm wells. After 48 h, cells were incubated with antibody and complement (NS) or PMA (250 ng/ml) for 6 h. Luciferase activity was measured in cell lysates. *P < 0.05, **P < 0.005, and ***P < 0.002 vs. HIS; N = 6 experiments conducted in duplicate.

Fig. 6. Complement-induced COX-2 upregulation is independent of the NF-κB pathway. A: GEC were incubated with complement (NS) in the presence of pyrrolidinedithiocarbamate (PDTC; 100 μM) or MG-132 (20 μM) for 1 h. Nuclear extracts were prepared and analyzed for NF-κB by EMSA. Arrows point to specific NF-κB bands. B: GEC (1.2 × 10⁶ cells) were transfected with the wild-type mouse COX-2 promoter-luciferase construct (pGC815; 15 μg) or a COX-2 promoter, where the NF-κB site is mutated (κB-M; 15 μg), and were plated into 35-mm wells. After 48 h, cells were incubated with antibody and complement for 6 h, and luciferase activity was quantified in cell lysates. *P < 0.05 vs. HIS; N = 3 experiments conducted in duplicate.
To further assess the role of NF-κB in complement-mediated COX-2 upregulation, we utilized mouse COX-2 promoter-reporter constructs with or without a mutation at the NF-κB binding site. GEC were transfected with the mouse COX-2 promoter-luciferase construct (pGC815) or with a promoter in which the NF-κB binding site was mutated (pGC815NF-κBM). After incubation with antibody and complement, luciferase activity increased ~1.6-fold compared with control in GEC expressing the wild-type promoter and ~1.7-fold in GEC expressing pGC815NF-κBM (Fig. 6B). Similar results were obtained with the use of rat COX-2 promoters with or without a mutation at the NF-κB binding site (data not shown).

It has been reported recently that NF-κB is activated in GEC of rats with PHN and that PDTC (200 mg·kg⁻¹·day⁻¹) effectively inhibits NF-κB activation (32). To determine whether NF-κB activation contributes to complement-mediated COX-2 upregulation in vivo, rats with PHN were treated with PDTC according to a similar protocol (200 mg·kg⁻¹·day⁻¹ from day 7 to 14). Glomerular COX-2 mRNA expression was evaluated on day 14 with the use of RNase protection assay. Densitometric analysis showed that COX-2 mRNA expression in glomeruli from rats with PHN treated with vehicle (water) was 1.5 ± 0.1-fold greater compared with normal rats (N = 3), consistent with our previous study (49). PDTC treatment of PHN rats did not affect the increase in COX-2 mRNA, i.e., COX-2 mRNA expression was 2.0 ± 0.2-fold greater compared with normal rats (N = 3). Taken together, the results show that NF-κB does not contribute to complement-induced COX-2 upregulation in GEC both in vitro and in vivo.

**DISCUSSION**

The present study characterizes the regulation of the COX-2 gene by C5b-9. We have previously shown that in GEC, complement activates protein kinases, including PKC and ERK. Our present study demonstrates that complement activates JNK (Fig. 2A). Moreover, JNK activity was stimulated by C5b-9 in GEC in vivo, i.e., in PHN (Fig. 2B). To our knowledge, the present study is the first to demonstrate C5b-9-mediated JNK activation in vivo. Complement-induced COX-2 mRNA was upregulated via PKC, whereas the ERK pathway was not involved (Fig. 1). The rat COX-2 promoter was activated by complement, and this activation was inhibited significantly by blocking of the JNK pathway by overexpression of a kinase-inactive mutant of JNK1 (Fig. 3A). Inhibition of the JNK pathway did not, however, affect PKC-mediated COX-2 promoter activation (Fig. 3A). Stimulation of the JNK pathway using a constitutively active mutant of MEKK1 resulted in COX-2 promoter activation and protein expression, which was independent of PKC (Fig. 4, A, B, and D). Thus the JNK and PKC pathways contribute to COX-2 upregulation in GEC independently and additively. It is interesting to note that in GEC, PKC is involved in the regulation of eicosanoid production at two levels, including stimulation of cPLA₂, thereby facilitating arachidonic acid release and induction of COX-2, which enhances arachidonic acid metabolism, whereas JNK, which does not activate cPLA₂ (unpublished observations), regulates only COX-2.

Our results are in keeping with studies of Xie and Herschman (53, 54), who showed that the Ras-MEK1-JNK signal transduction pathway contributed to v-src-, platelet-derived growth factor-, or serum-induced COX-2 promoter activation in mouse fibroblasts. The same investigators also reported a role for the JNK pathway in transcriptional regulation of the COX-2 gene in activated mast cells and the endotoxin-treated mouse macrophage cell line RAW 264.7 (39, 50). In these cells, the cAMP-response element (CRE) appears to be critical in COX-2 gene activation. Curiously, the rat COX-2 promoter does not contain the CRE, although the E-box element, which overlaps the CRE in mouse and human genes, is conserved in the rat and is critical in hormone-induced COX-2 expression in rat ovarian granulosa cells (31). The cis-acting elements required for the JNK pathway-mediated COX-2 regulation in rat GEC remain to be elucidated. Guan et al. (15) demonstrated that a constitutively active truncation mutant of MEKK1 increased COX-2 expression in mouse fibroblast cells (15). Although these results appear to be similar to our study, in that system, inhibition of p38 by SC-68376 completely abolished MEKK1-induced COX-2 induction, whereas in our study, SC-68376 inhibited neither MEKK1-induced COX-2 promoter activity nor complement-mediated COX-2 upregulation (Fig. 4, B and C). The reason for this discrepancy is not clear, but the effects of MEKK1 might be influenced by the cell type.

The mouse COX-2 gene was originally identified as a phorbol ester-inducible gene (24), and, since then, other studies have demonstrated that PKC is a potent inducer of COX-2 (17). However, surprisingly little is known about the mechanisms of PKC-induced COX-2 gene activation. In some reports, it was shown that PKC-dependent COX-2 upregulation may be mediated by activation of ERK (12, 19), although this is not the case in the present study (Fig. 1). Miller et al. (29) reported that overexpression of the atypical PKC-ζ isoform increased COX-2 expression in mouse fibroblast cells (15). Although these results appear to be similar to our study, in that system, inhibition of p38 by SC-68376 completely abolished MEKK1-induced COX-2 induction, whereas in our study, SC-68376 inhibited neither MEKK1-induced COX-2 promoter activity nor complement-mediated COX-2 upregulation (Fig. 4, B and C). The reason for this discrepancy is not clear, but the effects of MEKK1 might be influenced by the cell type.

The promoter region of the COX-2 gene in all species examined so far is known to contain NF-κB binding site(s) (9, 23, 46, 55). Therefore, it was surprising that although NF-κB was activated by complement in GEC (Fig. 5), NF-κB did not contribute to COX-2 upregulation. However, such a result is not without precedent. For example, the NF-κB binding site was not relevant to COX-2 regulation in endotoxin-treated mouse macrophages and activated mouse mast cells (39, 50). On the other hand, a number of studies demonstrate a
critical role for NF-κB in COX-2 regulation (57). One possible explanation for these conflicting results is that an extracellular stimulus activates a panel of transcription factors rather than a single transcription factor, and the stimulus-dependent combination of transcription factors would lead to a certain pattern of gene regulation. Transcription cofactors, such as p300/ CBP, may orient certain transcription factors to stimulate gene transcription (10). Promoter regions of the human (23), mouse (9), chicken (55), and rat (46) COX-2 gene have been shown to contain multiple regions of homology, including putative binding sites for transcription factors such as CRE, E-box, NF-interleukin-6/C/EBPβ, AP-2, SP1, and NF-κB. C5b-9 has been reported to induce expression of several growth factors, cytokines, and extracellular matrix proteins, suggesting that C5b-9 can potentially activate a variety of distinct transcription factors. A specific pattern of transcription factor induction could upregulate COX-2. There might also be a redundancy of certain induced transcription factors, such as NF-κB in the case of COX-2. Recent studies have reported that COX-2 expression may also be regulated by stabilization of mRNA (26, 56). Assembly of C5b-9 could thus potentially enhance COX-2 mRNA stability in GEC. This might account for the discrepancy between relatively modest COX-2 promoter activation (∼1.8-fold) and more pronounced mRNA upregulation (∼4-fold) (49). The potential effect of complement on RNA stability should be addressed in future studies.

A number of studies support an important role for prostanooids in the mediation of proteinuria in experimental (49) and human membranous nephropathy (11). The potential role for COX-2 in the pathogenesis of proteinuria was highlighted by our earlier study (49), and it was recently reported that a COX-2-selective inhibitor, flosulide, reduced proteinuria in PHN, although the inhibitory action of flosulide may not have occurred solely via COX-2 inhibition (2). Elucidation of the pathways of C5b-9-induced regulation of COX-2 gene expression will provide further insights into the pathogenesis of membranous nephropathy and other forms of glomerulonephritis, as well as different types of C5b-9-mediated tissue injury involving prostanooids (30, 34, 44), and will eventually allow for development of novel therapeutic approaches.

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