Role of calcium-independent phospholipase A_2 in complement-mediated glomerular epithelial cell injury

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Submitted 7 August 2007; accepted in final form 28 December 2007

Cohen D, Papillon J, Aoudjit L, Li H, Cybulsky AV, Takano T. Role of calcium-independent phospholipase A_2 in complement-mediated glomerular epithelial cell injury. Am J Physiol Renal Physiol 294: F469–F479, 2008. First published January 2, 2008; doi:10.1152/ajprenal.00372.2007. —In experimental membranous nephropathy, complement C5b-9-induced glomerular epithelial cell (GEC) injury leads to morphological changes in GEC and proteinuria, in association with phospholipase A_2 (PLA2) activation. The present study addresses the role of calcium-independent PLA2 (iPLA2) in GEC injury. iPLA2 short and iPLA2γ were expressed in cultured rat GEC and normal rat glomeruli. To determine whether iPLA2 is involved in complement-mediated arachidonic acid (AA) release, GEC were stably transfected with iPLA2γ or iPLA2β cDNAs (GEC-iPLA2γ; GEC-iPLA2β). Compared with control cells (GEC-Neo), GEC-iPLA2γ and GEC-iPLA2β demonstrated greater expression of iPLA2 proteins and activities. Complement-mediated release of [3H]AA was augmented significantly in GEC-iPLA2γ compared with GEC-Neo, and the augmented [3H]AA release was inhibited by the iPLA2-directed inhibitor bromoanol lactone (BEL). For comparison, overexpression of iPLA2γ also amplified [3H]AA release after incubation of GEC with H_2O_2, or chemical anoxia followed by reoxygenation to glucose (in vitro ischemia-reperfusion injury). In parallel with release of [3H]AA, complement-mediated production of prostaglandin E_2 was amplified in GEC-iPLA2γ. Complement-mediated cytotoxicity was attenuated significantly in GEC-iPLA2γ compared with GEC-Neo, and the cytoprotective effect of iPLA2γ was reversed by BEL, and in part by indomethacin. Overexpression of iPLA2β did not amplify complement-dependent [3H]AA release, but nonetheless attenuated complement-mediated cytotoxicity. Thus iPLA2γ may be involved in complement-mediated release of AA. Expression of iPLA2γ or iPLA2β induces cytoprotection against complement-dependent GEC injury. Modulation of iPLA2 activity may prove to be a novel approach to reducing GEC injury.

iPLA2β; iPLA2γ; cytoprotection

Members of the phospholipase A_2 (PLA2) family of enzymes hydrolyze fatty acids from the sn-2 position of phospholipids, generating free fatty acids and lysophospholipids (34, 43). Both products of this reaction have signaling properties. Moreover, arachidonic acid (AA) may be further metabolized by cyclooxygenase (COX) and lipoxygenase enzymes to bioactive eicosanoids. The PLA2s may be subdivided into three categories, including secretory PLA2s (sPLA2; groups I, II, III, V, VII–XIV), calcium-dependent PLA2s, e.g., cytosolic phospholipase A_2α (cPLA2, group IV), and the most recently discovered calcium-independent PLA2s (iPLA2s, group VI) (34, 43).

cPLA2 plays an important role in complement-mediated glomerular epithelial cell (GEC) injury in the passive Heymann nephritis (PHN) model of membranous nephropathy (19). GEC, otherwise known as podocytes, are an important component of the glomerular permselective barrier (33, 38, 42). In PHN and human membranous nephropathy, deposition of antibodies in the subepithelial region of the glomerulus leads to activation of complement and assembly of the C5b-9 membrane attack complex in GEC plasma membranes, leading to GEC injury and proteinuria. Studies in cultured GEC and in PHN indicate that C5b-9 induces sublethal GEC injury, which is associated with activation of protein kinases and phospholipases (including cPLA2), upregulation of COX-2, production of reactive oxygen species, induction of endoplasmic reticulum (ER) stress response, and other effects. These pathways ultimately contribute to changes in GEC lipid composition and function, reorganization of the actin cytoskeleton, and displacement of filtration slit diaphragm proteins (19).

Previous studies have demonstrated that assembly of C5b-9 in cultured GEC is linked to the activation of cPLA2 and that glomeruli of rats with PHN demonstrate increased cPLA2 activity compared with control glomeruli (23). Activation of cPLA2 leads to various cellular effects. First, in GEC, activation of cPLA2 perturbs the membrane of the ER and contributes to induction of ER stress. Preconditioning of rats with compounds that induce ER stress can limit proteinuria in PHN (22). Second, activation of cPLA2 may contribute directly to the exacerbation of C5b-9-induced GEC injury (18, 23, 37). Finally, the AA mobilized by cPLA2 is metabolized by COX enzymes (44) to bioactive eicosanoids. Glomeruli from rats with PHN show increased COX-1 and COX-2 protein expression and enhanced production of prostaglandin (PG) E_2 and thromboxane A_2. Blockade of COX enzymes reduces urinary protein excretion in PHN and in human membranous nephropathy (45, 46).

The purpose of the present study was to determine whether complement could stimulate the activity of iPLA2. Among the various members of the iPLA2 family, we focused on two isoenzyme subfamilies, so-called iPLA2-VIA-1 and -2 (iPLA2β short and iPLA2β long) and iPLA2-VIB (iPLA2γ), each with unique structural features, especially in the NH2 terminus (29, 31, 43). Both iPLA2β short and iPLA2β long are functional enzymes, which differ by a 54-amino acid proline-rich insertion sequence (13). Knockout mice lacking iPLA2β show only impaired sperm motility (7), while transgenic mice overexpressing the same isoform are prone to ventricular tachyarrhythmias.

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iPLA2γ is a membrane-bound enzyme, with competing peroxisomal (COOH terminal) and mitochondrial (NH2 terminal) localization signals (31). iPLA2γ also localizes in the ER (17). iPLA2γ gene transcription and translation are complex (31). iPLA2γ has multiple promoter sites, as well as a transcriptional repressor site in the 5’-untranslated region. The full-length 2.4-kb transcript contains 13 exons and includes four AUG translation initiation codons generating as many as 10 splice variants, which appear as 88-, 77-, 74-, and 63-kDa immunoreactive proteins on SDS-PAGE and immunoblotting (47). iPLA2γ enzymes are active in basal phospholipid turnover, phospholipid accumulation during the G1/M phase of mitosis, and are involved at various levels of apoptotic cascades (2, 5). iPLA2γ was shown to be cytoprotective against oxidant insults in renal cells, presumably by removing oxidized phospholipids (17). In contrast, inhibition of rabbit renal proximal tubule cell microsomal iPLA2γ during cisplatin-induced apoptosis reduced annexin V staining, chromatin condensation, and caspase-3 activation, indicating that iPLA2γ inhibition was cytoprotective (16). Mitochondrial iPLA2γ has also been shown to actively participate in the permeability pore transition of the mitochondria and the release of the proapoptotic cytochrome c (25). A recent study showed that iPLA2γ knockout mice display multiple bioenergetic dysfunctional phenotypes (32). In addition, several potential pathophysiological roles have been described for the iPLA2γs, including AA release for eicosanoid production, tumorigenesis, cell injury, apoptosis, chemotaxis, and others (2, 5, 35).

In the present study, we have used cultured GEC to demonstrate that C5b-9 can stimulate an iPLA2 to increase free [3H]AA. Overexpression of iPLA2γ in GEC augmented complement-dependent [3H]AA release, and production of PGE2. Furthermore, overexpression of iPLA2γ, as well as iPLA2β limited complement-induced GEC injury via eicosanoids, suggesting that activation of iPLA2γ is cytoprotective.

MATERIALS AND METHODS

Materials. Tissue culture media, zeocin, hygromycin, and Lipofectamine 2000 were from Invitrogen-Life Technologies (Burlington, ON). Electrophoresis reagents were from Bio-Rad Laboratories (Mississauga, ON). Mouse anti-iPLA2β, anti-COX-1, and anti-COX-2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) detection reagents were from Amersham Bioscience (Baie d’Urfe, QC). Bromoelenol lactone (BEL) and PGE2 enzyme immunoassay kits were purchased from Cayman Chemical (Ann Arbor, MI). [3H]AA (100 mCi/ml), [14C]arachidonyl-phosphatidylethanolamine (PE; 48 mCi/ml), and [14C]arachidonyl-phosphatidylcholine (PC; 53 mCi/ml), were purchased from PerkinElmer Life and Products (Boston, MA). Mouse iPLA2γ long and iPLA2γ short cDNAs subcloned into pcDNA 1.1 were kindly provided by Dr. Suzanne Jackowski (University of Tennessee Health Centre, Memphis, TN), while human iPLA2γ cDNA in pcDNA 1.1 was provided by Drs. Richard Gross and David Mancuso (Washington University School of Medicine, St. Louis, MO). iPLA2γ antibody was kindly provided by Dr. Makoto Murakami (Showa University, Tokyo, Japan). Unless otherwise specified, all other chemicals and biochemical reagents were purchased from Sigma-Aldrich Canada (Mississauga, ON).

Cell culture, radiolabeling, and transfection. Rat GEC culture and characterization have been described previously (14, 40). According to established criteria, the cells demonstrated cytotoxic susceptibility to low doses of aminonucleoside of puromycin, the presence of junctional complexes by electron microscopy, and expression of a variety of GEC antigens. GEC were maintained in K1 medium. COS cells and Chinese hamster ovary (CHO) cells were cultured in DMEM-10% fetal calf serum.

Phospholipids were radiolabeled to isotopic equilibrium by incubating cells with [3H]AA (0.125 μCi/ml K1 medium) for 72 h (20). At the end of incubations, supernatants and cells were collected, lipids were extracted with chloroform:methanol (1:1.2) and 0.2% formic acid, followed by chloroform, and were then dried under nitrogen. After reconstitution in chloroform:methanol (2:1) and addition of “cold” AA and 1,2-diacylglycerol (DAG) standards, the free AA and DAG were separated from other lipids by thin-layer chromatography in hexane:diethyl ether:acetic acid (80:20:2). Relevant bands were scraped, and radioactivity was quantified in a beta liquid scintillation counter (18, 37). It should be noted that when using this method, we cannot exclude some oxidation of [3H]AA.

For expression of iPLA2γ enzymes, plasmids containing iPLA2β short or iPLA2γ cDNAs were transfected in GEC using the Lipofectamine 2000 reagent. Following selection by neomycin and expansion, all subclones were tested for overexpression of the enzyme by immunoblotting. Confirmation of enhanced enzyme activity was determined using an in vitro PLA2 assay (described below). Characterization of GEC that overexpress cPLA2γ was published previously (18, 37).

Incubation of GEC with complement. GEC were incubated with rabbit anti-GEC antisera (5% vol/vol) in modified Krebs-Henseleit buffer containing (in mM) 145 NaCl, 5 KCI, 0.5 MgSO4, 1 Na3HPO4, 0.5 mM CaCl2, 5 glucose, and 20 HEPES, pH 7.4, for 30 min at 22°C. The cells were then incubated at 37°C with normal human serum (NS; with full complement activity) or heat-inactivated serum (HIS; incubated at 56°C for 30 min to inactivate complement) as indicated. In experiments designed to measure Ca2+-independent [3H]AA release, antibody-sensitized GEC were incubated with Ca2+-deficient human serum (CSDS) in buffer containing 0.5 mM Ca2+ for 40 min. Then, GEC were washed and incubated with or without purified C8 (2.0 μg/ml) and C9 (1.5 μg/ml) for 40 min in buffer containing 1 mM EGTA (18, 37).

Preparation of cell extracts and in vitro assay of PLA2 activity. Cells were collected in homogenization buffer containing 50 mM HEPES, 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 20 μM leupeptin, 20 μM pepstatin, and 0.1 mM PMSF, pH 7.4 (18, 37). Following centrifugation for 3 min at 1,500 g, the pellet was homogenized with 25 strokes in a Wheaton homogenizer, centrifuged for 5 min at 2,500 g, and the supernatant was collected. The remaining pellet was again homogenized and centrifuged for 5 min with the supernatant being pooled with that collected earlier. The supernatant was centrifuged at 15,000 g for 20 min to remove any remaining insoluble membrane fractions.

To assay PLA2 activity (18, 37), exogenous 2-14C-arachidonoyl-PE or PC (substrate; 3.5 μM) was dried under a stream of nitrogen and reconstituted in 2 μl of DMSO. Cell extracts diluted in homogenization buffer (48 μl) were incubated with substrate for 45 min at 37°C. In some experiments, PLA2 activity was measured after addition of CaCl2 to the homogenization buffer to a final Ca2+ concentration of 2 mM. The reaction was stopped by adding 50 μl of ethanol containing cold AA and acetic acid. [14C]AA was separated from other lipids by thin-layer chromatography as described above.

Immunoblotting. Immunoblotting was done as described previously (45). Glomeruli were isolated from rat kidney cortices by differential sieving (purity of glomeruli was >95%) (22). Briefly, cells or isolated glomeruli were lysed in buffer containing 10 mM sodium pyrophosphate, 25 mM NaF, 2 mM Na3VO4, 2% Triton X-100, 250 mM NaCl, 20 mM Tris, 2 mM EDTA, 2 mM EGTA, and a protease inhibitor cocktail (Roche Diagnostics), pH 7.4. After centrifugation at 14,000 g for 10 min, soluble components were collected and the protein concentration was quantified using the Bio-Rad assay. Equal quantities of proteins were separated by SDS-PAGE under reducing conditions. Proteins were then transferred to a nitrocellulose membrane, and the membrane was
blocked at room temperature for 60 min with 5% dry milk in TBS-T wash buffer containing 10 mM Tris, pH 7.5, 50 mM NaCl, 2.5 mM EDTA, and 0.05% Tween 20. The nitrocellulose membrane was subsequently incubated with primary antibody for 60 min at 22°C, washed three times in TBS-T wash buffer, and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase for 60 min at 22°C. Immunoreactive proteins were then visualized using ECL.

RT-PCR. RNA samples were isolated from cells or normal rat glomeruli using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions (3). RT-PCR was performed with an Invitrogen Superscript II One-Step PCR Kit. Primers designed for iPLA2 were flanked the insertion site of exon 9 (forward: TTGGGCCAGAAGTAGACACC, reverse: GTCAGCATCACCTTGGGTTT) to distinguish short and long isoforms. Primers for iPLA2 were ATTGATGGTG-GAGGAACAAGA (forward) and ATGGCCTGACCATTTATAC (reverse). PCR for iPLA2 and iPLA2 were performed with 35 cycles of 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C. Experimental conditions for EP4 and β-actin PCR were described previously (3). PCR products were separated by agarose gel electrophoresis and visualized after staining with ethidium bromide.

Measurement of intracellular Ca2+ concentration. The method has been described in detail (20). Briefly, GEC were loaded in suspension with 5 μM fura 2-AM in buffer containing 0.5 mM CaCl2 for 30 min at 37°C. After washing, aliquots of cells were placed into a spectrofluorometer in Ca2+-free Krebs-Henseleit buffer containing 2 mM EGTA. Fluorescence of fura 2 was monitored continuously (emission wavelength of 510 nm, excitation wavelengths alternating between 340 and 380 nm) at 37°C. Release of Ca2+ sites was initiated by the addition of 5 μM ionomycin.

Lactate dehydrogenase assay. GEC were incubated with antibody and complement as described above. At the end of the incubations, supernatants were collected and the remaining cell contents were solubilized with Triton-X 100 (1%). The supernatant and Triton fractions were assayed for lactate dehydrogenase (LDH) by incubating 100 μl of the sample in 1.5 ml glycine (58 mM)/lactate buffer (0.2 M), containing NAD (4.8 mg/ml) at 37°C and monitoring absorbance at 340 nm in a spectrophotometer. Specific LDH release was calculated using the formula (NS – HIS)/(100 – HIS) × 100 (40).

Statistics. Data are presented as mean ± SE. The t-statistic was used to determine significant differences between two groups. For more than 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 adjusting the critical value according to the Bonferroni method (see Figs. 5–8). Two-way ANOVA was used to determine significant differences in multiple measurements among groups (see Figs. 3B and 4B).

RESULTS

C5b-9 induces Ca2+-independent release of [3H]AA in GEC. Previous studies demonstrated that in GEC, assembly of C5b-9 leads to an increase in cytosolic Ca2+ concentration, which is primarily due to Ca2+ influx (20). Furthermore, Ca2+ influx activates the Ca2+-dependent cPLA2, leading to release of [3H]AA (37). In the first set of experiments, we assessed whether C5b-9 could also induce [3H]AA release in the absence of Ca2+ influx. Since antibody-dependent activation of complement (i.e., the classical pathway) is Ca2+-dependent, we incubated antibody-sensitized GEC with C8DS in the presence of Ca2+. As a second step, GEC were incubated with or without purified C8 and C9 without Ca2+ (i.e., in the presence of EGTA). The C8DS leads to assembly of the nascent C5b-7 complex, while addition of C8 and C9 allows assembly of the C5b-9 transmembrane channel (in the absence of Ca2+). Formation of C5b-9 resulted in a significant release of [3H]AA, suggesting activation of an iPLA2 (Fig. 1A). C5b-9 also increased [3H]DAG (Fig. 1A), which reflects activation of phospholipase C.

It should be noted that the incubation of GEC with C8 and C9 in buffer containing EGTA not only results in chelation of Ca2+.
extracellular Ca\(^{2+}\) but would most likely also result in depletion of intracellular Ca\(^{2+}\) stores. To verify this effect of EGTA, we measured changes in intracellular Ca\(^{2+}\) concentration by monitoring fluorescence of the Ca\(^{2+}\) indicator fura 2. Addition of ionomycin to GEC in buffer containing 2 mM EGTA resulted in a Ca\(^{2+}\) spike, reflecting release of Ca\(^{2+}\) from intracellular storage sites (Fig. 1B, top). In contrast, if GEC were preincubated with 2 mM EGTA for 20 min before the addition of ionomycin, the Ca\(^{2+}\) spike was almost completely abolished (Fig. 1B, bottom), indicating that intracellular Ca\(^{2+}\) stores were depleted. In preliminary studies, we determined that a 40-min incubation after the addition of C8 and C9 to the GEC with assembled C5b-7 complexes was required to increase free [\(^{3}H\)]AA significantly. Thus the effect of C5b-9 on [\(^{3}H\)]AA occurred while Ca\(^{2+}\) stores were largely depleted.

Expression of iPLA\(_2\) isoforms. Several isoforms of the group VI iPLA\(_2\) enzyme have been described, including iPLA\(_2\)β long, iPLA\(_2\)β short, and iPLA\(_2\)γ. To confirm endogenous expression of iPLA\(_2\) in cultured rat GEC, as well as in rat glomeruli, RT-PCR was performed using primers to regions flanking exon 9 of the iPLA\(_2\)β isoform, and the coding sequence of iPLA\(_2\)γ (Fig. 2). PCR products consistent with iPLA\(_2\)β short (Fig. 2A), as well as iPLA\(_2\)γ (Fig. 2B) were identified in GEC and glomeruli, and identities were confirmed by purification of bands and DNA sequencing. It should be noted that iPLA\(_2\)β RT-PCR in GEC and glomeruli showed additional faint bands, with a band that appeared to correspond to the predicted PCR product for iPLA\(_2\)β long. However, we were not able to confirm the identity of this band by purification and DNA sequencing, so it is not possible to make a firm conclusion regarding expression of iPLA\(_2\)β long.

iPLA\(_2\) protein expression was studied by immunoblotting. GEC and glomeruli express iPLA\(_2\)β and, on SDS-PAGE, migration of this protein was slightly retarded, compared with COS cells transfected with iPLA\(_2\)β short (Fig. 2C). Nevertheless, the protein migrated faster than the iPLA\(_2\)β long isoform. iPLA\(_2\)γ has four potential translational initiation sites, yielding proteins of 88, 77, 74, and 63 kDa, respectively (47). GEC and glomeruli predominantly express the 88-kDa isoform of iPLA\(_2\)γ (Fig. 2D).

Overexpression of iPLA\(_2\) enzymes. To strengthen the link between complement and iPLA\(_2\) activation, we transfected GEC with iPLA\(_2\)γ and iPLA\(_2\)β short cDNAs. Clones were screened for stable expression by immunoblotting. Several clones of GEC transfected with iPLA\(_2\)γ (GEC-iPLA\(_2\)γ) expressed the 88-, 77-, and 74-kDa isoforms of the enzyme (Fig. 3A). Clones that express iPLA\(_2\)β short (GEC-iPLA\(_2\)β) were also produced, and a representative clone is presented (Fig. 4A). It should be noted that the transfected enzyme migrates slightly faster than the endogenous isoform.

To ensure that the expressed iPLA\(_2\) proteins were enzymatically active, we further tested the extracts of stable subclones for iPLA\(_2\) activity, as monitored by release of [\(^{14}C\)]AA from exogenous [\(^{14}C\)]arachidonyl-PE. GEC transfected with iPLA\(_2\)γ (Fig. 3B) or iPLA\(_2\)β short (Fig. 4B) showed augmented [\(^{14}C\)]AA release in a concentration-dependent manner, compared with GEC-Neo. Interestingly, release of [\(^{14}C\)]AA from exogenous [\(^{14}C\)]PE was relatively weak (data not shown), suggesting that at least in our assay system, [\(^{14}C\)]PE is a preferred substrate for iPLA\(_2\). This observation is in keeping with an earlier study (35).

Stimulus-coupled [\(^{3}H\)]AA release in GEC stably transfected with iPLA\(_2\)γ. The effect of complement on [\(^{3}H\)]AA release was studied in GEC-iPLA\(_2\)γ and was compared with GEC-Neo. GEC-iPLA\(_2\)γ tended to have slightly increased basal levels of free [\(^{3}H\)]AA, in keeping with previous studies (35). Complement stimulated [\(^{3}H\)]AA release in GEC-Neo, whereas overexpression of iPLA\(_2\)γ enhanced complement-mediated [\(^{3}H\)]AA release significantly (Fig. 5A). As expected, complement-mediated [\(^{3}H\)]AA release in GEC-iPLA\(_2\)γ was inhibited significantly by the

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**Fig. 2. Expression of iPLA\(_2\). Endogenous expression of calcium-independent PLA\(_2\) (iPLA\(_2\)β; A) and iPLA\(_2\)γ mRNAs (B) in GEC and rat glomeruli was assessed using RT-PCR. Glomeruli were isolated from adult rat kidneys by differential sieving. Control lanes (−) were devoid of reverse transcriptase. A: GEC express iPLA\(_2\)β short mRNA and possibly some iPLA\(_2\)β long mRNA. iPLA\(_2\)β RT-PCR in Chinese hamster ovary cells (CHO), which express only the short isoform (577 bp), is shown for comparison. B: GEC express iPLA\(_2\)γ mRNA. Expression of iPLA\(_2\)β mRNAs in glomeruli is similar to expression in GEC (A and B). Expression of iPLA\(_2\)β (C) and iPLA\(_2\)γ proteins (D) in GEC and glomeruli was assessed by immunoblotting. COS cells transfected with iPLA\(_2\)β short and long isoforms (C) and COS cells transfected with iPLA\(_2\)γ (D) are shown for comparison. C: GEC express an immunoreactive iPLA\(_2\)β protein, whose molecular mass appears in between the long and short isoforms expressed by COS cells. D: GEC express iPLA\(_2\)γ. The lower molecular mass bands in glomeruli and GEC (C), and COS-iPLA\(_2\)γ (D) are probably nonspecific.
iPLA2γ-directed inhibitor BEL (1) (Fig. 5A). This result confirms that in these cells, a significant portion of complement-dependent [3H]AA release is due to iPLA2γ activity. BEL did not have a significant effect on [3H]AA release in control cells, suggesting that this [3H]AA release may have been mediated by another PLA2, e.g., cPLA2 (18, 37).

In previous studies, we demonstrated that in GEC, C5b-9 can induce [3H]AA release by activating cPLA2 (37). BEL was shown to have an inhibitory concentration (IC50) 250-fold greater for iPLA2 over cPLA2 (1); nevertheless, we wanted to rule out the possibility that BEL had inhibited cPLA2. To determine whether BEL could affect cPLA2 activity, we monitored PLA2 activity in COS cells that had been transiently transfected with cPLA2. In the presence of 1 mM EGTA (Ca2+-free buffer), PLA2 activity in COS cell extracts was low (Table 1). In the presence of 2 mM free Ca2+, PLA2 activity increased more than sixfold, reflecting Ca2+-dependent activation of cPLA2. PLA2 activity was not affected by BEL in Ca2+-replete buffer, indicating that BEL is not an effective inhibitor of cPLA2 activity (Table 1).

For comparison, we also studied how other stimuli that may injure GEC may affect [3H]AA. Overexpression of iPLA2γ enhanced release of [3H]AA by H2O2 (Fig. 5B), and by chemical anoxia/reoxygenation (Fig. 5C). These effects of H2O2 (Fig. 5B) and anoxia/recovery (Fig. 5C) in GEC-iPLA2γ were reduced by BEL, although the effect did not reach statistical significance in the case of H2O2. Thus iPLA2γ is involved in stimulus-coupled [3H]AA release, as the liberation of [3H]AA in response to complement, H2O2, and chemical anoxia/recovery is, at least in part, mediated by iPLA2γ.

In previous studies, we demonstrated that overexpression of cPLA2 in GEC (5- to 10-fold above endogenous levels) amplified the C5b-9-dependent release of [3H]AA (18, 37). In contrast, the increases in free [3H]AA induced by H2O2 or anoxia/recovery (Fig. 5, B and C) were not amplified in GEC that overexpress cPLA2 (Table 2), suggesting lack of involvement of cPLA2. Furthermore, these increases in [3H]AA were attenuated in the presence of BEL (Table 2). Together, the results suggest that iPLA2γ (and possibly other iPLA2γ), but not cPLA2, is activated by H2O2, oxygen, and anoxia/recovery, whereas both iPLA2γ and cPLA2 are activated by complement.

In some cells, AA may be derived from metabolism of DAG. Thus, in the above experiments, we also monitored changes in [3H]DAG. Complement (Fig. 1), H2O2, and anoxia/recovery (data not shown) increased [3H]DAG production, but the increases were not affected by iPLA2γ overexpression (data not shown). Furthermore, BEL did not reduce [3H]DAG production (data not shown), even though BEL is reported to inhibit phosphatidic acid phosphohydrolase, which mediates generation of DAG from phosphatic acid. Based on these results, it is unlikely that inhibition of DAG production was the mechanism by which BEL attenuated AA release in GEC.
iPLA2-dependent AA release is coupled to production of PGE2. The above experiments demonstrated that complement can induce release of [3H]AA via activation of iPLA2γ. In the next set of experiments, we investigated whether free AA could be metabolized via COX to eicosanoids. We monitored production of PGE2, which was shown to be a major metabolite of COX in GEC (45). GEC-Neo produced a small amount of PGE2 in response to complement, which appeared to be inhibited by indomethacin but not by BEL (HIS: 16 ± 9, HIS/BEL: 7 ± 4, NS: 46 ± 15, NS/BEL: 50 ± 14, NS/Indo: 18 ± 2 pg/ml) (Fig. 6). However, when analyzed together with GEC-iPLA2γ, these changes were not statistically significant.

Table 1. Effect of BEL on cPLA2 activity

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<th>Buffer</th>
<th>% [14C]AA Release</th>
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<tr>
<td>1 mM EGTA</td>
<td>8.3 ± 1.6</td>
</tr>
<tr>
<td>1 mM EGTA + BEL</td>
<td>6.8 ± 2.0</td>
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<tr>
<td>2 mM calcium</td>
<td>48.8 ± 0.5*</td>
</tr>
<tr>
<td>2 mM calcium + BEL</td>
<td>48.7 ± 2.0*</td>
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</tbody>
</table>

Values are means ± SE. Arachidonic acid, AA; BEL, bromoenol lactone. cPLA2 activity was measured in extracts of COS cells that had been transiently transfected with cPLA2 by monitoring release of [14C]AA from exogenous [14C]phosphatidylcholine (PC). cPLA2 activity in CsCl-replete buffer was not affected by BEL. *P < 0.0001 vs. 1 mM EGTA, 6 measurements.

Overexpression of iPLA2γ did not affect basal PGE2 production significantly during short-term incubation (Fig. 6, 60-min incubation), while there was a small increase in PGE2 in culture media of GEC-iPLA2γ, compared with GEC-Neo, after 2 days of cell culture (GEC-Neo: 284 ± 47 pg/ml, GEC-iPLA2γ: 519 ± 8 pg/ml, P < 0.05, 3 experiments). However, after incubation with complement, PGE2 production was ~20-fold greater in GEC-iPLA2γ relative to GEC-Neo (Fig. 6). The effect of complement was inhibited significantly by BEL and by the nonselective COX inhibitor indomethacin (Fig. 6). Thus complement-induced production of PGE2 parallels the release of [3H]AA (Fig. 5A).

Overexpression of iPLA2γ limits complement-mediated GEC injury. We reported previously that overexpression of cPLA2 in GEC increased the susceptibility to complement-mediated cytotoxicity (18). We next tested whether overexpression of iPLA2γ has a similar effect. Complement-mediated cytotoxicity was monitored as the release of LDH. Antibody-sensitized GEC-Neo or GEC-iPLA2γ were incubated with normal serum at serially increasing concentrations (to assemble Csb-9) for 100 min. The protocol allows for complement to activate iPLA2γ, but with increasing incubation time and complement dose, a portion of the cells will undergo lysis. LDH release was attenuated significantly in GEC-iPLA2γ, compared with GEC-Neo (Fig. 7A). Further studies in GEC-iPLA2γ showed that the cytoprotective effect of iPLA2γ was reversed by the addition of BEL, and to a lesser extent by the addition of indomethacin (Fig. 8A). Indomethacin did not reverse the cytoprotective effects of iPLA2γ.

Table 2. Effects of H2O2 or anoxia/recovery on free [3H]AA

<table>
<thead>
<tr>
<th>Condition</th>
<th>[3H]AA Release</th>
</tr>
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<tbody>
<tr>
<td>Untreated</td>
<td>100</td>
</tr>
<tr>
<td>BEL</td>
<td>139 ± 19</td>
</tr>
<tr>
<td>H2O2</td>
<td>470 ± 64*</td>
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<tr>
<td>H2O2 + BEL</td>
<td>196 ± 17†</td>
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Values are means ± SE representing [3H]AA release in treated/un-treated cells (%). Overexpression of cPLA2 in glomerular epithelial cells (GEC-GEC-cPLA2) does not enhance [3H]AA release after incubation with H2O2 or anoxia/recovery (A/R). *P < 0.0001 H2O2 vs. untreated (GEC-Neo and GEC-cPLA2), †P < 0.0001 H2O2 + BEL vs. H2O2 (GEC-Neo and GEC-cPLA2), 3 experiments. ‡P < 0.0001 A/R vs. untreated (GEC-Neo and GEC-cPLA2), §P < 0.01 A/R + BEL vs. A/R (GEC-Neo), 4 experiments. There are no significant differences between GEC-Neo and GEC-cPLA2 after treatment with H2O2 or A/R.
effect of iPLAγ completely, despite complete inhibition of PGE2 production (Fig. 6). Therefore, part of the cytoprotective effect of iPLAγ may have been independent of eicosanoid production. In keeping with previous results, GEC-Neo tended to be protected by the addition of indomethacin, since the activation of other PLA2s by complement was shown to involve mobilization of AA and downstream metabolism to bioactive eicosanoids with a cytotoxic effect (46).

Fig. 6. Complement-induced production of PGE2 is enhanced in GEC that overexpress GEC-iPLAγ. Incubation with complement is described in Fig. 5.

In some experiments, BEL (25 μM) or the COX inhibitor indomethacin (10 μM) were included in the incubations. GEC-iPLAγ produced significantly more PGE2 than GEC-Neo in response to complement, and this increase was attenuated by the addition of BEL or indomethacin. *P < 0.0001 GEC-iPLAγ vs. GEC-Neo (NS), **P < 0.0001 NS/BEL vs. NS (GEC-iPLAγ), +P < 0.0001 NS/indomethacin vs. NS (GEC-iPLAγ), 5 experiments.

Fig. 7. Overexpression of iPLAγ (A), or iPLAβ (B) limits complement-mediated GEC injury. GEC-iPLAγ, GEC-iPLAβ, and GEC-Neo were incubated with anti-GEC antiserum and then with 2.5, 5.0, or 7.5% NS for 100 min (HIS in control incubations). Cell injury was monitored as release of LDH. *P < 0.0001 GEC-iPLAγ vs. GEC-Neo (5.0% NS), **P < 0.025 GEC-iPLAγ vs. GEC-Neo (7.5% NS), 3 experiments. Similar results were obtained when these experiments were performed using another GEC-Neo clone. B: *P < 0.005 GEC-iPLAβ vs. GEC-Neo (2.5% NS), **P < 0.0001 GEC-iPLAβ vs. GEC-Neo (5.0% NS), +P < 0.0001 GEC-iPLAβ vs. GEC-Neo (7.5% NS), 4 experiments.

Fig. 8. Protective effect of iPLAγ, but not iPLAβ on complement-mediated injury is prostaglandin dependent. A: GEC-iPLAγ and GEC-Neo were preincubated with or without BEL (25 μM) or indomethacin (10 μM) for 30 min. Cells were then incubated with anti-GEC antiserum and 5% NS (HIS in control) for 100 min. *P < 0.0001 GEC-iPLAγ vs. GEC-Neo (NS), **P < 0.0001 NS/BEL vs. NS (GEC-iPLAγ), +P < 0.0001 NS/indomethacin vs. NS (GEC-iPLAγ), 3 experiments. B: GEC-iPLAγ and GEC-Neo were preincubated with or without NS 398 (10 μM) or SC560 (10 μM) for 30 min. Cells were then incubated as described above. *P < 0.0001 GEC-iPLAγ vs. GEC-Neo (NS), **P < 0.04 NS/NS398 vs. NS (GEC-iPLAγ), +P < 0.002 NS/SC560 vs. NS (GEC-iPLAγ), 4 experiments. C: GEC-iPLAβ and GEC-Neo were treated as in A. *P < 0.01 GEC-iPLAβ vs. GEC-Neo (NS), 4 experiments.
To further elucidate which COX enzyme was responsible for the cytoprotective effect of iPLA2γ, we included isoform-specific COX inhibitors NS 398 (COX-2) and SC560 (COX-1) in incubations with complement (Fig. 8B). Both inhibitors partially reversed the cytoprotective effect of iPLA2γ, suggesting that both COX isoforms were involved. Previously, we showed that C5b-9 upregulates the expression of COX-2 in GEC, although expression of COX-1 was not affected (45). Interestingly, COX-2 was upregulated in resting GEC-iPLA2γ, i.e., independently of a complement effect (Fig. 9A). In contrast, expression of COX-1 was not significantly different between GEC-iPLA2γ and GEC-Neo (Fig. 9B). These results provide further support for the view that following activation, LDH release was attenuated significantly in GEC-iPLA2γ with serially increasing concentrations of complement (Fig. 8B). Both iPLA2γ and PL-A2γ, in contrast to GEC-iPLA2γ, neither BEL nor indomethacin reversed the protective effect of iPLA2γ (Fig. 8C). Thus, while overexpression of iPLA2γ in GEC was not involved in complement-induced [3H]AA release, it was nonetheless cytoprotective.

**Role of iPLA2β in complement-dependent GEC injury.** By analogy to the studies that addressed iPLA2γ (described above), we employed GEC-iPLA2β to study complement-mediated effects on [3H]AA release and cell injury. In contrast to GEC-iPLA2γ, there was no amplification of complement-mediated [3H]AA release in GEC-iPLA2β. Levels of free [3H]AA in GEC-Neo and GEC-iPLA2β after exposure to HIS were 1.5 ± 0.2 and 2.5 ± 0.3% of total radioactivity, respectively, while after exposure of GEC-iPLA2β to 4.1 ± 0.7 (P < 0.0001) and 3.3 ± 0.2% of total radioactivity, respectively (the increase in GEC-iPLA2β was not statistically significant; 3 experiments). After incubation of antibody-sensitized GEC with serially increasing concentrations of complement, LDH release was attenuated significantly in GEC-iPLA2β, compared with GEC-Neo (Fig. 7B). However, in contrast to GEC-iPLA2γ, neither BEL nor indomethacin reversed the protective effect of iPLA2β (Fig. 8C). Thus, while overexpression of iPLA2β in GEC was not involved in complement-induced [3H]AA release, it was nonetheless cytoprotective.

**DISCUSSION**

The present study demonstrates that in GEC, assembly of C5b-9 resulted in a significant release of [3H]AA in Ca2+-free medium, suggesting activation of an iPLA2 (Fig. 1). GEC endogenously express iPLA2β short, although on SDS-PAGE, the protein appeared to be slightly greater in molecular mass, compared with transfected iPLA2β short in COS cells (Fig. 2). Perhaps the endogenous iPLA2β short undergoes a posttranslational modification in GEC, which accounts for the slight increase in size. GEC also express the 88-kDa isoform of iPLA2γ, although expression of COX-1 was not affected (45). Overexpression of iPLA2γ in GEC augmented complement-dependent [3H]AA release, compared with GEC-Neo, and furthermore the [3H]AA release in GEC-iPLA2γ was attenuated by BEL (Fig. 5). These results indicate that activation of complement is coupled with the activation of iPLA2γ. It should also be noted that the AA mobilized by iPLA2γ was accessible to COX and could be metabolized to prostanooids (Fig. 6). An earlier study also showed coupling of iPLA2γ with COX-1 in HEK293 cells (35). In contrast to iPLA2γ, overexpression of iPLA2β in GEC did not amplify complement-dependent [3H]AA release.

By analogy to iPLA2γ, overexpression of cPLA2 in GEC was previously shown to augment complement-dependent [3H]AA release and prostanooid production, compared with GEC-Neo. However, overexpression of group IIA sPLA2 in GEC was similar to iPLA2β, i.e., neither enzyme amplified complement-dependent [3H]AA release (37). Previous studies also demonstrated that complement induces serine phosphorylation of cPLA2 in GEC, in association with an increase in enzymatic activity (18) and that glomerular cPLA2 is phosphorylated in PHN (21). This posttranslational modification of cPLA2, which is generally associated with activation, has been described with multiple agonists (27). iPLA2γ activity has been reported to increase after stimulation with PMA or ATP, but the mechanism is unknown at present and will require further study (16, 28). An alternate mechanism of iPLA2γ activation may involve modification of phospholipids (e.g., by C5b-9-induced deformability of the membrane lipid bilayer, or by lipid peroxidation) (9, 36), such that there is increased substrate availability to iPLA2γ. The overexpression models of the various PL-A2s are invaluable in defining links of C5b-9 assembly with PLA2 isoform activation. We have expressed
these PLA2s at levels modestly above endogenous, but the results need to be interpreted with some caution. Furthermore, overexpression does not allow for a direct comparison of the amounts of [3H]AA release by cPLA2 vs. iPLA2. Nevertheless, release of [3H]AA by complement in the presence of Ca2+ was substantially greater than that in the absence of Ca2+ (37), and overexpression of cPLA2 generally resulted in a greater amplification of complement-dependent [3H]AA release, compared with iPLA2γ. Thus it is likely that cPLA2 is a quantitatively more important enzyme than iPLA2γ.

To further address the potential functional importance of iPLA2 in GEC, we examined the effects of H2O2 and anoxia/recovery on [3H]AA release. Both of these stimuli modulated effects of ischemia-reperfusion injury in cultured cells (24). By analogy to complement, overexpression of iPLA2γ enhanced release of [3H]AA by H2O2 and by anoxia/recovery (Fig. 5), providing further support for the view that iPLA2γ activity can be enhanced by specific stimuli, including ischemia-reperfusion. In contrast to complement, the increases in free [3H]AA induced by H2O2 or anoxia/recovery (Fig. 5, B and C) were not amplified in GEC that overexpress cPLA2 (Table 2). Other studies have demonstrated that release of AA in response to H2O2 was mediated by cPLA2 in renal tubular epithelial and mesangial cells (26, 41). Thus H2O2-coupled AA mobilization may be cell specific (10).

Various PLA2 enzymes have been shown to regulate pathways of cell injury in several experimental disease models (5, 11, 12, 15, 39). In vivo, assembly of Cs-b-9 in GEC is associated with sublethal GEC injury and proteinuria; however, it is not practical to assay proteinuria in cell culture models. Instead, in cultured GEC, we have assayed Cs-b-9-mediated injury as cytolysis (LDH release), and it has been shown that cytolysis in culture correlates with proteinuria in vivo, in the context of injury associated with generation of prostanoids (46), p38 activation (3), and ER stress (22). The present study demonstrates that overexpression of iPLA2γ attenuated complement-induced GEC injury, and the effect was reversed by BEL (Fig. 8). In addition, the attenuation of complement-induced GEC injury was partially reversed in the presence of a nonselective COX inhibitor, as well as with selective inhibitors of the COX-1 and -2 isozymes (Fig. 8). Thus cytoprotection was, at least in part, mediated via generation of prostanoids, most likely through both COX-1 and COX-2. In support of this view are our earlier results, which showed COX inhibitors protected the cells from complement-induced cell death. Finally, it should be noted that inhibition of COX in PHN in vivo with nonselective or with COX-2-selective inhibitors reduced proteinuria (45, 46).

There would seem to be an apparent contradiction in that production of prostanoids was cytoprotective in GEC-iPLA2γ but exacerbated cytotoxicity in other GEC lines (46). Possibly, individual prostanoids downstream of COX (and/or intracellular localization of their production) are responsible for inducing a cytoprotective vs. cytotoxic effect. For example, in earlier studies in GEC-Neo, where COX inhibition protected the cells from complement-induced injury, the protective effect of the COX inhibitor could be reversed by the addition of a thromboxane A2 analog, but not PGE2 (46). Among the PGE2 receptors, GEC predominantly express EP4 as well as some EP1 receptors (3). Previously, it was demonstrated that exogenous PGE2 was protective against apoptosis induced by serum deprivation and that these effects were reversed by an EP4 receptor antagonist. Moreover, exogenous PGE2 could also attenuate purinergic aminonucleoside-induced GEC injury, indicating that PGE2 is cytoprotective (3). In the present study, we have shown that GEC-iPLA2γ express EP2 receptor mRNA, at levels similar to GEC-Neo (Fig. 10). Thus a PGE2-EP2 receptor pathway could mediate the cytoprotective effect of iPLA2γ in complement-dependent injury. Further studies will be required to confirm this hypothesis more directly.

In GEC-iPLA2γ, BEL reversed the cytoprotective effect of iPLA2 to a greater extent than indomethacin (Fig. 8), suggesting that mechanisms distinct from AA metabolism, but nonetheless related to iPLA2 catalytic activity, also contributed to the cytoprotective effects of iPLA2. One possible mechanism is that the iPLA2 enzyme facilitates activation of the ER stress response, a cytoprotective response shown to be activated in GEC exposed to complement Cs-b-9 (22). At least some iPLA2γ isoforms may be localized at the membrane of the ER (17); consequently, iPLA2γ could perturb the ER membrane sufficiently to initiate the ER stress response, which may limit complement-induced injury. Additional studies will be required to determine the subcellular localization of iPLA2γ in GEC, in particular, whether iPLA2γ is found at the ER (17); of interest, iPLA2γ protected mitochondria in renal proximal tubular cells from oxidant-induced lipid peroxidation and dysfunction (28), and iPLA2γ knockout mice show defects in mitochondrial functions and mitochondrial lipid composition (32). Thus mitochondria are another potential target of iPLA2γ in GEC.

The present study demonstrates that overexpression of iPLA2β in GEC also attenuated complement-induced GEC injury (Fig. 7), and this effect occurred in the absence of stimulated [3H]AA release and was not blocked by acute incubation with BEL and indomethacin (Fig. 8). Another study failed to demonstrate stimulus-coupled AA release with iPLA2β, but there are also reports of protein kinase C and calmodulin involvement in iPLA2β activation (2, 5). In the absence of complement-induced [3H]AA release or acute inhibition, our results suggest that overexpression of iPLA2β may have induced “preconditioning” of GEC, such that the cells were able to resist complement-mediated injury. There were no significant differences in basal free [3H]AA levels between GEC-Neo and GEC-iPLA2β, but it is possible that there was enhanced turnover of [3H]AA in the latter. This view is consistent with earlier studies, which have determined that
iPLA$_2$ may be involved in both deacylation of membrane phospholipids and production of lysosphospholipid acceptors (4, 6, 8). The mechanisms of phospholipid metabolism and cytoprotection in GEC-iPLA$_2$ will require further study.

While the role of complement in the PHN model of membranous nephropathy is appreciated, the ensuing biological cytoprotection in GEC-iPLA$_2$ family enzymes may represent a mechanism of limiting both cell culture and in vivo models. Further studies will be required to delineate these effects more precisely and define the interplay between the various PLA$_2$ isoforms. Current treatment approaches to idiopathic membranous nephropathy are nonspecific and not very effective. Modulation of specific PLA$_2$ family enzymes may represent a mechanism of limiting GEC injury and maintaining the integrity of the permselective barrier.

GRANTS

This study was supported by grants from the Canadian Institute of Health Research (A. V. Cybulsky, T. Takano) and Kidney Foundation of Canada (A. V. Cybulsky, T. Takano), scholarships from the Fonds de la Recherche en Santé du Québec (A. V. Cybulsky, T. Takano), and the Catherine McLaughlin Hakim Chair (A. V. Cybulsky).

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