Role of calcium-independent phospholipase A$_2$ gamma (iPLA$_2$$\gamma$) in glomerular epithelial cell injury

Hanan Attia Elimam

Faculty of Medicine,
Division of Experimental Medicine
McGill University, Montréal, Québec
April 2014

A THESIS SUBMITTED TO MCGILL UNIVERSITY
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS OF THE DEGREE OF THE
DOCTORATE OF PHILOSOPHY

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Abstract

In experimental membranous nephropathy, complement C5b-9 induces glomerular epithelial cell (GEC)/podocyte injury and proteinuria. The effects of C5b-9 are mediated via signaling pathways, including calcium-independent phospholipase A2γ (iPLA2γ), and mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. The iPLA2γ pathway is cytoprotective. First, we studied the mechanisms of iPLA2γ activation and cytoprotection. iPLA2γ activation was monitored by quantifying prostaglandin E2 (PGE2) production. In GECs, iPLA2γ localized at the endoplasmic reticulum and mitochondria, which was dependent on the N-terminal region of iPLA2γ. Complement-mediated production of PGE2 was amplified in GECs that overexpress iPLA2γ, compared with control cells, and was blocked by the iPLA2γ inhibitor bromoenol lactone in both iPLA2γ-overexpressing and control GECs. Complement-induced activation of iPLA2γ was mediated via ERK and p38 pathways, but not JNK pathway. In COS-1 cells that overexpress iPLA2γ and cyclooxygenase-1, PGE2 production was induced by co-expression of constitutively active MEK1 or MAPK interacting kinase 1 (MNK1) as well as by stimulation with epidermal growth factor (EGF) + ionomycin. Complement- and EGF + ionomycin-stimulated iPLA2γ activity was attenuated by the S511A/S515A double mutation. Moreover, complement and EGF + ionomycin enhanced phosphorylation of Ser-511. Thus, stimulation of iPLA2γ was dependent on an increase in cytosolic Ca2+ concentration and phosphorylation of Ser-511 and/or Ser-515 via MNK1. Phosphorylation of Ser-511 and/or Ser-515 plays a key role in the catalytic activity and signaling of iPLA2γ.

Next, we addressed the cytoprotective function of iPLA2γ by examining if iPLA2γ is involved in the adaptive unfolded protein response (UPR). In GECs, iPLA2γ amplified tunicamycin-induced activating transcription factor-6 (ATF6) activation and upregulated the ER chaperones, grp94 and grp78, which are downstream of ATF6 and enhance protein-folding capacity of the ER. These effects were dependent on iPLA2γ catalytic activity, but not on prostanoids. Furthermore,
ATF6 amplification occurred only when the full length iPLA2γ was expressed, but not an N-terminally truncated mutant, which does not associate with the membrane of the ER. Induction of the ATF6 pathway of the UPR and its amplification by iPLA2γ limited tunicamycin-induced GEC injury.

Finally, to better understand the role of iPLA2γ in normal podocyte function and in podocyte injury in vivo, we employed iPLA2γ knockout (KO) mice. Deletion of iPLA2γ caused neither albuminuria nor morphological changes in the glomerulus. However, after induction of anti-glomerular basement membrane nephritis, iPLA2γ KO mice exhibited significantly increased levels of albuminuria, compared to wild type (WT) mice. Furthermore, in contrast to WT mice, iPLA2γ KO mice exhibited a marked loss of podocytes, implying that iPLA2γ has a protective role in glomerulonephritis.

Collectively, this work characterizes the mechanism of iPLA2γ activation in complement-mediated GEC injury, shows that the cytoprotective effect of iPLA2γ involves the unfolded protein response, ATF6, and that iPLA2γ has a protective role in glomerulonephritis. Defining the role of iPLA2γ provides opportunities for development of novel therapeutic approaches to GEC injury and proteinuria.
Résumé

Dans la néphropathie membraneuse expérimentale, le complément C5b-9 induit des dommages aux cellules épithéliales glomérulaires (CGE)/podocytes et cause la protéinurie. Les effets du C5b-9 sont médiés par des voies de signalisation, y compris la phospholipase A₂γ indépendante du calcium (iPLA₂γ), et des protéines kinases activées par les mitogènes (MAPK) telles que ERK, JNK et p38. La voie iPLA₂γ est cytoprotectrice. Tout d'abord, nous avons étudié les mécanismes d'activation de et de cytoprotection de iPLA₂γ. L'activation de iPLA₂γ a été suivie par la quantification de la production de prostaglandine E₂ (PGE₂). Dans les CGE, iPLA₂γ est localisée au réticulum endoplasmique (RE) et aux mitochondries. Cette localisation était dépendante de la région N-terminale de iPLA₂γ. La production de la PGE₂ induite par le complément était amplifiée dans les CGE qui surexpriment iPLA₂γ, par rapport aux cellules de contrôle. Cette amplification était bloquée par un inhibiteur de iPLA₂γ (bromoenol lactone) à la fois dans les CGE surexprimant iPLA₂γ et les CGE de contrôle. L'activation du iPLA₂γ induite par le complément était médiée par des voies de signalisation ERK et p38, mais pas par JNK. Dans les cellules COS-1 qui surexpriment iPLA₂γ et la cyclooxygénase-1, la production de PGE₂ était induite par la co-expression de la kinase constitutivement active MEK1 ou de MNK1 ainsi que par la stimulation par le facteur de croissance épidémique (EGF) + ionomycine. L’activité iPLA₂γ stimulé par le complément, EGF et ionomycine, était atténuée par la double mutation, S511A/S515A. En outre, la stimulation par le complément et par la combinaison EGF + ionomycine augmentait la phosphorylation de Ser-511. Ainsi, la stimulation de iPLA₂γ était dépendant d'une augmentation de la concentration de calcium cytosolique et de la phosphorylation de Ser-511 et/ou Ser-515 par l'intermédiaire de MNK1. La phosphorylation de Ser-511 et/ou Ser-515 joue un rôle clé dans l'activité catalytique et la signalisation de iPLA₂γ.

Ensuite, nous avons examiné la fonction cytoprotectrice de iPLA₂γ en examinant si iPLA₂γ est impliqué dans la “unfolded protein response” ou UPR. Dans les
CGE, iPLA2γ amplifiait l’activation transcription factor 6 (ATF6) induite par la tunicamycine et a augmentait l'expression des chaperons du RE grp94 et grp78, qui sont en aval du ATF6 et améliorent la capacité de pliage des protéines dans ce compartiment. Ces effets étaient dépendants de l'activité catalytique de iPLA2γ, mais pas des prostanoïdes. En plus, l’amplification de ATF6 se produisait seulement en présence d’une forme intacte de iPLA2γ, mais pas en présence d’un mutant portant une troncature N-terminale et incapable de s’associer avec la membrane du RE. L’induction de la voie de ATF6 de l’UPR et son amplification par iPLA2γ limitait les dommages induits par la tunicamycine dans les CGE.

Enfin, pour mieux comprendre le rôle de iPLA2γ dans la fonction normale des podocytes et dans les dommages aux podocytes in vivo, nous avons utilisé des souris knock-out iPLA2γ (KO). La déplétion de iPLA2γ ne cause pas d’albuminurie, ni de changements morphologiques dans le glomérule. Cependant, après l’induction de néphrite par anticorps anti-membrane glomérulaire basale, les souris KO iPLA2γ démontraient une albuminurie augmentée par rapport aux souris de type sauvage (WT). De plus, contrairement aux souris WT, les souris KO iPLA2γ présentaient une diminution marquée des podocytes, ce qui implique que iPLA2γ a un rôle protecteur dans la glomérulonéphrite.

Mis ensemble, ces travaux caractérisent le mécanisme d'activation des iPLA2γ dans le dommage aux CGE induit par le complément. Ils montrent que l’effet cytoprotecteur de iPLA2γ implique l’UPR, ATF6, et que iPLA2γ a un rôle protecteur dans la glomérulonéphrite. Définir le rôle de iPLA2γ offre des possibilités pour le développement de nouvelles approches thérapeutiques pour les dommages aux CGE et pour la protéinurie.
Acknowledgements

I would like to take this opportunity to thank all who have encouraged and supported me throughout the preparation and completion of my thesis.

To my supervisors, Dr. Andrey Cybulsky and Dr. Tomoko Takano, thank you for your support, honesty, patience and guidance throughout this thesis project. I am truly indebted to you for your generosity and academic experience that have been invaluable to me. I am grateful for the opportunity you have given me and for your endless assistance during my research. I have learned a great deal from each of you to use as I start my next phase of my career.

I am forever grateful to my family, for their continuous assistance, and enthusiasm throughout my studies, they have always supported and encouraged me throughout my life. Special thanks for my husband, Dr. Sabri Moussa, and my bright kids, Mahmoud, Rewan, and Karim for their patience, listening to my science-related ramblings, and help during my studies. I am also grateful to my previous supervisors, Dr. Tarek Moustafa and Dr. Sylvie Marleau, for their inspiration, motivation, and for providing a solid background that allowed me to pursue further study.

I would like to thank my committee members, Dr. Jean-Jacques Lebrun, Dr. Giovanni DiBattista, Dr. William S. Powell, and Dr. John Filep for their guidance, suggestions, and encouragement. I would like to thank Joan Papillon, Julie Guillemette, Lamine Aoudjit and Cindy Baldwin for helping me with lab techniques and experiments. I would like also to thank my colleagues who have assisted me with their useful comments and suggestion. De plus, j’apprécie beaucoup l’aide de Dr. Serge Lemay pour la traduction de mon résumé. Merci.
Finally, I am also grateful to everyone in the Nephrology division, for their collaboration and for creating an interesting work environment that enhances the greater part of the learning experience. It has been a real pleasure to work among them for the past four years.
Preface and Contribution of Authors:

In experimental membranous nephropathy, complement C5b-9 induces glomerular epithelial cell (GEC) injury and proteinuria. The Cybulsky and Takano laboratories previously identified that iPLA\(_2\)\(\gamma\) is cytoprotective in C5b-9-mediated GEC injury (1). The present work characterizes the mechanism of iPLA\(_2\)\(\gamma\) activation in complement-mediated GEC injury, shows that the cytoprotective effect of iPLA\(_2\)\(\gamma\) involves the ATF6 branch of the unfolded protein response, and that iPLA\(_2\)\(\gamma\) has a protective role in glomerulonephritis. Defining the role of iPLA\(_2\)\(\gamma\) provides opportunities for development of novel therapeutic approaches to GEC injury and proteinuria. Characterization of the mechanism of iPLA\(_2\)\(\gamma\) activation in complement-mediated GEC injury, demonstration that the cytoprotective effect of iPLA\(_2\)\(\gamma\) involves the ATF6 unfolded protein response pathway, and demonstration that iPLA\(_2\)\(\gamma\) has a protective role in glomerulonephritis in vivo are new contributions to knowledge.

This is a manuscript (article)-based style thesis.

In Chapter 2 (first manuscript), the text and figures are reproduced from “Elimam, H., Papillon, J., Takano, T., and Cybulsky, A. V. (2013) Complement-mediated activation of calcium-independent phospholipase A\(_2\) gamma: role of protein kinases and phosphorylation. J Biol Chem 288, 3871-3885.” I performed the majority of experiments in this paper. Co-author Joan Papillon contributed to the iPLA\(_2\) activity assay shown in Figure 3, as well as constructing the iPLA\(_2\)\(\gamma\) S168A mutant shown in Figure 9.

In Chapter 3 (second manuscript), I contributed most of the experiments. Immunoblotting in Figure 5, as well as the luciferase assays in Table II were completed by co-author Joan Papillon.
In Chapter 4 (third manuscript), most of the experiments were performed by myself. Preparation of electron microscopy (EM) samples was performed by co-author Lamine Aoudjit. EM pictures were taken by myself together with Dr. Andrey Cybulsky.

Experimental design, and data analyses were done cooperatively by myself and co-authors Drs. Andrey Cybulsky and Tomoko Takano. I authored the initial versions of each manuscript, and revisions were performed together with Drs. Andrey Cybulsky and Tomoko Takano.
# Table of abbreviations

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<th>A</th>
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<th>Arachidonic acid</th>
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<td></td>
<td>ATF6</td>
<td>Activating transcription factor 6</td>
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<td></td>
<td>bip</td>
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CHAPTER 1: INTRODUCTION
1.1 Glomerular filtration barrier

The kidneys are the primary organs of the urinary system. The kidney glomerulus filters blood into an ultrafiltrate that will ultimately become urine (2). Over the human lifetime, the kidneys produce more than 4 million litres of virtually protein-free primary urine. Despite this immense workload, the glomerulus remains intact year after year. The glomerular capillary wall is a size-selective barrier, consists of three major components: the capillary endothelium, the glomerular basement membrane (GBM), and the visceral glomerular epithelial cells (GECs) also called podocytes with their slit diaphragms. The glomerular capillary is supported by mesangium, which is composed of mesangial cells and their surrounding matrices. All cell types and constituents of the filtration apparatus participate in maintaining its integrity (3). The glomerulus is susceptible to both immunological and hemodynamic injury because of its specialized structure and abundant blood flow. Injury of the filtration apparatus can pathogenetically result from acquired and inherited causes. In the inherited and acquired diseases that are characterized by leakage of large plasma proteins (such as albumin and immunoglobulins) into the urine, the glomerular filtration barrier is affected (4).

Several studies, in vivo, have elucidated the sieving properties of the glomerular capillary wall responsible for size selectivity. It is freely permeable to water, and midsized solutes in plasma, yet maintains considerable size selectivity for larger molecules and proteins (mw>100kDa). The sieving coefficient describes the transport of a solute through the glomerular filtration barrier in relation to that of a freely filtered solute, which behaves similarly to water (e.g. inulin). Albumin has a low sieving coefficient of approximately 0.0003.

The filtration barrier does not discriminate only on the basis of molecular size but also on the net molecular electrical charge on the glomerular filtration of circulating macromolecules. The use of cationic ferritin first demonstrated the anionic nature of the GBM (5); anionic ferritin was restricted from entering the
GBM, whereas cationic ferritin reached the podocyte slit diaphragm. The GBM consists predominantly of type IV collagen, laminin 521 (α5β2γ1), nidogen, and heparan sulfate proteoglycans. GBM heparan sulfate contributes to the charge selectivity of the glomerular capillary wall. A previous study demonstrated that anti-heparan sulfate antibody administration to rats resulted in massive proteinuria (6). Podocytes have also been demonstrated to have anionic sites (see below) (7). The GBM thickening is due to the accumulation of extracellular matrix. The GBM structural changes may affect the adjacent cellular elements and reduce cell binding to GBM therefore promoting cellular detachment.

Podocytes are negatively charged on their apical membrane domain due to the presence of surface anionic proteins such as anionic glycocalyx (constituted mainly by podocalyxin), podoplanin, and podoendin. The negative charges limit passage of negatively charged molecules (like albumin) and maintain separation of adjacent podocytes. Collectively, the glomerular capillary wall is a size- and charge-selective filter that is critical for ultrafiltration and restricting passage of proteins (4). Changes in size and charge properties of podocytes occurring in kidney diseases contribute to the development of proteinuria (7). Abnormalities of proteins important for the maintenance of podocyte cytoskeleton and GBM integrity are involved in inherited human glomerular filtration barrier diseases (8).

1.2 Podocyte structure and function

Podocytes are highly specialized polarized cells that serve as an important component of the filtration barrier in the glomerulus. The podocyte is considered as a primary target in most forms of inherited and acquired glomerular disease. They are highly differentiated cells with limited ability to proliferate (9). Architecturally, they possess a complex cell body from which a network of interdigitating cellular extensions (primary, secondary, and tertiary) extends toward the capillary forming the foot processes. These foot processes support the glomerular capillary loop from the visceral aspect of the Bowman’s space. The foot processes contain a cytoskeleton that is composed of microfilaments
comprised of actin, α-actinin-4, myosin, paxillin, talin, and vinculin. The cytoskeleton serves as a podocyte backbone by maintaining the shape of podocytes and also enables its continuous formation and adaptation. This cytoskeleton supports the glomerular capillary wall and opposes the high hydrostatic pressure necessary for glomerular filtration. The narrow gaps (30–40 nm) between adjacent processes (filtration slits) are bridged by glomerular slit diaphragms (7,10).

The slit diaphragm is the final filtration barrier. The size selectivity of the filtration barrier is largely dependent on the slit diaphragm, as it prevents the passage of macromolecules. The slit diaphragm is bound to the cytoskeleton and anchored to the lipid raft domain of the podocyte plasma membrane by a protein complex containing podocin, CD2AP, and Zona Occludens-1 (ZO-1) (9). Slit diaphragm proteins are typically found in tight junctions (ZO-1) and adherens junction (catenins, P-cadherin) (11). Structurally, the slit diaphragm is multicomponent, and includes interactions between nephrin and neph1 and possibly cadherins. Homologous interactions of nephrin from an adjacent podocyte or heterologous interaction with neph-1 is hypothesized to form the “pore” of the slit diaphragm (12). In addition, the nephrin intracellular domain interacts with podocin and other regulatory proteins to facilitate actin polymerization. The specific arrangement of actin filaments, as a result of actin polymerization, is the key to foot process structure (12). The cytoplasmic tyrosine kinase, focal adhesion kinase (FAK) is essential in connecting the actin cytoskeleton with the foot process anchor on the GBM. Podocyte-specific FAK-knockout mice appear to protect podocytes from developing proteinuria and foot processes effacement in response to lipopolysaccharide (LPS) administration (13). Podocin is also important for the integrity of the slit diaphragm as it stabilizes nephrin and slit diaphragms in the lipid raft domain in podocyte plasma membrane. Dissociation of nephrin from podocin and actin will allow dislocation of slit diaphragms and nephrin will be shed into the urine (9). Several studies have showed that mutations in nephrin or podocin lead to nephrotic syndrome and
proteinuria, mutations in actinin-4 lead to focal segmental glomerular sclerosis (FSGS), whereas mutations in podocin (NPHS2) account for up to 30% of steroid-resistant idiopathic FSGS in children (14-16). Moreover, the intracellular domain of nephrin contains serine and tyrosine residues that are potential phosphorylation sites, indicating the possibility that nephrin is involved in signaling (4). The signaling properties of the slit diaphragm complex is mediated in part by Src family kinases (9).

Foot processes are functionally defined as having three membrane domains: the apical membrane domain, the slit diaphragm, and the basal membrane domain associated with the GBM. The function of podocytes is based largely on maintaining the complex cell architecture, especially the foot process structure (10,17). The unique shape of podocyte foot processes is supported by the actin cytoskeleton, which allows the podocyte to continuously, and dynamically, alter shape. Distortion of foot process architecture results in foot process flattening, in most cases, or effacement with presence of proteinuria. Podocytes are attached to the GBM by integrins (predominantly the α3β1 integrin) and α- and β-dystroglycans (7). The important part of podocyte-GBM interaction includes interaction between α3β1 integrin and β2 laminin, and its link to the podocyte actin cytoskeleton. α3β1 integrin has a critical role in the development and maintenance of the glomerular filter (11).

1.3 Podocyte injury

Podocyte injury is the hallmark of glomerular diseases such as minimal change disease, FSGS, membranous glomerulopathy, diabetic nephropathy, and lupus nephritis (18,19). The best-characterized pattern of podocyte injury is foot processes effacement, due to gradual simplification of the interdigitating foot process that involves rearrangement of the foot processes actin cytoskeleton, and slit diaphragm disruption (Figure 1A and C). Experimental data showed that foot
process length decreases by up to 70% and the width increases by up to 60% compared to normal (20). Effacement is an active process that is energy dependent, not simply a passive phenomenon, and is initiated by changes in the podocyte’s cytoskeleton (20). The frequency of filtration slits along the GBM is reduced due to the effacement and effacement has been associated with narrowing of filtration slits and development of actual tight junctions between foot processes (21).

Proteinuria arises from the disruption of any of the three foot processes domains with the concomitant transformation of the actin cytoskeleton (22). Increased glomerular capillary hydrostatic pressure cause proteinuria/albuminuria. Chronic
increases in intraglomerular pressure leads to progressive glomerular injury, thereby augmenting proteinuria by further damage of the filtration barrier. Acute podocyte injury is reversible if the actin cytoskeleton is repaired, allowing foot processes to branch once again into their interdigitating pattern. However, chronic podocyte injury can lead to the loss of glomeruli and ultimately kidney failure (7).

The question whether foot processes effacement is a cause or a result of proteinuria is debated and its answer is not decisive yet. Three views have been discussed; first, effacement itself is sufficient to cause proteinuria. Data show that effacement indeed precedes proteinuria in experimental models such as puromycin aminonucleoside nephrosis (23). A second scenario is that effacement itself does not cause proteinuria, but it is a ‘marker’ for the actual cause of proteinuria. Therefore, podocyte effacement is not required for proteinuria as shown by Liu and co-workers, who showed proteinuria with preserved foot processes when disrupting the Neph1–nephrin complex with specific antibodies (24). Third, effacement can occur in the absence of proteinuria as studied by Van den Berg and coworkers. They showed significant differences in the degree of effacement in patients with minimal change nephrotic syndrome, IgA, and, membranous nephropathy, and that the differences were independent of proteinuria levels (25).

Nephron loss occurs by three pathways, the dysregulated pathway, inflammatory pathway, and the degenerative pathway (26). These pathways represent prototypes of damage development in the glomerulus. Dysregulated mechanisms are characterized by dedifferentiation of podocytes leading to regulatory defect followed by cell proliferation within Bowman’s space and collapse of glomerular tuft. The most commonly observed mechanism is the degenerative pathway, in which the persistence of podocyte injury can cause cell body attenuation, podocyte hypertrophy, detachment from the GBM, and fixation of parietal epithelial cell to GBM. Significant podocyte loss and misdirected filtration into the periglomerular interstitium lead to proteinaceous crescents, tubular destruction.
and the progression of kidney failure (26,27). In the inflammatory mechanism, abnormal activity of podocytes leads to fixation to their parietal basement membranes followed by establishment of tuft adhesions to Bowman’s capsule (26).

The mechanism underlying proteinuria is due to a lack of charge and size selectivity in areas of podocyte loss. Studies have shown that proteinuria increases as podocyte number decreases. Podocyte number reflects the balance between processes that favor a decrease in cell number, and those that favor an increase in cell number. The most common process occurs in glomerular disease that favors decreases in cell number. Multiple factors cause a decrease of podocyte number (podocytopenia); podocyte apoptosis, podocyte detachment, and inability to proliferate and restore podocyte number (Figure 1 B and D). Podocytes must be attached to the GBM to survive and once they detach, apoptosis increases significantly.

The mechanisms of podocyte detachment have not been well understood. Recalling that integrins and dystroglycans are adhesion molecules by which podocytes attach to the GBM. Reduction of α3β1 integrin (the predominant integrin tethering the podocyte to the underlying GBM) expression appears to contribute to podocyte detachment (28). Genetic inactivation of α3 or β1 integrin results in podocyte foot process effacement and kidney failure in newborn mice (29,30). In cultured GEC, anti-Fx-1A antibody recognizes α3β1 integrin and reduces cell adhesion to extracellular matrix (31).

Experimental and clinical literature have shown that apoptosis is a major cause of reduced podocyte number, leading to proteinuria and/or glomerulosclerosis (32). TGF-β and its receptors increase in membranous nephropathy, diabetic nephropathy, and FSGS. In addition to its profibrotic effects, Schiffer et al. showed that TGF-β also induces podocyte apoptosis (33). They showed that SMAD-7 increases TGF-β induced apoptosis. In addition, cyclin-dependent
kinase (CDK) inhibitor p21 is necessary for TGF-β-induced apoptosis. Moreover, apoptosis is also augmented when podocytes proliferate in the absence of CDK inhibitors p21 and p27 (20). CD2AP is one of nephrin’s binding partners; CD2AP-null mice die of massive proteinuria and proteinuria rise in patients who have CD2AP haploinsufficiency (34). CD2AP absence is associated with increased susceptibility to podocyte apoptosis. It selectively engages the phosphatidylinositol 3-kinase (PI3K)/AKT antiapoptotic signaling pathway. Apoptosis has been difficult to document in all podocyte diseases as apoptotic podocytes are flushed out in the urine, making it technically difficult to detect these cells (35).

Podocytes are terminally differentiated cells and thus typically do not proliferate in vivo. They do not change their phenotype in response to injury, and they are unable to proliferate in certain diseases such as membranous nephropathy, minimal change disease, classic idiopathic FSGS, and diabetic nephropathy. However, there are experimental data showing that podocytes can proliferate and change their phenotype in experimental HIV nephropathy and crescentic glomerulonephritis. In podocytes, the state of differentiation and proliferation are very closely linked. During glomerulogenesis, immature undifferentiated podocytes have a proliferative phenotype. In contrast, mature adult podocytes have a quiescent phenotype (i.e. cells are in the G0 phase of the cell cycle).

Specific cell-cycle regulatory protein alteration might prevent podocyte proliferation (20). In the passive Heymann nephritis (PHN) model of membranous nephropathy, cyclin D1 and the protein levels of cyclin A and its partner CDK2 increase following podocyte injury. In experimental crescentic glomerulonephritis, CDK2 activity also is markedly increased. Therefore, in the majority of podocyte diseases the lack of podocyte proliferation is not due to the lack of the cell cycle machinery (i.e. cyclins and CDKs). Despite an increase in cyclin–CDK levels, cyclin activity is reduced due to the marked increase in CDK inhibitors. p21 was shown to bind to and inhibit cyclin A–CDK2. In PHN, marked
increase in p21 and p27 levels in podocytes coincide with the lack of proliferation. (36). Moreover, p27 is a critical regulator of podocyte proliferation following injury. Its level decreases in collapsing FSGS, but not in classic idiopathic FSGS, minimal change, and membranous nephropathy (non proliferative podocytes diseases); this corresponds to increased podocyte proliferation in collapsing FSGS (37). Collectively, a change in podocyte number, whether a decrease (podocytopenia) by cell detachment, apoptosis or lack of proliferation (as occurs in most glomerular diseases) or an increase, is detrimental to normal glomerular function.

1.4 Complement system

The complement system acts as a rapid and efficient immune system, eliminates cellular debris and infectious microbes, contributes substantially to homeostasis, and even may be involved in the repair and regeneration of damaged tissues (38-40). Complement-mediated cellular injury has been implicated in various diseases including glomerulonephritis, sepsis, lupus, rheumatoid arthritis, myocardial infarction, multiple sclerosis, myasthenia gravis, organ transplant rejection, and, more recently, osteoarthritis and age-related macular degeneration (39,41). The complement system is composed of three pathways, namely the classic, alternative, and mannose-binding lectin (MBL) pathways, which contain more than 30 proteins involved in activation and regulation (Figure 2). Each pathway differs in the requirements for their activation. Classic pathway activation is initiated by binding of C1q to its ligands (IgG or IgM immune complexes), which induces conformational changes of the C1 complex and leads to activation of its subunits (C1r and C1s). C1s activates C4 and C2 resulting in the formation of the C4bC2a complex (C3 convertase). C3 convertase exists in two forms (C4bC2a and C3bBb). The alternative pathway activation is initiated by activating surfaces such as bacterial cell wall components and dimeric or polymeric IgA. Properdin binds directly to alternative pathway activators, the only positive regulator of the complement system, and then binds either C3(H2O) or C3b (42). When C3
convertase (C3bBb) is formed, it can be further stabilized by properdin. Therefore, properdin has two functions in the amplification of the alternative pathway (44). The lectin pathway of complement activation is initiated by binding of MBL and ficolins to its ligands (carbohydrate containing surfaces and IgA). This binding results in activation of the mannose-binding lectin-associated serine proteases (MASP-1, MASP-2 and MASP-3). MASP-2 cleaves C4 then C2, leading to the formation of the same C3 convertase. Although the three pathways differ in the way of activation, they share the essential complement component C3 convertase (43).
Activation of the complement system through any of the three pathways results in activation of C3. C3 convertase cleaves complement C3 into C3a and C3b, which allow opsonization of bacteria and activation of the terminal pathway. The three pathways converge at the activation of C5 to form a potent chemoattractant C5a and the membrane attack complex (C5b-9). Assembly of C5b-9 may lead to lysis of pathogenic cells (Figure 2). The chemoattractant recruits the inflammatory cells, which may contribute to innate immune functions or tissue damage (44,45). New evidence by Selander et al. indicates the presence of a fourth pathway that activates complement. These authors showed that MBL can bind to serogroup O antigen-specific Salmonella oligosaccharides, which activate C3 in the absence of C2, C4 and even MBL associated MASP-2 (46).

In summary, complement has several functions, including cytolysis, opsonization, enhancing phagocytosis, chemotaxis, solubilization of immune complexes and regulation of the acquired immune response. In normal physiology, complement activation is beneficial, and it is important for natural defense against pathogens. The complement system has regulatory roles in immune system by bridging innate and adaptive immunity, and disposal of immune complexes, inflammatory products and apoptotic cells. However, inappropriate activation of complement can mediate cellular injury and contribute to the pathogenesis of various diseases (43).

1.5 Complement-mediated renal injury

Deficiencies of complement regulatory components are associated with renal disease directly or indirectly. Defective regulation of C3 is typically associated with glomerulonephritis (47). Both C3 glomerulopathy (a form of membranoproliferative glomerulonephritis) and atypical hemolytic uremic syndrome (HUS) are associated with factor H deficiency (48). Systemic lupus erythematosus (SLE) can develop due to deficiency of C1q, C4 and C2 via mechanisms relating to defective clearance of apoptotic material. Mice deficient
in the complement regulator, clusterin, which inhibits the assembly of C5b-9, also develop renal disease (49). Complement deficiencies can cause renal disease, by uncontrolled complement activation or secondary to the development of SLE.

Glomerulonephritis may also be caused by abnormal complement activation, i.e. characterized by localization of antibodies in the glomerulus with subsequent activation of complement. Normally, nucleated cells are equipped with several mechanisms that support resistance to complement-dependent cytotoxicity. Thus, multiple pathways may be activated simultaneously by sublytic C5b-9 in nucleated cells, and there is equilibrium among pathways that lead to cellular injury with those that are cytoprotective (40). In glomerulonephritis, there are three possible mechanisms that explain immune deposit formation in the glomerulus (Figure 3). First, is the deposition of circulating immune complexes. This mechanism has been established in a rabbit model of chronic serum sickness. Second, in situ formation of immune complexes on the glomerular capillary wall where antigens are endogenous constituents of the podocyte membrane and the immune complex is initiated by binding of the circulating antibodies to those antigens. For example, anti-GBM nephritis is one of the few autoimmune diseases in which the pathogenic autoantigen has been identified. The anti-GBM autoantibodies target the non-collagenous domain 1 of the α3 chain of type IV collagen in the GBM (44). Third, antibodies react with molecules that are planted on the GBM or the podocyte within the glomerulus (50). Cationized bovine serum albumin (BSA) is an example of a planted antigen that triggers an immune response (51). The sites of the immune complex deposition could lead to different consequences. If immune complexes deposit on the GBM at the side of endothelial cells, complement activation products may be released into the bloodstream (e.g. C5a), which will attract and activate the leukocytes. If immune complexes are generated on the outer aspect of the GBM (in the subepithelial space near the podocytes), complement activation products are released into the urine, and there does not appear to be any attraction of circulating leukocytes (43).
Membranous nephropathy (MN) is a common cause of nephrotic syndrome in adults, accounting for 20% of cases. The disease presentation can be idiopathic (unknown cause) or secondary to other conditions, including infection (hepatitis B or syphilis), SLE, drug and cancer (52). Morphologically, MN characterized by the deposition of immune complexes in subepithelial space, thickening of the GBM, podocyte foot processes effacement, and proteinuria (50,53). Three antigenic targets have been identified in experimental and human MN; megalin (Heyman nephritis), neutral endopeptidase (NEP), and secretory phospholipase A2 receptor (PLA2R1). Megalin is a large protein (approximately 600 kDa) and a member of the low-density lipoprotein receptor family. It constitutes the target antigen complex in HN in rats. A small fragment of megalin is important for induction of active HN (54). NEP is an antigen on the podocyte foot processes in human MN. It was reported in alloimmune neonatal MN (51,55). The M-type PLA2R1 is a major target antigen in idiopathic MN in adults. Autoantibodies to this receptor are detected in serum samples from majority of the patients with MN
before immunosuppressive treatment and were mainly IgG4. In addition, anti-PLA2R1 antibodies can be valuable tools to monitor response to treatment (50,56).

The role of complement in experimental MN was overlooked initially because of the absence of glomerular inflammation. Active and passive HN in rats are the best and most widely employed experimental models for human MN, as the immunohistological features of HN are similar to the human MN disease (53). Deposition of antibodies in the subepithelial region of the glomerulus leads to activation of complement and assembly of C5b-9 in GEC plasma membranes, leading to GEC injury and proteinuria. Injury is associated with activation of diverse signaling pathways, which include phospholipases, as well as protein kinases, proteinases, COX2, endoplasmic reticulum (ER) stress, reactive oxygen species, and others. These pathways contribute to changes in GEC lipid structure and function, actin cytoskeleton reorganization, and displacement of filtration slit diaphragm proteins, ultimately resulting in proteinuria (9).

Anti-GBM glomerulonephritis is a well characterized but rare human autoimmune disease. Binding of autoantibodies to the GBM leads to autoimmune injury with complement activation and increased C3 deposition (44). Complement activation plays a key role in the pathogenesis of human anti-GBM disease. Based on work in experimental animals, early complement components are involved in recruitment of leukocytes. There is also assembly of the C5b-9 membrane attack complex in podocytes. Together, these mediators lead to damage of the GBM, glomerular capillary endothelium and podocytes. The injury is manifested by leukocyte infiltration, proteinuria, foot process effacement, rupture of the GBM, crescent formation, scarring, and ultimately, loss of renal function. It is important to note that injury in anti-GBM nephritis is potentially mediated by early and late complement components. For example, previous studies have shown that C6-deficient mice (which can not assemble C5b-9) are protected from injury induced by anti-GBM antibodies (57).
Activation of the terminal complement pathway is associated with the assembly of C5b-9 (Figure 4). The pathogenic role of C5b-9 in experimental models of glomerulonephritis (i.e. in the development of proteinuria) was confirmed in vivo, by administration of neutralizing antibodies to C6, and in an ex vivo model of PHN (isolated perfused kidney) by perfusing kidneys with serum deficient in C8 and C6 (terminal complement components) (53). Consistent with induction of proteinuria, C5b-9 was also localized with the immune deposits on GEC plasma membranes in the PHN model (58).

1.6 C5b-9-induced responses in GEC

Assembly of C5b-9 in GEC plasma membrane leads to formation of transmembrane channels or rearrangement of membrane lipids with loss of membrane integrity. Sublytic (sublethal) injury is induced by small doses of C5b-9. Similarly, in vivo complement-mediated tissue injury generally associated with sublytic amount of complement. In GECs, sublytic doses of C5b-9 do not disrupt the membrane, but rather “activate” the cells to stimulate specific signaling pathways (Figure 4). As already mentioned above, these pathways include protein kinases, phospholipases transcription factor, etc. While these signals injure the cells, there are other signals activated in parallel to limit the complement-induced injury i.e. promote cell recovery (Figure 4) (53).

In cultured GEC and PHN, C5b-9 resulted in transactivation of receptor tyrosine kinases including epidermal growth factor receptor (EGFR) (59). C5b-9 activates EGFR by tyrosine phosphorylation, which was able to bind to adaptor protein Grb2 and a peptide containing SH2 and SH3 regions of PLC-γ1 followed by activation of Ras-extracellular signal-regulated kinase (ERK) (60). In addition, activation of receptor tyrosine kinase may also activate downstream effector protein kinase C (PKC) in conjunction with increase of cytosolic Ca²⁺ concentration. Cytosolic Ca²⁺ can also be elevated independent on PKC activation due to Ca²⁺ influx.
Figure 4. Pathogenesis of experimental MN. Binding of antibody leads to assembly of C5b-9, with associated consequences; adapted from (53). See text for details.

In addition to aforementioned protein kinases, C5b-9 activates p38 kinase in GECs (Figure 4). The functional role of p38 appears rather complex. In GEC, p38 pathway reduced complement mediated cytotoxicity and the cytoprotective effect may involve Heat shock protein 27 (HSP27) (61). By analogy, p38 activity increased in glomeruli from rats with PHN, and treatment of the rats with p38 inhibitor exacerbate proteinuria. Moreover, complement induced phosphorylation of mitogen-activated protein kinase-associated protein kinase 2 (MAPKAPK-2 or
MK2) (62). MK2 is regarded primarily as a substrate of p38 kinase but is also reported to be a substrate of ERK (63). In a previous study Cybulsky et al. demonstrated that complement induced a robust activation-specific phosphorylation of MK2 (~4-fold above control) and that activation of MK2 was mediated by both ERK and p38 kinase pathways (64). In addition to ERK and p38 pathways, complement can activate C-Jun N-terminal kinase (JNK) in GEC culture and in vivo, by inducing superoxide production. JNK activation was associated with cytoprotection (65). In contrast, other studies showed that reactive oxygen species are produced in PHN at high amount and that reactive oxygen species are associated with lipid peroxidation and modification of GEC membrane proteins and GBM components by malondialdehyde adducts (66). Release of arachidonic acid (AA) by phospholipase A₂ is another important pathway activated by C5b-9 in GEC (Figure 4, and see below).

1.7 Phospholipase A₂ classification and structure

Phospholipases A₂ (PLA₂s) comprise a family of enzymes that hydrolyze the acyl bond at the sn-2 position of phospholipids to yield free fatty acids such as AA, and lysophospholipids (67,68). In the beginning of the twentieth century, PLA₂ was known to be a major component of snake venoms, and it was later recognized that PLA₂ from old world snakes (group I) differed in their disulfide bond pattern from new world snakes (group II). The more complicated PLA₂ from bee venom is group III. In 1991 the human cytosolic calcium-dependent PLA₂ was isolated from macrophages (group IVA). As the discovery of additional PLA₂s continued, the need for a group numbering system became obvious. In 2000, Six and Dennis expanded the PLA₂ superfamily into 15 groups based on their sequence homologies, and from a functional point of view these groups can be recombined into five principal types of PLA₂s, secretory PLA₂s (sPLA₂, groups I, II, III, V, VII–XIV), cytosolic PLA₂α (cPLA₂, group IV), calcium-independent PLA₂s (iPLA₂, group IV), platelet activating factor (PAF) acetyl hydrolases, and lysosomal PLA₂. iPLA₂s are members of group VI family of PLA₂ enzymes (69). sPLA₂ is characterized by low molecular weight (13-15 kDa), an active site
histidine residue adjacent to a Ca\(^{2+}\)-binding loop, and six conserved disulfide bonds. cPLA\(_2\) is the only PLA\(_2\) with a preference for AA in the sn-2 position of phospholipids and its structure contains Ca\(^{2+}\) dependent lipid binding C2 domain, and a catalytic \(\alpha/\beta\) hydrolase domain. cPLA\(_2\) is characterized by an active site serine and aspartic acid dyad and requirement for Ca\(^{2+}\) for activity (70). cPLA\(_2\) is activated when recruited to the membrane by a Ca\(^{2+}\) dependent translocation of the C2 domain and through phosphorylation on residues 505, 515, and 727 (71).

iPLA\(_2\) enzymes are characterized by not requiring Ca\(^{2+}\) for catalytic activity. iPLA\(_2\) is involved in signal transduction pathways that include mitogen-activated protein kinase (MAPK) p38, epidermal growth factor (EGF) receptor, the tumor suppressor gene, p53, and cell cycle-regulator, p21 (72). A diverse group of cellular processes has been proposed to be regulated by iPLA\(_2\), including cellular proliferation (73), assembly of very low density lipoprotein (74), apoptosis, tumorigenesis, cell injury, and chemotaxis (75). Inhibition of iPLA\(_2\) in HEK293 and INS-1 cells altered the amounts of several phospholipids and resulted in decreased cell growth and p53 activation (76-78).

The first and most extensively studied enzyme in group VI is iPLA\(_2\)-VIA, which has two isoforms, iPLA\(_2\)-VIA-1 and -2 (iPLA\(_2\)\(\beta\) short and iPLA\(_2\)\(\beta\) long). The two isoforms of iPLA\(_2\)\(\beta\) are composed of 7-8 ankyrin repeats, a linker region and a catalytic domain. Both iPLA\(_2\)\(\beta\) short and iPLA\(_2\)\(\beta\) long are functional enzymes, which differ by a 54-amino acid proline-rich insertion sequence. The activity of the iPLA\(_2\)\(\beta\) has been suggested to be regulated through many different mechanisms, including ATP binding, caspase cleavage, calmodulin, and possible ankyrin repeat mediated protein aggregation (71). In resting cells, iPLA\(_2\)s, including iPLA\(_2\)\(\beta\), are involved in the maintenance of membrane phospholipids by generating lysophospholipid acceptors that are reacylated with fatty acids. Therefore, iPLA\(_2\)\(\beta\) plays a housekeeping role by facilitating phospholipid remodeling (79). iPLA\(_2\)\(\beta\) KO mice show impaired sperm motility (80). In addition, smooth muscle cell AA and PGE\(_2\) release are attenuated in iPLA\(_2\)\(\beta\) KO
mice, leading to modulation of vascular tone, proliferation and migration (81). The second iPLA₂ isoform, group VIB (iPLA₂γ), is homologous to iPLA₂β in the C-terminal catalytic domain but shows no similarities in N-terminal region; accordingly, iPLA₂γ may have distinct regulatory properties from those of iPLA₂β (82,83). This isoform is further discussed below.

A membrane-bound, calcium-independent PLA₂, cPLA₂γ, has also been characterized with significant homology to the catalytic domain of the 85-kDa cPLA₂ but does not contain the regulatory calcium-dependent lipid binding domain in cPLA₂ (84). PAF acetyl hydrolase, also called oxidized lipid lipoprotein associated PLA₂, is involved in diverse kinds of lipid metabolism. The C-terminal regions of this enzyme are required for binding to HDL and LDL. This enzyme was cloned in 1995 from human plasma, and was shown to have anti-inflammatory activity in vivo (85).

1.8 Phospholipase A₂γ

Mancuso et al in 2000 and Tanaka et al. in 2000 cloned a human cDNA encoding a protein of 782 amino acids, which contains a region with a homology to the catalytic domain of cytosolic iPLA₂. This region contains the lipase consensus sequence Gly-Xaa-Ser-Xaa-Gly and several stretches surrounding the motif but lacks ankyrin repeats (82,83). The protein is predominantly present in the membrane fraction and exhibits a calcium-independent PLA₂ activity. They tentatively designated this membrane-associated enzyme as iPLA₂γ. A database search has yielded a putative iPLA₂γ homologue in the nematoda Caenorhabditis elegans gene, suggesting that this type of iPLA₂ participates in biologically important processes. Tanaka et al. expressed the protein with a FLAG-tag in COS-7 cells. iPLA₂γ was detected in the membrane fraction, by western blot, at the expected molecular mass of approximately 90 kDa. iPLA₂γ-N-terminal region lacks the ankyrin repeats and displays no homology with known proteins, but it is
rich in Ser and Thr residues, and some of them can be phosphorylated by protein kinases (83).

iPLA\(_2\)\(_\gamma\) is a membrane-bound enzyme that is reported to localize at the ER, peroxisomes, and mitochondria (86). These distinct sites of localization may be a result of specific domains in the structure of the enzyme (87). Mancuso et al. demonstrated that iPLA\(_2\)\(_\gamma\) is synthesized from a 3.5 kb mRNA containing a putative 2.4 kb coding region which was most prominent in heart tissue (82). The full-length 2.4 kb transcript contains 13 exons and includes four potential AUG translation initiation codons generating as many as 10 splice variants (88). iPLA\(_2\)\(_\gamma\) gene transcription and translation appear complex, as distinct translation initiation sites, resulting in the production of 88-, 77-, 74-, and 63-kDa forms of the enzyme were reported (89). iPLA\(_2\)\(_\gamma\) contains a consensus site for nucleotide binding and a lipase consensus motif in its C-terminal half, as well as potential cAMP-dependent protein kinase, protein kinase C, and extracellular signal-regulated kinase (ERK) phosphorylation sites (87). The lipase consensus motif GVSTG (amino acids 481–485 in the C-terminal region) is essential for Ca\(^{2+}\)-independent PLA\(_2\) catalytic activity, and substitution of Ala for Ser-483 or Asp-627 results in loss of PLA\(_2\) activity. By comparing this catalytic domain with those of the mouse homologue, human cPLA\(_2\), and the plant PLA\(_2\) patatin, Tanaka et al. found that the amino acid sequence of a short segment around Asp-627 of iPLA\(_2\)\(_\gamma\) is conserved among these PLA\(_2\)s. Ser-483 and Asp-627 of human iPLA\(_2\)\(_\gamma\) constitute an active site similar to the Ser-Asp dyad in cPLA\(_2\) and patatin. (90). Tanaka et al. (83) suggested that iPLA\(_2\)\(_\gamma\) may have multiple potential phosphorylation sites. In addition, a recent phosphoproteomic analysis of mitochondrial proteins in murine heart revealed two iPLA\(_2\)\(_\gamma\) phosphorylation sites, Ser-505 and Thr-509, corresponding to Ser-511 and Ser-515 in human iPLA\(_2\)\(_\gamma\). The authors suggested that the two phosphorylation sites (RKLGpSDVFpSQNV) may be in the context of MK2 or casein kinase I substrate motifs (91).
The catalytic domain of iPLA\(_{2}\)\(_\gamma\) is located in the C-terminal region of the protein, whereas the role of the N-terminal region is poorly defined. Indeed, a previous study showed that iPLA\(_{2}\)\(_\gamma\) contains four N-terminal methionine residues that may act as translation initiation sites (89). Tanaka et al. showed that deletion of a 362-amino acid N-terminal region of iPLA\(_{2}\)\(_\gamma\) caused a portion of the enzyme to shift from the membrane to the cytosol in a cell fractionation study. Moreover, the N-terminal fragment localized solely in the membrane, but not in the cytosolic fraction, indicating that the N-terminal region is involved in the membrane association of iPLA\(_{2}\)\(_\gamma\) (90). Despite the Ca\(^{2+}\)-independent catalytic properties of iPLA\(_{2}\)\(_\gamma\) in vitro, two findings suggest that activation of iPLA\(_{2}\)\(_\gamma\) in agonist-stimulated cells may involve a Ca\(^{2+}\)-regulated process (82,83), possibly activation of Ca\(^{2+}\)-dependent protein kinases, such as calmodulin (92). Alternatively, Ca\(^{2+}\) may enhance activation of iPLA\(_{2}\)\(_\gamma\) directly. A recent study showed that divalent cations (Ca\(^{2+}\) or Mg\(^{2+}\)) can activate iPLA\(_{2}\)\(_\gamma\) in heart mitochondria, leading to release of eicosanoids and lysolipids, possibly by facilitating mitochondrial phospholipid hydrolysis by iPLA\(_{2}\)\(_\gamma\) (93).

During oxidative stress, an ER-associated iPLA\(_{2}\) in renal proximal tubular cells (iPLA\(_{2}\)\(_\gamma\)) recognizes, cleaves, and removes oxidized phospholipids from the ER membrane; thus, iPLA\(_{2}\)\(_\gamma\) may act to repair or prevent lipid peroxidation during oxidative stress (86). Another study demonstrated that expression of shRNA against iPLA\(_{2}\)\(_\gamma\) increased lipid peroxidation and induced apoptosis in renal cells (94). In addition, inhibition of microsomal iPLA\(_{2}\)\(_\gamma\) in rabbit renal proximal tubule cell during cisplatin-induced apoptosis reduced annexin V staining, chromatin condensation, and caspase-3 activation, indicating that iPLA\(_{2}\)\(_\gamma\) inhibition was cytoprotective (95). Mitochondrial iPLA\(_{2}\)\(_\gamma\) has been shown to actively participate in the permeability transition pore opening of the mitochondria and the release of the proapoptotic cytochrome c (96).
Certain functions of iPLA\(_{2\gamma}\) have been delineated using KO mice. Genetic ablation of iPLA\(_{2\gamma}\) in mice resulted in the generation of viable progeny that demonstrated reduced growth rate, cold intolerance due to impaired fat burning in brown adipose tissue, and a defect in mitochondrial cytochrome oxidase (Complex IV) function when stimulated with ascorbate. Several studies showed that iPLA\(_{2\gamma}\) KO mice display multiple bioenergetic dysfunctional phenotypes (97-100). For example, iPLA\(_{2\gamma}\) deletion results in profound alterations in hippocampal phospholipid metabolism and mitochondrial phospholipid homeostasis resulting in enlarged and degenerating mitochondria, leading to autophagy and cognitive dysfunction (97). Another recent study showed that genetic ablation of iPLA\(_{2\gamma}\) prevents obesity and insulin resistance during high fat feeding by mitochondrial uncoupling and increased adipocyte fatty acid oxidation (100). These reports identified an obligatory role for iPLA\(_{2\gamma}\) in mitochondrial lipid metabolism and membrane structure, perturbation of which may profoundly influence fatty acid β-oxidation, oxygen consumption, energy expenditure, and thus, tissue homeostasis.

Despite the recent advance in studying the pathophysiological role of iPLA\(_{2\gamma}\) in the brain, liver (98), heart and skeletal muscles (99), the pathophysiological roles of iPLA\(_{2\gamma}\) in the kidney are poorly understood.

### 1.9 Phospholipases in GEC injury

In addition to loss of membrane integrity associated with assembly of sublytic C5b-9 in complement–mediated glomerular disease, there is activation of a number of biochemical pathways (Figure 5). One of the main pathways involves upregulation of protein kinase activity leading to phospholipase C (PLC) activation (101). Moreover, assembly of C5b-9 in cultured GEC results in Ca\(^{2+}\) influx and an increase in cytosolic free Ca\(^{2+}\) concentration which promotes activation of cPLA\(_2\) (102). Activation of cPLA\(_2\) with subsequent release of AA is compartmentalized to the ER. cPLA\(_2\), a mediator of C5b-9-dependent GEC injury, directly induces membrane phospholipid hydrolysis and disruption of ER membrane integrity, stimulates ER stress protein upregulation, and exacerbates
proteinuria. Also cPLA₂ activation mediates GEC injury by providing AA for metabolism via COX-1 and -2 to produce prostanoids and thromboxane A₂ (Figure 5) (9). Collectively, activation of cPLA₂ leads to various cellular effects. First, activation of cPLA₂ perturbs the membrane of the ER and contributes to induction of ER stress, in GEC. Second, activation of cPLA₂ may contribute directly to the exacerbation of C5b-9-induced GEC injury. Prostanoids may increase glomerular transcapillary pressure, therefore proteinuria is increased in PHN on hemodynamic basis. In addition, production of prostanoids may directly exacerbate complement-induced GEC injury (103).

![Figure 5](image)

**Figure 5.** Activation of signaling pathways leading to GEC injury and proteinuria; adapted from (9). See text for details.

Recently, it has been shown that C5b-9 stimulated a Ca²⁺-independent PLA₂ activity in cultured GECs and that complement-dependent AA release in GEC can be mediated by iPLA₂γ (Figure 5) (9). The stimulation of iPLA₂γ activity was associated with phosphorylation of the enzyme. In addition, C5b-9 can induce deformability of the membrane lipid bilayer (104), such that there would be increased substrate availability to iPLA₂γ. Therefore, C5b-9-mediated iPLA₂γ stimulation may involve modification of phospholipid substrate. Overexpression
of iPLA₂ attenuated complement-induced GEC lysis (measured by lactate dehydrogenase release), and this effect was reversed by the iPLA₂-directed inhibitor, bromoenol lactone (BEL) as well as indomethacin, suggesting that the cytoprotective effect of iPLA₂γ was at least in part mediated by generation of prostanooids (1). However, BEL reversed the cytoprotective effect of iPLA₂γ to a greater extent than indomethacin, suggesting that factors other than prostanooids may also be involved. The cytoprotection mechanisms of iPLA₂γ are related to iPLA₂ catalytic activity, and are distinct from AA metabolism. In GEC exposed to complement C5b-9, one of the possible cytoprotective mechanisms is activation of the ER stress response (105). Given the location of iPLA₂γ at the ER, the enzyme could potentially perturb the ER membrane to initiate the ER stress response, which may limit C5b-9-induced cytolysis.

1.10 Kidney injury and ER stress

Exposure of GEC to C5b-9 perturbs the integrity of the ER and induces ER stress and these effects are dependent, in part, on the activation of cPLA₂ (102). The ER is an organelle where secretory, luminal and membrane proteins, are processed after translation. About one-third of the newly synthesized proteins translocate into the ER lumen where nascent proteins are folded with the assistance of molecular chaperones and folding enzymes. Proper maturation of proteins relies on the oxidative environment of the ER lumen, which favors disulfide bond formation and protein folding. Only correctly folded proteins are transported to the Golgi apparatus (106). In addition to protein maturation, the ER is also responsible for synthesis of cholesterol, steroids, and other lipids, and is also a major store for calcium (107). Maintenance of the homeostasis within the ER, by maintaining the appropriate level of calcium, glucose, and ATP, is essential for its functionality. Disruption of this homeostasis results in misfolding of proteins in the ER, which exceeds the capacity of the folding apparatus and ER-associated degradation (ERAD) machinery. The misfolded protein accumulation leads to ER stress and activation of the unfolded protein response (UPR) (Figure 6) (108-111).
1.11 Unfolded protein response

The UPR is activated to rescue misfolded proteins and improve protein folding. There are three major UPR pathways, activated via protein sensors located in the ER membrane, including activating transcription factor-6 (ATF6), inositol-requiring enzyme 1α (IRE1α), and protein kinase RNA-like ER kinase (PERK) (Figure 6) (108-110). In resting cells, the three sensors are inactive, and are believed to be in association with the ER chaperone, glucose-regulated protein (grp) 78 (bip). Accumulation of misfolded proteins leads to release of ATF6 from bip and translocation to the Golgi, where it is cleaved by site 1 and 2 proteases. The cleaved cytosolic fragment of ATF6, which has a DNA-binding domain, migrates to the nucleus to activate transcription of ER chaperones. Upregulation of ER chaperones, including grp94 and bip, enhances ER protein folding capacity and may limit cytotoxicity (referred to as an “adaptive” UPR). The IRE1α endoribonuclease cleaves X box–binding protein-1 (xbp1) mRNA and changes the reading frame to yield a potent transcriptional activator, which works in
parallel with ATF6 to activate transcription of ER chaperone genes. The third aspect of the UPR involves PERK, whose activation phosphorylates the eukaryotic translation initiation factor-2α subunit (eIF2α), which reduces the general rate of translation and aims at decreasing the protein load on a damaged ER. The three UPR pathways are often activated together, but selective activation of some pathways together with suppression of others can occur (111,112).

The UPR plays an important role in normal physiological function in certain types of cells, such as pancreatic β-cells and plasma cells. These particular cells have a high rate of protein synthesis (112). bip appears to be required for the maintenance of normal glomerular and tubular structures in the kidney (107). bip and grp94 bind to misfolded (abnormal) proteins and prevent their aggregation by rescuing them from irreversible damage, or by increasing their susceptibility to proteolytic degradation. Induction of the UPR may be protective to additional insult (Figure 6) (109,110). However, prolonged ER stress may be cytotoxic, for example, leading to apoptosis. The apoptotic pathways include PERK, leading to the induction of CHOP, as well as IRE1α kinase activity (Figure 6). The latter can lead to activation of apoptosis signal–regulating kinase-1 and JNK, and/or decay of ER-localized mRNAs. Sustained ER stress was also reported to result in necrotic cell death (113).
Objectives

A previous study demonstrated that C5b-9 stimulated a Ca$^{2+}$-independent PLA$_2$ activity in GECs, and complement-induced release of AA and prostaglandin E$_2$ (PGE$_2$) were amplified in GECs that overexpress iPLA$_2$$^\gamma$. Furthermore, overexpression of iPLA$_2$$^\gamma$ attenuated complement-induced GEC lysis, and this effect was reversed by the iPLA$_2$-directed inhibitor, bromoenol lactone (BEL), suggesting that activation of iPLA$_2$$^\gamma$ is cytoprotective in C5b-9-mediated GEC injury (1). Therefore, the goal of the present study was to further characterize the activation and functional role of iPLA$_2$$^\gamma$ in complement-induced GEC injury. The first aim was to determine the upstream mediator potentially involved in iPLA$_2$$^\gamma$ activation by complement (Chapter 2). Second, membranous nephropathy and anti-GBM nephritis are complement-mediated diseases, which result in the upregulation of ER stress (9). iPLA$_2$$^\gamma$ is reported to localize at the ER (86). Therefore, the second aim was to address the role of iPLA$_2$$^\gamma$ in ER stress, specifically, if the cytoprotective effect of iPLA$_2$$^\gamma$ involves the UPR (Chapter 3). Modulation of ER stress is potentially a novel target for therapy in glomerular disease. Third, although we and others have provided considerable insight into the functions of iPLA$_2$$^\gamma$ in cultured cell lines, but information on the functional role of iPLA$_2$$^\gamma$ in the kidney is lacking. The third aim was to employ iPLA$_2$$^\gamma$ KO mice to better understand the role of iPLA$_2$$^\gamma$ in normal podocyte function, and in podocyte injury (Chapter 4).
CHAPTER 2: PUBLISHED ARTICLE
COMPLEMENT-MEDIATED ACTIVATION OF CALCIUM-INDEPENDENT PHOSPHOLIPASE A₂γ; ROLE OF PROTEIN KINASES AND PHOSPHORYLATION*

Hanan Elimam, Joan Papillon, Tomoko Takano, and Andrey V. Cybulsky

Department of Medicine, McGill University Health Centre, McGill University, Montreal, Quebec, Canada

Running title: Activation of calcium-independent phospholipase A₂γ

Address for correspondence: Andrey V. Cybulsky, MD, Division of Nephrology, Royal Victoria Hospital, 687 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1. Tel: 1-514-398-8148. Fax: 1-514-843-2815. E-mail: andrey.cybulsky@mcgill.ca; Tomoko Takano, MD, PhD, Division of Nephrology, McGill University, 3775 University Street, Rm 236, Montreal, Quebec, Canada H3A2B4. Tel: 1-514-398-2171, Fax: 1-514-843-2815. E-mail: tomoko.takano@mcgill.ca

This research was originally published in the Journal of Biological Chemistry, volume 288, pages 3871-3885, 2013 (114). © the American Society for Biochemistry and Molecular Biology.
**Background:** Calcium-independent phospholipase A$_{2\gamma}$ (iPLA$_{2\gamma}$) is a mediator of complement-induced glomerular injury.

**Results:** Complement stimulated iPLA$_{2\gamma}$ through activation of mitogen-activated protein kinases.

**Conclusion:** Phosphorylation of iPLA$_{2\gamma}$ plays a role in activation and signaling.

**Significance:** Understanding the regulation of iPLA$_{2\gamma}$ activity is essential for developing novel therapeutic approaches to glomerular injury and proteinuria.

**ABSTRACT**

In experimental membranous nephropathy, complement C5b-9 induces glomerular epithelial cell (GEC) injury and proteinuria. The effects of C5b-9 are mediated via signaling pathways, including calcium-independent phospholipase A$_{2\gamma}$ (iPLA$_{2\gamma}$), and mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. The iPLA$_{2\gamma}$ pathway is cytoprotective. The present study addresses the mechanisms of iPLA$_{2\gamma}$ activation. iPLA$_{2\gamma}$ activity was monitored by quantifying prostaglandin E$_2$ (PGE$_2$) production. In GEC, iPLA$_{2\gamma}$ localized at the endoplasmic reticulum and mitochondria. Complement-mediated production of PGE$_2$ was amplified in GEC that overexpress iPLA$_{2\gamma}$, compared with control cells, and was blocked by the iPLA$_{2\gamma}$ inhibitor, bromoenol lactone (BEL) in both iPLA$_{2\gamma}$-overexpressing and control GEC. In GEC that overexpress iPLA$_{2\gamma}$, complement-mediated PGE$_2$ production was reduced by inhibitors of MAP/ERK kinase-1 (MEK1) and p38, but not JNK. In COS-1 cells that overexpress iPLA$_{2\gamma}$ and cyclooxygenase-1, PGE$_2$ production was induced by co-expression of constitutively active MEK1 or MAPK-interacting kinase 1 (MNK1), as well as by stimulation with epidermal growth factor (EGF) + ionomycin. Complement- and EGF + ionomycin-stimulated iPLA$_{2\gamma}$ activity was attenuated by the S511A/S515A double mutation. Moreover, complement- and EGF + ionomycin enhanced phosphorylation of S511. Thus, complement-mediated activation of iPLA$_{2\gamma}$ is mediated via ERK and p38 pathways, and phosphorylation of S511
and/or S515 plays a key role in the catalytic activity and signaling of iPLA$_2$$. Defining the mechanisms by which complement activates iPLA$_2$$\gamma$ provides opportunities for development of novel therapeutic approaches to GEC injury and proteinuria.

**INTRODUCTION**

Phospholipases A$_2$ (PLA$_2$) comprise a family of enzymes that hydrolyze the acyl bond at the sn-2 position of phospholipids to yield free fatty acids, such as arachidonic acid (AA), and lysophospholipids (67,68). Both products represent precursors for signaling molecules that can exert multiple biological functions. AA can be converted into bioactive eicosanoids by the effect of cyclooxygenases (COX), lipoxygenases and cytochrome P450 (115). Schaloske and Dennis (69) classified PLA$_2$s enzymes into 15 groups based on their sequence homologies, and from a functional point of view, these groups can be regrouped into five principal types of PLA$_2$s, secreted PLA$_2$, cytosolic PLA$_2$ (cPLA$_2$), calcium-independent PLA$_2$ (iPLA$_2$), platelet-activating factor acetylhydrolases, and lysosomal PLA$_2$. iPLA$_2$s are members of group VI family of PLA$_2$ enzymes (69). The first and most extensively studied enzyme in this group is VIA, which has two isoforms, iPLA$_2$-VIA-1 and -2 (iPLA$_2$$\beta$ short and iPLA$_2$$\beta$ long). The second iPLA$_2$ isoform, Group VIB (iPLA$_2$$\gamma$), is homologous to iPLA$_2$$\beta$ in the C-terminal catalytic domain, but shows no similarities in N-terminal region; accordingly, iPLA$_2$$\gamma$ may have distinct regulatory properties from those of iPLA$_2$$\beta$ (82,83).

Various PLA$_2$ enzymes have been shown to mediate pathways of cell injury in experimental disease models (75,116-119). For example, cPLA$_2$$\alpha$ and its products are important mediators of complement-induced glomerular epithelial cell (GEC; podocyte) injury in the passive Heymann nephritis model of membranous nephropathy (9,53). In passive Heymann nephritis, GEC (an important component of the glomerular permselectivity barrier) (3,120) are targeted by the complement C5b-9 membrane attack complex, which leads to noncytolytic GEC injury (9,53).
Injury is associated with activation of diverse signaling pathways, which include phospholipases, as well as protein kinases, proteinases, COX2, endoplasmic reticulum (ER) stress, reactive oxygen species, and others. These pathways contribute to changes in GEC lipid structure and function, actin cytoskeleton reorganization and displacement of filtration slit diaphragm proteins, ultimately resulting in proteinuria (1,9,53). Recently, we demonstrated that C5b-9 stimulated a Ca^{2+}-independent PLA\(_2\) activity in GEC, and complement-induced release of \([^3\text{H}]\text{AA}\) and prostaglandin (PG) \(\text{E}_2\) was amplified in GEC that overexpress iPLA\(_{2\gamma}\) (1,53). Furthermore, overexpression of iPLA\(_{2\gamma}\) attenuated complement-induced GEC injury, and this effect was reversed by the iPLA\(_2\)-directed inhibitor, bromoenol lactone (BEL), as well as indomethacin, suggesting that the cytoprotective effect of iPLA\(_{2\gamma}\) was, at least in part, mediated by generation of prostanoids (1).

Although we and others have shown functional coupling of iPLA\(_{2\gamma}\) with COX1, leading to prostanoid production (121), the functions of iPLA\(_{2\gamma}\) have not been fully delineated (122). In resting cells, iPLA\(_2\)s, including iPLA\(_{2\beta}\), are involved in the maintenance of membrane phospholipids, by generating lysophospholipid acceptors that are reacylated with fatty acids. Therefore, iPLA\(_2\) plays a housekeeping role by facilitating phospholipid remodeling (79,123,124). Inhibition of iPLA\(_2\) in HEK293 and INS-1 cells altered the amounts of several phospholipids, and resulted in decreased cell growth and p53 activation (76–78). During oxidative stress, an ER-associated iPLA\(_2\) in renal proximal tubular cells (iPLA\(_{2\gamma}\)) recognizes, cleaves, and removes oxidized phospholipids from the ER membrane (86); thus, iPLA\(_{2\gamma}\) may act to repair or prevent lipid peroxidation during oxidative stress (86). Another study demonstrated that expression of shRNA against iPLA\(_{2\gamma}\) increased lipid peroxidation and induced apoptosis in renal cells (94). iPLA\(_2\) is involved in signal transduction pathways that include mitogen-activated protein kinase (MAPK) p38, epidermal growth factor (EGF) receptor, the tumor suppressor gene, p53, and cell cycle-regulator, p21 (72). A diverse array of cellular processes has been proposed to be regulated by iPLA\(_{2\gamma}\),
including cellular proliferation (73), assembly of very low density lipoprotein (74), apoptosis (125), endothelial cell platelet activating factor synthesis (126), tumorigenesis, cell injury, and chemotaxis (75).

iPLA\textsubscript{2}\gamma is a membrane-bound enzyme, which is reported to localize at the ER, peroxisomes and mitochondria (86). These distinct sites of localization may be a result of specific domains in the structure of the enzyme (87). iPLA\textsubscript{2}\gamma gene transcription and translation appear complex, as distinct translation initiation sites, resulting in the production of 88, 77, 74, and 63 kDa forms of the enzyme were reported (89). iPLA\textsubscript{2}\gamma contains a consensus site for nucleotide binding and a lipase consensus motif in its C-terminal half, as well as potential cAMP-dependent protein kinase, protein kinase C, and extracellular signal-regulated kinase (ERK) phosphorylation sites (87). The lipase consensus motif, GVSTG (amino acids 481-485, in the C-terminal region), is essential for Ca\textsuperscript{2+}-independent PLA\textsubscript{2} catalytic activity, and substitution of Ala for Ser-483 or Asp-627 results in loss of PLA\textsubscript{2} activity (90). To date, it is not known if/how phosphorylation would affect iPLA\textsubscript{2}\gamma activity.

The goal of the present study was to further characterize the activation of iPLA\textsubscript{2}\gamma in complement-induced GEC injury. Specifically, we addressed the role of various kinases known to be activated by complement. In GEC, we demonstrate the subcellular localization of iPLA\textsubscript{2}\gamma at the ER and mitochondria, which was dependent on the N-terminal region of iPLA\textsubscript{2}\gamma. Complement-induced activation of iPLA\textsubscript{2}\gamma was mediated via ERK and p38 pathways. Stimulation of iPLA\textsubscript{2}\gamma was dependent on phosphorylation of S511 and/or S515 via MAPK-interacting kinase 1 (MNK1).

**EXPERIMENTAL PROCEDURES**

**Materials.** Tissue culture media, G418 (geneticin), plasmid pRc/RSV, and Lipofectamine 2000 were from Invitrogen-Life Technologies (Burlington, ON).
Electrophoresis reagents were from Bio-Rad Laboratories (Mississauga, ON). Mouse monoclonal anti-green fluorescent protein (GFP), sheep anti-COX1, rabbit anti-MEK1 (C-18) and rabbit anti-MAP/ERK kinase kinase-1 (MEKK1) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-calnexin was from Assay Designs and Stressgen (Ann Arbor, MI). Mouse monoclonal anti-α-tubulin was from Sigma-Aldrich Canada (Mississauga, ON). Rabbit phospho-p44/42 MAPK (Thr-202/Tyr-204), rabbit anti-phospho-p38 (Thr-180/Tyr-182), rabbit anti-phospho-JNK (Thr-183/Tyr-185), and rabbit anti-phospho-Ser/Thr Akt substrate antibody (which recognizes the sequence R/KXXpS/T) were from Cell Signaling Technology (Danvers, MA). MitoTracker Red CMXRos was from Molecular Probes (Eugene, OR). Enhanced chemiluminescence (ECL) reagents were from GE Healthcare (Baie d’Urfé, QC). BEL, CGP57380, PLA₂ assay kits and prostaglandin E₂ (PGE₂) enzyme immunoassay kits were from Cayman Chemical (Ann Arbor, MI). PD98059 was from Calbiochem (La Jolla, CA). Human iPLA₂γ wild type (WT) in pcDNA 1.1 was kindly provided by Drs. Richard Gross and David Mancuso (Washington University School of Medicine, St. Louis, MO) (1). R4F-MEK cDNA was provided by Dr. Natalie Ahn (University of Colorado, Boulder, CO) (127). pFC-MEKK, the constitutively active form of MEKK1, and pFC-MKK3, the constitutively active form of MKK3, were from Stratagene (La Jolla, CA). C8 and C8-deficient serum were from Complement Technology (Tyler, TX). pcDNA3-myc-MAPK activated protein kinase-2 (MK2) WT, and constitutively active mutant, pcDNA3-myc-MK2-EE, were kindly provided by Professor Matthias Gaestel (Institute of Biochemistry, Medical School, Hannover, Germany) (128). Constructs encoding glutathione S-transferase (GST)-tagged MNK1 (pEBG-MNK1), a constitutively active form of MNK1 (pEBG-T332D), dominant-interfering MNK1 mutant (pEBG-T2A2) and pEBG empty vector were kindly provided by Dr. Jonathan Cooper (Fred Hutchinson Cancer Research Center, Seattle, WA) (129). Other reagents were from Sigma-Aldrich Canada (Mississauga, ON).
Cloning and construction of iPLA2γ mutants. WT, full-length (782 amino acid; M1) human iPLA2γ in pcDNA 1.1 was digested with SalI and SacII, and was subcloned into pEGFP-C1 vector (Clontech) at SalI and SacII restriction sites to produce M1 GFP-iPLA2γ WT. (M1 denotes that the iPLA2γ cDNA sequence begins at the codon for the first methionine.) Mutations in potential ERK phosphorylation sites (Ser-271 and Ser-168 to Ala, as well as the double mutation) were constructed by PCR-based mutagenesis (primers are presented in Table I). A double mutation in two other potential phosphorylation sites (Ser-511 and Ser-515 to Ala) was constructed using an analogous approach (Table I). For construction of N-terminally truncated (M4) GFP-iPLA2γ (i.e. iPLA2γ cDNA sequence beginning at the codon for the fourth methionine, amino acid 221), PCR reactions were performed with primers M4-F1 in combination with R1 (Table I). All GFP-iPLA2γ mutant cDNAs were verified by DNA sequencing.

Cell culture and transfection. Rat GEC culture and characterization have been described previously (130). GEC were maintained in K1 medium on plastic substratum. Cells were stably transfected with M1 or M4 GFP-iPLA2γ WT plasmids using Lipofectamine 2000 reagent, according to the manufacturer’s instructions. Following selection with G418 and expansion, cells were sorted by flow cytometry to obtain cells with the highest expression of GFP-iPLA2γ WT. Fluorescence microscopy and immunoblotting were used to confirm GFP-iPLA2γ overexpression. A clone of GEC containing the neomycin-resistance gene was used as a control (GEC-Neo). COS-1 cells were cultured in DMEM-10% fetal bovine serum, and were transfected transiently with GFP-iPLA2γ and/or COX1 cDNAs using Lipofectamine 2000.

Incubation of GEC with complement. GEC in monolayer culture were washed twice, and were incubated with rabbit anti-GEC antiserum (5% vol/vol) in modified Krebs-Henseleit buffer containing 145 mM NaCl, 5 mM KCl, 0.5 mM MgSO4, 1 mM Na2HPO4, 0.5 mM CaCl2, 5 mM glucose, and 20 mM Hepes, pH 7.4, for 30 min at 22°C. The cells were then incubated for 40 min at 37°C with
normal human serum (NS, 2% vol/vol; with full complement activity) or heat-inactivated (decomplemented) human serum (HIS, 2% vol/vol; incubated at 56°C for 60 min) in controls (105,131).

**PGE$_2$ assay.** Stimulated iPLA$_2$ enzymatic activity was monitored by measuring PGE$_2$ production. After incubation, supernatants were collected to quantify PGE$_2$. The amount of PGE$_2$ released into supernatants was equivalent to that from cells plus supernatants, indicating that most PGE$_2$ was released from cells into supernatants. PGE$_2$ was quantified using an enzyme immunoassay kit, according to the manufacturer’s instructions. The range of the standard curve in the assay was 4 to 1000 pg PGE$_2$/100µl of sample (132). PGE$_2$ concentration was calculated according to standard formulas.

**PLA$_2$ assay.** PLA$_2$ activity was measured in COS-1 cell extracts using a PLA$_2$ activity assay kit, according to the manufacturer’s instructions, and as described previously (133). In this assay, hydrolysis of arachidonoyl thio-phosphatidylcholine at the sn-2 position by PLA$_2$ releases a free thiol, which is detected by 5,5’-dithio-bis-2-nitrobenzoic acid. Briefly, cells were homogenized in 50 mM Hepes, pH 7.4, containing 1 mM EDTA. Cell homogenates were cleared by centrifugation at 8,500 g for 10 min at 4°C. The reaction was initiated by the addition of 2-arachidonoyl thio-phosphatidylcholine to cell extracts in buffer containing 80 mM Hepes, pH 7.4, 150 mM NaCl, 4 mM Triton X-100, 30% glycerol, and 1 mg/ml BSA. Duplicate samples were incubated with and without 10 µM BEL. After 60 min at 22°C, the reaction was terminated by the addition of 1 mM 5,5’-dithio-bis-2-nitrobenzoic acid, and the absorbance was measured at 450 nm. To determine iPLA$_2$ activity, the optical density obtained in the presence of BEL was subtracted from the total optical density (133) (in control cells, ~20% of PLA$_2$ activity was inhibited by BEL). The value of the group with maximum iPLA$_2$ activity was set to 1.0, and the iPLA$_2$ activities of the other groups were calculated as percent of maximum.
Immunoblotting. Cells were lysed in ice-cold buffer containing 1% Triton X-100, 125 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EGTA, 2 mM Na$_3$VO$_4$, 10 mM sodium pyrophosphate, 25 mM NaF, and protease inhibitor cocktail (Roche Diagnostics). Equal amounts of lysate proteins were dissolved in Laemmli buffer and were subjected to SDS-PAGE under reducing conditions. Proteins were then electrophoretically transferred onto a nitrocellulose membrane and blocked at room temperature for 60 min with 5% dry milk in buffer containing 10 mM Tris, pH 7.5, 50 mM NaCl, 2.5 mM EDTA, and 0.05% Tween 20. The membrane was then incubated with primary and secondary antibodies, and developed with ECL.

Immunofluorescence microscopy. GEC expressing GFP-iPLA$_{2\gamma}$ WT (M1 or M4) and GEC-Neo (control) were cultured on glass coverslips for 24 h. All reactions were carried out at 22°C. To examine the localization of GFP-iPLA$_{2\gamma}$ WT at the ER, cells were fixed with 3% paraformaldehyde in PBS for 30 min and were permeabilized with 0.1% Triton-X-100 in PBS for 30 min. After washing with PBS, GEC were incubated with rabbit anti-calnexin antiserum or normal rabbit serum (negative control) diluted in 3% BSA for 30 min. Cells were washed and incubated with rhodamine-conjugated goat anti-rabbit IgG in 3% BSA for 30 min. Nuclei were counter-stained with 4’6-Diamidino 2-phenylindole (DAPI, 30 nM) in PBS for 4-5 min just before mounting the coverslips onto glass slides. Staining was visualized with a Zeiss AxioObserver fluorescence microscope with visual output connected to an AxioCam digital camera. To visualize mitochondria, GEC expressing GFP-iPLA$_{2\gamma}$ WT (M1 or M4) and GEC-Neo (control), on coverslips, were incubated for 15 min at 37°C with MitoTracker Red CMXRos (25 nM). Cells were then fixed with 3% (w/v) paraformaldehyde in PBS for 30 min. After washing, coverslips were mounted onto glass slides and visualized with a fluorescence microscope.

Statistics. Data are presented as mean ± SE. 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 Tukey’s or Bonferroni’s method. Statistical significance was considered to be $P<0.05$.

RESULTS

*M1 GFP-iPLA$_{2\gamma}$ WT is enzymatically active.* To study the effect of complement on iPLA$_{2\gamma}$ activation, we first established a subclone of GEC that stably overexpress M1 GFP-iPLA$_{2\gamma}$ WT (M1 GEC-iPLA$_{2\gamma}$). By immunoblotting, M1 GEC-iPLA$_{2\gamma}$ was expressed as a 115 kDa protein, consistent with GFP (27 kDa) fused with the 88 kDa isoform of iPLA$_{2\gamma}$ (Fig. 1A). When M1 GFP-iPLA$_{2\gamma}$ WT or untagged iPLA$_{2\gamma}$ was transfected in COS-1 cells together with COX1, PGE$_2$ production was markedly increased, compared with untransfected control (Fig. 1B) or cells transfected with COX1 alone (Fig. 1C). This increase was attenuated by the addition of BEL (Fig. 1C). Together the results indicate that M1 GFP-iPLA$_{2\gamma}$ WT is enzymatically active.

*iPLA$_{2\gamma}$ localizes at the ER and mitochondria.* The expression and localization of iPLA$_2$ isoforms varies considerably among different cell types and the role of iPLA$_2$ may be determined by the isoform present in a specific cell or organelle (134). We examined the localization of M1 GFP-iPLA$_{2\gamma}$ WT in stably transfected GEC. By fluorescence microscopy, confluent monolayers of resting GEC demonstrated green fluorescent staining mainly in the perinuclear region (Fig. 2A, C). A significant portion of the M1 GFP-iPLA$_{2\gamma}$ WT co-localized with calnexin (Fig. 2B, D), indicating localization at the ER. In resting cells not expressing M1 GFP-iPLA$_{2\gamma}$ WT, calnexin staining showed a similar distribution in the perinuclear region, indicating that expression of M1 GFP-iPLA$_{2\gamma}$ WT did not affect the structure of the ER (data not shown).

A portion of the M1 GFP-iPLA$_{2\gamma}$ WT did not appear to co-localize with calnexin, but localized at the mitochondria (Fig. 2E-G). The appearance of the mitochondria
was unaffected by the expression of M1 GFP-iPLA₂γ WT (data not shown). Finally, we treated the GEC expressing M1 GFP-iPLA₂γ WT with digitonin to permeabilize the plasma membranes and release cytosolic proteins into cell supernatants. Distribution of the GFP fluorescence in the digitonin-treated cells was similar to that seen in untreated (intact) cells (Fig. 2H), further supporting the association of iPLA₂γ with intracellular organelles. Together, the results shown in Fig. 2 indicate that in GEC, M1 GFP-iPLA₂γ WT is associated with at least two organelles, the ER and mitochondria.

N-terminally truncated iPLA₂γ (M4 GFP-iPLA₂γ) is mislocalized. The catalytic domain of iPLA₂γ is located in the C-terminal region of the protein, while the role of the N-terminal region is poorly defined. Indeed, a previous study showed that iPLA₂γ contains four N-terminal methionine residues, which may act as translation initiation sites, resulting in 88, 77, 74, and 63 kDa forms of iPLA₂γ in SF9 insect cells (89). To determine if the N-terminal region may be involved in localization and/or regulation of iPLA₂γ catalytic activity, we deleted the 220 N-terminal amino acids (spanning between the first and fourth methionine) to generate a short form of iPLA₂γ in which the GFP-iPLA₂γ fusion would be at the fourth methionine (M4 GFP- iPLA₂γ). Expression of M4 GFP-iPLA₂γ in COS-1 cells showed a prominent band at ~92 kDa (Fig. 3A), which represents GFP (27 kDa) fused with the 63 kDa form of iPLA₂γ. When both M1 and M4 isoforms were expressed at serially-increasing concentrations in COS-1 cells (together with COX1), PGE₂ production by M1 GFP-iPLA₂γ WT was markedly greater, compared with M4 GFP-iPLA₂γ, despite weaker expression (Fig. 3A-C). PGE₂ production by M4 GFP-iPLA₂γ was trivial, as it was not significantly greater, compared with control cells.

We also employed an in vitro assay to measure iPLA₂ enzymatic activity in extracts of control COS-1 cells, and COS-1 cells expressing M1 GFP-iPLA₂γ WT or M4 GFP-iPLA₂γ. Using 2-arachidonoyl phosphatidylcholine as substrate, M1 GFP-iPLA₂γ WT activity was significantly greater than control. M4 GFP-iPLA₂γ
also demonstrated significant activity (Fig. 3D). Thus, M4 GFP-iPLA$_{2\gamma}$ is active in vitro, but not in intact cells.

In the next set of experiments, we employed GEC stably transfected with M4 GFP- iPLA$_{2\gamma}$ (Fig. 3C) to examine the localization. M4 GFP-iPLA$_{2\gamma}$ was found mainly in the cytosol, and only a minor portion was co-localized with calnexin (Fig. 2I-L). Some cells contained what appeared to be aggregates of M4 GFP-iPLA$_{2\gamma}$ (Fig. 2I). M4 GFP-iPLA$_{2\gamma}$ did not co-localize with Mitotracker red (Fig. 2M-P). Thus, deletion of the N-terminal region of iPLA$_{2\gamma}$ leads to mislocalization of the enzyme away from the ER and mitochondria. Taken together, M4 GFP-iPLA$_{2\gamma}$ is enzymatically active, but may not be functional in releasing AA and PGE$_2$ in intact cells due to the mislocalization of the enzyme from the membrane containing the substrate.

**Complement induces release of PGE$_2$ in GEC that overexpress iPLA$_{2\gamma}$.** A previous study demonstrated that in GEC, complement can induce release of [$^3$H]AA via activation of iPLA$_{2\gamma}$ (1). Consistent with the previous results, when GEC-Neo were incubated with antibody and sublytic NS (to form C5b-9) or HIS in controls, PGE$_2$ production increased significantly, which was inhibited by R-BEL, a specific inhibitor of iPLA$_{2\gamma}$ (Fig. 4A). Next, we compared PGE$_2$ release in M1 GEC-iPLA$_{2\gamma}$ and GEC-Neo. Overexpression of M1 GFP-iPLA$_{2\gamma}$ WT did not affect the basal PGE$_2$ production (during incubation with HIS). However, after incubation with complement, PGE$_2$ release was significantly amplified in M1 GEC-iPLA$_{2\gamma}$, compared with GEC-Neo (Fig. 4B), and the complement-mediated PGE$_2$ release in M1 GEC-iPLA$_{2\gamma}$ was almost completely inhibited by BEL (racemic mixture, non-selective inhibitor of iPLA$_{2\beta}$ and $\gamma$) (Fig. 4B), in keeping with earlier results (1). Therefore, activation of complement is coupled with stimulation of iPLA$_{2\gamma}$ activity. To verify that the PGE$_2$ release produced by antibody and NS was actually due to formation of C5b-9, antibody-sensitized M1 GEC-iPLA$_{2\gamma}$ were exposed to C8-deficient serum or C8-deficient serum reconstituted with C8. C8-deficient serum alone had no significant effect on PGE$_2$
production, while C8-deficient serum reconstituted with C8 increased PGE$_2$ release significantly (Fig. 4C).

We also tested the effect of complement on PGE$_2$ release in the GEC stably expressing M4 GFP-iPLA$_{2\gamma}$. In contrast to M1 GEC-iPLA$_{2\gamma}$, the effect of complement on PGE$_2$ production in the M4-expressing cells was not significantly different from GEC-Neo (Fig. 4D). The result indicates that M4 GFP-iPLA$_{2\gamma}$ is less active in the cell, and is in keeping with the experiments involving transient transfection of the M1 and M4 forms of iPLA$_{2\gamma}$ in COS-1 cells (Fig. 3B).

**Role of MAPKs in iPLA$_{2\gamma}$ activation.** The C5b-9 complex is assembled in the plasma membranes of cells, while iPLA$_{2\gamma}$ is localized at the ER and mitochondria, i.e. at organelles generally separated from the plasma membrane. It is therefore unlikely that C5b-9 interacts with iPLA$_{2\gamma}$ directly. Instead, it is reasonable to propose that C5b-9 may activate iPLA$_{2\gamma}$ via intermediary signals. C5b-9 has been shown to increase the cytosolic Ca$^{2+}$ concentration, and activate MAPKs, including ERK, JNK and p38 (131). In the next series of experiments, we examined if complement-induced activation of iPLA$_{2\gamma}$ was mediated through these MAPK pathways. We employed several MAPK pathway inhibitors, including the MEK1 inhibitor, PD98059, the p38 inhibitor, SB203580, and the JNK inhibitor, SP600125 (63). M1 GEC-iPLA$_{2\gamma}$ were preincubated with each inhibitor, and were then incubated with antibody and complement (Fig. 5A). The complement-induced release of PGE$_2$ was inhibited significantly by SB203580 and PD98059. SP600125 tended to decrease the complement-mediated production of PGE$_2$, but the effect was not significant. PD98059 and SB203580 were reported to cross-react and inhibit COX1 and COX2 activities (135). We therefore tested FR167653 and U0126, inhibitors of p38 and MEK1, respectively, which do not inhibit COX1 and COX2 (136), and are structurally distinct from SB203580 and PD98059. Both FR167653 and U0126 inhibited the complement-mediated production of PGE$_2$ (Fig. 5B), confirming a role for p38 and ERK pathways in iPLA$_{2\gamma}$ activation by complement.
**MAPKs enhance iPLA$_2\gamma$ activity in COS-1 cells.** To confirm the role of MAPK pathways in the regulation of iPLA$_2\gamma$ activity, we transiently co-transfected COS-1 cells with M1 GFP-iPLA$_2\gamma$ WT, COX1 and constitutively active mutants of MEK1 (kinase upstream of ERK), MEKK1 (kinase upstream of JNK and possibly p38), and MKK3 (kinase upstream of p38). All three constitutively active mutants enhanced PGE$_2$ production, compared with control (vector) (Fig. 6).

The expression and function of the constitutively active mutants were evaluated in the same sets of experiments. Constitutively active MEK1 migrated slightly faster than the endogenous MEK1 (45 kDa) (Fig. 7A). ERK phosphorylation (which reflects ERK activation) was increased 1.7-fold by the constitutively active MEK1 (Fig. 7A and B), confirming functional activity. Constitutively active MEKK1 was expressed as a protein of ~35 kDa (Fig. 7C), and it stimulated phosphorylation of JNK as expected by ~3-fold (Fig. 7C and D). In addition, the constitutively active MEKK1 activated the ERK pathway by 2.5-fold (Fig. 7B), and showed modest, but significant activation of the p38 pathway (Fig. 7E and F), consistent with earlier studies showing that when overexpressed, the MEKK1 mutant can activate ERK and p38 (62). Unexpectedly, we could not detect phosphorylation of p38 after transfection of the constitutively active mutant of MKK3 (Fig. 7E and F), even though MKK3 increased PGE$_2$ release (Fig. 6). Possibly, phosphorylation of p38 was very transient, limiting its detectability. In summary, the results support a role for ERK in the activation of iPLA$_2\gamma$, as constitutively active protein kinases that stimulated ERK phosphorylation also stimulated iPLA$_2\gamma$ activity, and complement-stimulated iPLA$_2\gamma$ activity was blocked by ERK pathway-directed inhibitors. By analogy, p38 may also stimulate iPLA$_2\gamma$, as constitutively active MEKK1 stimulated iPLA$_2\gamma$ and p38 phosphorylation, and complement-stimulated iPLA$_2\gamma$ activity was blocked by p38 inhibitors. However, the role of MKK3 could not be established definitively, and will require additional study. Finally, the results do not support a role for JNK in the activation of iPLA$_2\gamma$. 

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*EGF together with ionomycin enhances iPLA₂γ activity in COS-1 cells.* EGF is a well-known activator of the ERK pathway, and COS-1 cells express abundant EGF receptor. To further substantiate the role of the ERK pathway in the activation of iPLA₂γ, we investigated the effect of EGF on PGE₂ release in COS-1 cells that were transiently transfected with M1 GFP-iPLA₂γ WT (and COX1). Treatment of COS-1 cells with EGF alone did not affect PGE₂ release (Fig. 8A). Although iPLA₂ is Ca²⁺-independent (does not require Ca²⁺ for its catalytic activity), iPLA₂ activity may nonetheless be regulated by Ca²⁺ or a Ca²⁺-dependent factor (137). For this reason, we used the Ca²⁺ ionophore, ionomycin to induce a Ca²⁺ influx. Interestingly, stimulation with EGF in the presence of ionomycin increased PGE₂ release by more than 4-fold, compared with EGF alone, ionomycin alone or untreated (Fig 8A). Furthermore, BEL inhibited iPLA₂γ activity induced by EGF + ionomycin significantly (Fig. 8B). Finally, we confirmed that incubation of COS-1 cells with EGF + ionomycin induced ERK phosphorylation (Fig. 8C). Thus, the effect of ionomycin + EGF on iPLA₂γ activation is analogous to the effect of C5b-9, which also induces a Ca²⁺ influx and activation of ERK.

*Mutations in putative ERK phosphorylation sites do not affect iPLA₂γ activity.* Analysis of the iPLA₂γ protein sequence by the Scansite program (138) suggested that S168 (EKSP amino acid motif) and S271 (PTSP motif) may be ERK phosphorylation sites. Given the substantial evidence for the activation of iPLA₂γ via the ERK pathway, in the next series of the studies, we examined if iPLA₂γ may be a direct target of ERK. We constructed three mutant forms of M1 GFP-iPLA₂γ, including S168A, S271A, and S168A/S271A double mutation, and tested their activities in COS-1 cells. PGE₂ production stimulated by EGF + ionomycin with the iPLA₂γ mutants did not differ from the WT (Fig. 9A), while the expression levels of all constructs were comparable (Fig. 9B). Thus, single or double mutations of putative ERK phosphorylation sites did not affect iPLA₂γ activation by EGF + ionomycin, suggesting that the effect of ERK on the stimulation of iPLA₂γ is indirect.
Mutations in S511 and S515 inhibit iPLA₂γ activation. A recent phosphoproteomic analysis of mitochondrial proteins in murine heart revealed two iPLA₂γ phosphorylation sites, S505 and T509, corresponding to S511 and S515 in human iPLA₂γ (91). The authors suggested that the two phosphorylation sites (RKLGpSDVFpSQNV) may be in the context of MK2, or casein kinase I substrate motifs (91). Based on these results, we constructed a S511A/S515A double mutant form of M1 GFP-iPLA₂γ and tested its activity in COS-1 cells. The in vitro enzymatic activity of M1 GFP-iPLA₂γ S511A/S515A in COS-1 cells tended to be greater, compared with control, but was lower, compared with M1 GFP-iPLA₂γ WT (Fig. 3D). In addition, PGE₂ production stimulated by EGF + ionomycin was attenuated significantly in the COS-1 cells expressing the S511A/S515A double mutant, compared with WT (Fig. 10A), while the expression levels of WT and double mutant were comparable (Fig. 10B). Basal PGE₂ levels in iPLA₂γ WT and S511A/S515A expressing cells appeared comparable (Fig. 10A).

Next, we tested if S511 in iPLA₂γ WT was phosphorylated by EGF + ionomycin stimulation, using an antibody that identifies the R/KXXpS/T motif, corresponding to KLGpS in iPLA₂γ. Phosphorylation of S511 was evident in stimulated cells; in some experiments, faint phosphorylation was detected in unstimulated cells (Fig. 10C). In contrast, the S511A/S515A mutant iPLA₂γ was not phosphorylated by EGF + ionomycin (Fig 10C), confirming that in the WT enzyme, S511 is the relevant phosphorylation site. These results suggest that the iPLA₂γ activation by EGF + ionomycin is mediated by the direct phosphorylation of iPLA₂γ by a kinase, which is downstream of ERK. Phosphorylation of S515 in iPLA₂γ WT was not monitored due to the unavailability of an antibody that identifies the DVFpSQ motif.

In a previous study, we demonstrated that complement induced a robust activation-specific phosphorylation of MK2 (~4-fold above control), and that activation of MK2 was mediated by both ERK and p38 kinase pathways (64).
Indeed, MK2 is regarded primarily as a substrate of p38 kinase, but is also reported to be a substrate of ERK (63,64). Given that S511 and S515 may represent a MK2 phosphorylation motif, we investigated if expression of MK2 would stimulate the activity of iPLA$_2^{\gamma}$ WT. By analogy to constitutively active MEK1 and MEKK1 (as shown in Fig. 6), WT and constitutively active MK2 were expressed with iPLA$_2^{\gamma}$ WT in COS-1 cells. Despite robust expression, neither WT nor constitutively active MK2 stimulated PGE$_2$ production consistently (data not shown).

Another protein kinase that is activated by ERK and p38 is MNK1 (63). To determine if the effect of the ERK pathway on iPLA$_2^{\gamma}$ activation was mediated by MNK1, we expressed GFP-iPLA$_2^{\gamma}$ in COS-1 cells, and examined PGE$_2$ production after stimulation with EGF + ionomycin, in the presence or absence of the MNK1-directed inhibitor, CGP57380 (63). Stimulated PGE$_2$ production was blocked completely by CGP57380 (Table II). CGP57380 was reported to have some inhibitory activity against MEK1 (63); however, we verified that EGF-induced phosphorylation of ERK was not reduced in the presence of CGP57380 (result not shown). Therefore, inhibition of the EGF + ionomycin-induced activation of iPLA$_2^{\gamma}$ was most likely due to the inhibition of MNK1.

To confirm that MNK1 can activate and phosphorylate iPLA$_2^{\gamma}$, COS-1 cells were transfected with WT GFP-iPLA$_2^{\gamma}$ and COX1 in the presence of WT, constitutively active (T332D), or dominant interfering (T2A2) forms of MNK1, or with empty vector. Both MNK1 WT and T332D enhanced PGE$_2$ production, compared with control (vector), whereas MNK1 T2A2 did not show a significant effect (Fig. 11A and B). We tested S511 phosphorylation by constitutively active MNK1 in iPLA$_2^{\gamma}$ WT using the anti-R/KXXpS/T antibody. Phosphorylation of S511 was enhanced when COS-1 cells were cotransfected with MNK1 T332D, compared with empty vector (Fig. 11C and D). Together, these results support the view that phosphorylation of iPLA$_2^{\gamma}$ on S511 in response to EGF stimulation is mediated by MNK1.
**Complement induces phosphorylation of iPLA₂γ on S511.** In these experiments, we assessed if complement-mediated activation of iPLA₂γ involves phosphorylation. First, we tested if S511 in iPLA₂γ WT is phosphorylated by complement in GEC, using the anti-R/KXXpS/T antibody. Phosphorylation of S511 was stimulated in complement-treated GEC (NS), compared with control (HIS; Fig. 12A). In some experiments, ionomycin was included together with NS and HIS incubations; however, ionomycin did not modulate S511 phosphorylation neither in complement-treated cells nor control (Fig. 12A). Since complement increases the cytosolic Ca²⁺ concentration (1,101), ionomycin would not be expected to provide any additional stimulatory effect. Second, we compared complement-induced PGE₂ release in GEC expressing M1 GFP-iPLA₂γ WT, or GFP-iPLA₂γ S511A/S515A, and GEC-Neo. Overexpression of M1 GFP-iPLA₂γ WT or the S511A/S515A mutant did not affect the basal PGE₂ production (during incubation with HIS). After incubation with complement (NS), PGE₂ release was significantly amplified in cells expressing M1 GFP-iPLA₂γ, compared with GEC-Neo (Fig. 12B), in keeping with previous experiments (Fig. 4B and D). PGE₂ release in complement-treated cells expressing the S511A/S515A mutant was significantly lower, compared with WT (Fig. 12B), despite comparable levels of expression (Fig. 12C). Together, these results indicate that complement is, at least in part, activating iPLA₂γ via direct phosphorylation on S511, most likely by MNK1.

**DISCUSSION**

The present study demonstrates that complement C5b-9 activates endogenous and ectopic iPLA₂γ (Fig. 4). Activation of iPLA₂γ occurs via ERK and p38 pathways (Fig. 5-9), and is dependent on an increase in cytosolic Ca²⁺ concentration and phosphorylation of the enzyme on S511 and/or S515, most likely via MNK1 (Fig. 11, Table II). To our knowledge, this is the first demonstration of phosphorylation-dependent activation of iPLA₂γ. Stimulation of iPLA₂γ by complement was coupled with production of PGE₂. Similar to this result in the
In the present study, we examined the cellular localization of iPLA$_{2\gamma}$ to better understand the mechanisms by which iPLA$_{2\gamma}$ hydrolyzes membrane phospholipids. M1 GFP-iPLA$_{2\gamma}$ WT was found mainly in the perinuclear region and co-localized with markers of the ER and mitochondria (Fig. 2). Deletion of the 220 amino acid N-terminal region (M4 GFP-iPLA$_{2\gamma}$) altered the localization of the enzyme, such that a significant portion of M4 GFP-iPLA$_{2\gamma}$ shifted to the cytosol (Fig. 2). Our result is in keeping with the study of Tanaka et al. (90), which showed that deletion of a 362 amino acid N-terminal region of iPLA$_{2\gamma}$ caused a portion of the enzyme to shift from the membrane to the cytosol in a cell fractionation study. Moreover, the N-terminal fragment localized solely in the membrane, but not in the cytosolic fraction (90). Deletion of the N-terminal region of iPLA$_{2\gamma}$ did not abolish iPLA$_{2\gamma}$ enzymatic activity in vitro (Fig. 3D), consistent with the study by Tanaka et al. (90). However, the N-terminal truncated form of iPLA$_{2\gamma}$ was unable to induce significant PGE$_2$ production when expressed in cells (Fig. 3). Taken together, these results indicate that the N-terminal region is involved in the membrane association of iPLA$_{2\gamma}$, thereby allowing the enzyme access to phospholipid substrate intracellularly. In addition, the N-terminal region may have positive regulatory elements that could enhance iPLA$_{2\gamma}$ activity. Mislocalization of M4 GFP-iPLA$_{2\gamma}$ from the membrane may be expected to reduce PGE$_2$ production, as COX1 is localized in the ER membrane, and would couple with iPLA$_{2\gamma}$-mediated AA release (121).

In previous studies, it was demonstrated that C5b-9 can activate cPLA$_{2\alpha}$ to augment production of [$^3$H]AA and prostanoids (9,53). Complement did not stimulate group IIA secreted PLA$_2$, and in contrast to iPLA$_{2\gamma}$, overexpression of iPLA$_{2\beta}$ in GEC did not amplify complement-dependent release of [$^3$H]AA.
Thus, both cPLA$_2$$^\alpha$ and iPLA$_2$$^\gamma$ can contribute to complement-dependent release of AA. Previous studies in GEC demonstrated that complement induced an increase in cPLA$_2$$^\alpha$ catalytic activity, in association with S505 phosphorylation, although this phosphorylation was not essential for cPLA$_2$$^\alpha$ activation (9,53). In addition, glomerular cPLA$_2$$^\alpha$ was phosphorylated in vivo, in passive Heymann nephritis (64).

By analogy to cPLA$_2$$^\alpha$, complement activated iPLA$_2$$^\gamma$ through intermediate signals, including protein kinases. C5b-9 can activate MAPK pathways in GEC (9,53). In GEC overexpressing GFP-iPLA$_2$$^\gamma$ WT, the complement-induced release of PGE$_2$ was blocked by two distinct chemical inhibitors of both the ERK and p38 pathways, but not JNK (Fig. 5). Conversely, constitutively active MAPK pathway mutants (in particular MEK and MEKK1) increased iPLA$_2$$^\gamma$-dependent PGE$_2$ production (Fig. 6). Both MEK and MEKK1 induced activation-specific phosphorylation of ERK (Fig. 7). Taken together, the ERK and p38 pathways can mediate the activation of iPLA$_2$$^\gamma$ by complement. A role for MAPKs in the activation of iPLA$_2$ has been reported previously. Thrombin stimulated both ERK and p38, and iPLA$_2$ activity in vascular smooth muscle cells and ventricular myocytes (139,140). In mouse neural cells, p38 facilitated iPLA$_2$ activity during hypoxia (141). These studies did not, however, differentiate between iPLA$_2$$^\beta$ and iPLA$_2$$^\gamma$. To further substantiate the role of the ERK pathway in the activation of iPLA$_2$$^\gamma$, we showed that EGF + ionomycin stimulated PGE$_2$ release in COS-1 cells expressing GFP-iPLA$_2$$^\gamma$ WT, in association with ERK activation (Fig. 8 and 9). Interestingly, unlike the constitutively active mutants of MEK1, MKK3, or MEKK1, the stimulatory effect of EGF in cells required addition of ionomycin (to increase the cytosolic Ca$^{2+}$ concentration), despite the Ca$^{2+}$-independent catalytic properties of iPLA$_2$$^\gamma$ in vitro. In keeping with previous reports, this finding suggests that activation of iPLA$_2$$^\gamma$ in agonist-stimulated cells may involve a Ca$^{2+}$ regulated process (82,83), possibly activation of Ca$^{2+}$-dependent protein kinases, such as calmodulin (92). Alternatively, Ca$^{2+}$ may enhance activation of iPLA$_2$$^\gamma$ directly. A recent study showed that divalent cations (Ca$^{2+}$ or Mg$^{2+}$) can activate
iPLA2γ in heart mitochondria, leading to release of eicosanoids and lysolipids, possibly by facilitating mitochondrial phospholipid hydrolysis by iPLA2γ (93).

Tanaka et al. (83) suggested that iPLA2γ may have multiple potential phosphorylation sites. We carried out a mutagenesis analysis of iPLA2γ to determine the regulation of iPLA2γ activity by the ERK pathway. Mutation of two putative ERK phosphorylation sites, i.e. S168A, S271A, and S168A/S271A double mutation did not abrogate the stimulated activity of iPLA2γ WT (Fig. 9), implying that iPLA2γ was not a direct target of ERK. Another report suggested that the MAPK pathway might be involved in iPLA2γ activation indirectly, but no supporting data were presented (121). Based on a phosphoproteomic analysis of murine cardiac mitochondrial proteins (91), we then mutated S511 and S515 to Ala, and showed that this double mutation significantly attenuated EGF + ionomycin-, as well as complement-stimulated iPLA2γ-dependent PGE2 production (Fig. 10 and 12). Moreover, phosphorylation of S511 was induced by EGF + ionomycin and by complement (Fig. 10 and 12). Phosphorylation of S511 and/or S515 could induce a conformational change in the enzyme, leading to an increase in catalytic activity. Further studies will be required to define the mechanism more precisely.

The S511 and S515 phosphorylation sites were proposed to be a phosphorylation motif for MK2 (91), and given that complement was shown to activate MK2 via the ERK or p38 pathway (64), it was reasonable to examine if MK2 may be the kinase downstream of ERK, which phosphorylates iPLA2γ. However, we were not able to show stimulation of iPLA2γ following MK2 overexpression. The protein kinase MNK1 is also activated by ERK and p38, and the amino acid sequence preceding S511 in iPLA2γ (KLGS) resembles the MNK1 phosphorylation motif in eukaryotic translation initiation factor 4E (KSGS) (63). In the present study, the EGF + ionomycin-stimulated activity of iPLA2γ was blocked by a MNK1-directed inhibitor (Table II). Expression of WT and constitutively active MNK1 stimulated PGE2 release via iPLA2γ, and constitutively active MNK1 enhanced S511 phosphorylation (Fig. 11). Thus, activation of iPLA2γ by complement most
likely involves an ERK-MNK1 pathway, although an additional role of another kinase downstream of ERK remains a possibility. Interestingly, a previous study has shown that phosphorylation and activation of cPLA$_2$ can occur via p38 and MNK1(142).

Various PLA$_2$ enzymes have been shown to regulate pathways leading to cell injury in experimental disease models. These effects may be associated with generation of prostanoids (103), p38 activation (143), and induction of ER stress (105). There are both cytoprotective and injurious consequences related to the complement-mediated activation of PLA$_2$ enzymes and production of prostanoids (1,53). Overexpression of iPLA$_2$$\gamma$ attenuated complement-mediated injury in cultured GEC, and the cytoprotective effect was, in part, mediated through prostaglandin production (1). Further studies will be required to determine if activation of iPLA$_2$$\gamma$ is cytoprotective in C5b-9-mediated GEC in vivo, i.e. in attenuating development of proteinuria in experimental membranous nephropathy. Another potential mechanism of iPLA$_2$$\gamma$ cytoprotection may be related to the localization and action of iPLA$_2$$\gamma$ at the ER (Fig. 2). Such actions could include changes in ER membrane lipid composition, alterations in ER Ca$^{2+}$ transporters, or modification of ER Ca$^{2+}$ stores. Moreover, iPLA$_2$$\gamma$ could perturb the ER membrane to initiate an adaptive ER stress response as a feedback mechanism to limit complement-induced cell injury.

In GEC, a portion of iPLA$_2$$\gamma$ was localized at the mitochondria (Fig. 2). iPLA$_2$$\gamma$ may protect renal cortical mitochondria from oxidant-induce lipid peroxidation and dysfunction (134). Thus, in the presence of oxidized phospholipid acyl chains, iPLA$_2$$\gamma$ may hydrolyze damaged acyl chains, and allow for reesterification with normal fatty acids, thereby repairing mitochondrial membrane phospholipids. So far, we have not conclusively defined the site of phospholipid hydrolysis by complement-stimulated iPLA$_2$$\gamma$. Both ER and mitochondrial lipids in GEC contain AA (144); however, since COX isoenzymes are localized at the ER and the nuclear membranes, but not at the mitochondria, the production of
PGE_2 suggests a coupling of AA release with COX at the ER. Definition of the subcellular sites of phospholipid hydrolysis and the functional role, including the cytoprotective mechanisms of iPLA_2γ will require further investigation.

FOOTNOTES

* This work was supported by Research Grants from the Canadian Institutes of Health Research (MOP-53264 to A.V.C., and MOP-53335 to T.T.), and the Kidney Foundation of Canada (to A.V.C.). A.V. Cybulsky was supported by the Catherine McLaughlin Hakim Chair.

1. Abbreviations: AA, arachidonic acid; BEL, bromoenol lactone; COX, cyclooxygenase; cPLA_2, cytosolic phospholipase A_2; DAPI, 4’6-Diamindino 2-phenylindole; EGF, epidermal growth factor; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; GEC, glomerular epithelial cell; GFP, green fluorescent protein; GST, glutathione S-transferase; HIS, heat-inactivated human serum; iPLA_2, calcium-independent phospholipase A_2; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK1, MAP/ERK kinase-1; MEKK1, MAP/ERK kinase kinase-1; MK2, MAPK activated protein kinase-2; MKK3, MAPK kinase-3; MNK1, MAPK-interacting kinase-1; NS, normal human serum; PG, prostaglandin; WT, wild type.
FIGURES:

Figure 1

[Diagram A] COX 1 and PGE$_2$ levels in GEC and COS-1 cells transfected with GFP-rac and GFP-iPLA$_{2y}$

[Diagram B] Effect of COX 1 on PGE$_2$ levels in iPLA$_{2y}$ transfected cells

[Diagram C] Comparison of PGE$_2$ levels between different groups

* indicates p < 0.05
** indicates p < 0.01
Figure 1. Expression and activity of M1 GFP-iPLA$_{2\gamma}$ WT. A. GEC were stably transfected and COS-1 cells were transiently transfected with M1 GFP-iPLA$_{2\gamma}$ WT, or a GFP-rac fusion protein for comparison. Lysates were immunoblotted with antibody to GFP. * denotes M1 GFP-iPLA$_{2\gamma}$ in GEC, which migrated slower than M1 GFP-iPLA$_{2\gamma}$ in COS-1 cells, possibly because of differential post-translational modifications in the two cell lines. B. COS-1 cells were transiently transfected with untagged full-length iPLA$_{2\gamma}$, M1 GFP-iPLA$_{2\gamma}$ WT, and COX1. PGE$_2$ production was measured in cell supernatants 24 h after transfection. Both GFP-tagged and untagged enzymes increased PGE$_2$ release. *$P<0.001$ iPLA$_{2\gamma}$ + COX1 and GFP-iPLA$_{2\gamma}$ + COX1 vs untransfected (Untransf) cells, 3 experiments. C. COS-1 cells were untransfected, or were transiently transfected with vector, M1 GFP-iPLA$_{2\gamma}$ WT, COX1, or M1 GFP-iPLA$_{2\gamma}$ WT + COX1. Expression of COX1 alone increased PGE$_2$ release, compared with control, whereas GFP-iPLA$_{2\gamma}$ + COX1 substantially amplified the increase in PGE$_2$ production. This increase was inhibited by coincubation with 30 µM BEL (6 h). *$P<0.001$ GFP-iPLA$_{2\gamma}$ + COX1 vs untransfected or vector, $P<0.001$ GFP-iPLA$_{2\gamma}$ + COX1 vs GFP-iPLA$_{2\gamma}$, $P<0.05$ GFP-iPLA$_{2\gamma}$ + COX1 vs COX1, **$P<0.001$ GFP-iPLA$_{2\gamma}$ + COX1 + BEL vs GFP-iPLA$_{2\gamma}$ + COX1, 7 experiments.
Figure 2. Subcellular localization of M1 GFP-iPLA$_{2}\gamma$ WT and N-terminally truncated GFP-iPLA$_{2}\gamma$ (M4). GEC stably transfected with M1 GFP-iPLA$_{2}\gamma$ WT show predominantly perinuclear green fluorescent staining (A). Cells were labeled with antibody to calnexin (red staining; B) to localize the ER, while nuclei were counterstained with DAPI (blue fluorescence; C). Calnexin staining was perinuclear (B). Panel D shows co-localization of M1 GFP-iPLA$_{2}\gamma$ WT and calnexin (yellow-orange staining). GEC that express M1 GFP-iPLA$_{2}\gamma$ WT (E) were stained Mitotracker red, a marker of the mitochondria (F). Panel G shows co-localization of GFP-iPLA$_{2}\gamma$ with Mitotracker red. GEC expressing M1 GFP-iPLA$_{2}\gamma$ WT were treated with digitonin to permeabilize the plasma membranes (H). Digitonin treatment did not affect the localization of M1 GFP-iPLA$_{2}\gamma$ WT. GEC stably transfected with M4 GFP-iPLA$_{2}\gamma$ mainly show cytoplasmic green fluorescent staining (I and M). There is some minor perinuclear accentuation, and there appear to be aggregates in occasional cells. GEC were stained with anti-calnexin antibody (red staining; J), Mitotracker red (N), and DAPI (blue fluorescence; K and O). Panel L (merge of I-K) shows only minor co-localization of M4 GFP-iPLA$_{2}\gamma$ with calnexin. Panel P (merge of M-O) shows an absence of co-localization of M4 GFP-iPLA$_{2}\gamma$ with Mitotracker red.
Figure 3

A

Untransf | M1 (µg) | M4 (µg)
------- | ------ | ------
    -   | 1.0    | 0.5    |
    -   | 0.7    | 0.5    |
    -   | 0.5    | 0.5    |
    -   | 0.3    | 0.3    |

M1 (115 kDa)  M4 (92 kDa)

- α-Tubulin

B

PGE₂ (µg/ml)

Untransf | M1 (µg) | M4 (µg)
------- | ------ | ------
    -   | 0.0    | 0.0    |
    -   | 0.7    | 0.5    |
    -   | 0.5    | 0.5    |
    -   | 0.3    | 0.3    |

M1 (µg)  M4 (µg)

C

GEC  COS-1

Ne0  M4  Untransf  M1  M4

- M1 (115 kDa)  M4 (92 kDa)
- α-Tubulin

D

iPLA² Activity (Fraction)

Untransf  M1-WT  M1-S511A/S515A  M4

- M1-WT  M1-S511A/S515A  M4

E

Untransf  M1-WT  M1-S511A/S515A  M4

- M1 (115 kDa)  M4 (92 kDa)
- Actin  Calnexin
**Figure 3.** Expression and activity of GFP-iPLA<sub>2γ</sub> WT and mutants. COS-1 cells were co-transfected with N-terminally truncated (M4) GFP-iPLA<sub>2γ</sub> (0.1-0.5 µg plasmid DNA), and for comparison with M1 GFP-iPLA<sub>2γ</sub> WT (0.5-1.0 µg plasmid DNA), both with COX1. A. Anti-GFP antibody immunoblot shows greater expression of M4 GFP-iPLA<sub>2γ</sub>, compared with M1 GFP-iPLA<sub>2γ</sub> WT. Expression tended to increase with increasing doses of plasmid DNA. The lower band in the M1 lanes is nonspecific. B. PGE<sub>2</sub> release in COS-1 cells expressing M4 GFP-iPLA<sub>2γ</sub> or M1 GFP-iPLA<sub>2γ</sub> WT (both with COX1) was normalized for corresponding protein expression. In these experiments, basal PGE<sub>2</sub> release (untransfected cells) was 38.2 pg/ml. PGE<sub>2</sub> production by M1 GFP-iPLA<sub>2γ</sub> WT was markedly greater, compared with M4 GFP-iPLA<sub>2γ</sub> *P<0.0001 M4 + COX1 (0.5, 0.3 and 0.1 µg) vs M1 + COX1 (1, 0.7 and 0.5 µg), 3 experiments. C. GEC were stably transfect and COS-1 cells were transiently transfect with M4 GFP-iPLA<sub>2γ</sub>. GEC-Neo and COS-1 cells transiently transfected with M1 GFP-iPLA<sub>2γ</sub> WT are presented for comparison. Lysates were immunobloted with antibody to GFP. * denotes M4 GFP-iPLA<sub>2γ</sub> in GEC. The bands in GEC-Neo and untransfected COS-1 cells are nonspecific. D. PLA<sub>2</sub> activity in untransfected (control) COS-1 cells, and COS-1 cells expressing M1 GFP-iPLA<sub>2γ</sub> WT, M1 GFP-iPLA<sub>2γ</sub> S511A/S515A double mutant, and M4 GFP-iPLA<sub>2γ</sub>. Cell extracts were prepared 24 h after transfection, and iPLA<sub>2</sub> activity was monitored by release of AA from 2-arachidonoyl-phosphatidylcholine (Experimental Procedures). iPLA<sub>2</sub> activities of M1 GFP-iPLA<sub>2γ</sub> WT and M4 GFP-iPLA<sub>2γ</sub> were significantly greater, compared with control. iPLA<sub>2</sub> activity of M1 GFP-iPLA<sub>2γ</sub> S511A/S515A tended to be greater than control. *P<0.01 M1 GFP-iPLA<sub>2γ</sub> WT vs control, **P<0.05 M4 GFP-iPLA<sub>2γ</sub> WT vs control, 4 experiments. In these experiments, basal iPLA<sub>2</sub> activity (control cells) was 0.69 nmol/min/ml. E. Cell lysates were immunoblotted with antibodies to GFP (iPLA<sub>2γ</sub>), actin, or calnexin (marker of ER).
Figure 4

![Bar charts showing PGE2 levels](image)

- **A**: GEC Neo and GEC GFP-iPLA2γ
- **B**: C8DS and C8DS+C8
- **C**: HIS, NS, C8DS, and C8DS+C8
- **D**: HIS and NS with Neo, M1, and M4 treatments
Figure 4. Complement induces production of PGE₂ via iPLA₂γ. A. Role of endogenous iPLA₂γ. Neo GEC were incubated with anti-GEC antiserum for 30 min at 22°C in the presence or absence of the iPLA₂γ-directed inhibitor R-BEL (10 µM). Cells were then incubated at 37°C with 2% NS (to form C5b-9) or HIS in controls, with or without R-BEL, for 40 min. Then, PGE₂ production was measured in cell supernatants. Complement stimulated PGE₂ production, and the increase was significantly attenuated by R-BEL. *P<0.0001 NS vs HIS and P<0.001 NS vs NS/R-BEL. 3 experiments. B. Complement-induced production of PGE₂ is amplified in GEC that overexpress M1 GFP-iPLA₂γ WT (GEC GFP-iPLA₂γ). GEC that express M1 GFP-iPLA₂γ WT were incubated with antibody and complement, with or without BEL, as above. M1 GFP-iPLA₂γ WT markedly amplified complement-induced PGE₂ production, and the increase was attenuated by BEL (30 µM). *P<0.001 GEC-GFP-iPLA₂γ (NS) vs GEC-Neo (NS), P<0.001 GEC-GFP-iPLA₂γ (NS/BEL) vs GEC-GFP-iPLA₂γ (NS), 3 experiments. C. PGE₂ production is dependent on C5b-9 assembly. GEC that express M1 GFP-iPLA₂γ WT were incubated with antibody and C8-deficient serum (C8DS) with or without purified C8. When C8DS was reconstituted with C8, PGE₂ production amplified significantly. *P<0.0001 NS vs HIS and **P<0.001 C8DS+C8 vs C8DS, 3 experiments. D. M4 GFP-iPLA₂γ is inactive in intact GEC. Neo GEC, or GEC that stably express M1 GFP-iPLA₂γ WT or M4 GFP-iPLA₂γ were incubated with antibody and complement, as above. *P<0.001 GEC-M1 GFP-iPLA₂γ WT (NS) vs GEC-Neo (NS) and P<0.001 GEC-M1 GFP-iPLA₂γ WT (NS) vs GEC-M4 GFP-iPLA₂γ (NS), 3 experiments.
Figure 5

A

![Bar graph A showing PGE2 levels with vehicle, SB203580, SP600125, and PD98059 treatments.](image)

B

![Bar graph B showing PGE2 levels with vehicle, FR167653, and U0126 treatments.](image)
Figure 5. Complement-induced PGE$_2$ production in GEC that stably express M1 GFP-iPLA$_{2\gamma}$ WT is mediated by ERK and p38. A. GEC were incubated with antibody and complement (as in Fig. 4) in the presence of inhibitors of p38 (SB203580; 10 µM), MEK1 (PD98059; 50 µM), or JNK (SP600125; 10 µM). Complement-induced PGE$_2$ production was reduced significantly by the p38 and MEK1 inhibitors. *$P<0.0001$ NS vs HIS (vehicle), **$P<0.01$ SB203580 vs vehicle (NS), +$P<0.001$ PD98059 vs vehicle (NS), 6 experiments. B. GEC were incubated with antibody and complement in the presence of inhibitors of p38 (FR167653; 10 µM) and MEK1 (U0126; 50 µM). Complement-induced PGE$_2$ production was reduced significantly by both drugs. *$P<0.0001$ NS vs HIS (vehicle), **$P<0.001$ FR167653 vs vehicle (NS), +$P<0.0001$ U0126 vs vehicle (NS), 4 experiments.
Figure 6. Activation of MAPK pathways stimulates iPLA$_{2\gamma}$-mediated PGE$_2$ production. COS-1 cells were co-transfected with M1 GFP-iPLA$_{2\gamma}$ WT, COX1, and constitutively active mutants of MEK1, MKK3, or MEKK1, or with empty vector. Untransfected cells are additional controls. PGE$_2$ release was measured 48 hours after transfection. *$P<0.01$, **$P<0.0001$ vs vector, 3 experiments.
Figure 7
Figure 7. Effects of constitutively active kinases on ERK, JNK, and p38 phosphorylation. COS-1 cells were co-transfected with M1 GFP-iPLA₂γ WT, COX1 and constitutively active mutants of MEK1, M KK3, and MEKK1, or with empty vector. A. Lysates were immunoblotted with antibodies to MEK1 (upper panel) or phospho-ERK (pERK; lower panel). Endogenous MEK1 is present in all samples; constitutively active MEK1 (R4F-MEK1) migrates ahead of endogenous MEK1 (MEK1; 45 kDa). Both constitutively active MEK1 and MEKK1 stimulated ERK phosphorylation. B. Densitometric quantification of pERK. *P<0.01 MEK1 vs vector, **P<0.0001 MEKK1 vs vector, 3 experiments. C. Lysates were immunoblotted with antibodies to MEKK1 (upper and middle panels) or phospho-JNK (pJNK; lower panel). Endogenous MEKK1 is present in all samples; constitutively active MEKK1 migrates at 35 kDa. Constitutively active MEKK1 stimulated JNK phosphorylation. D. Densitometric quantification of pJNK. *P<0.0001 MEKK1 vs vector, 3 experiments. E. Lysates were immunoblotted with antibodies to phospho-p38 (pp38; upper panel) or tubulin (lower panel). Constitutively active MEKK1 stimulated p38 phosphorylation. F. Densitometric quantification of pp38. *P<0.001 MEKK1 vs vector, 3 experiments.
Figure 8

A

B

C

Figure 8
**Figure 8.** EGF together with ionomycin enhances iPLA$_{2\gamma}$ activity. COS-1 cells were co-transfected with M1 GFP-iPLA$_{2\gamma}$ WT and COX1. After 24 h, COS-1 cells were incubated with EGF (100 ng/ml) and/or ionomycin (Iono, 1.0-1.5 µM) for 40 min. A. Stimulation by EGF and ionomycin significantly increased PGE$_2$ release, compared with unstimulated cells or cells stimulated with each agonist alone. *$P<0.001$ EGF + Iono vs untreated, $P<0.05$ EGF + Iono vs Iono (alone), $P<0.001$ EGF + Iono vs EGF (alone), 4 experiments. B. COS-1 cells were co-transfected with M1 GFP-iPLA$_{2\gamma}$ WT and COX1. After 24 h, one group of cells was preincubated with BEL (30 µM) for 6.5 h. Then, cells were incubated for 30 min with EGF and ionomycin, with or without BEL. The increase of PGE$_2$ release was inhibited almost completely in presence of BEL. *$P<0.01$ EGF + Iono vs untreated *$P<0.01$ EGF + Iono vs EGF + Iono + BEL, 3 experiments. C. Cell lysates were immunoblotted with antibodies to phospho-ERK (pERK) or tubulin. EGF and ionomycin enhanced phosphorylation of ERK.
Figure 9
Figure 9. Mutations in putative ERK phosphorylation sites do not affect iPLA$_2^\gamma$ activity. COS-1 cells were transiently transfected with M1-GFP-iPLA$_2^\gamma$ WT, the S168A and S271A mutants, or the S168A/S271A double mutant, together with COX1. A. After 24 h, cells were untreated, or were incubated with EGF (100 ng/ml) + ionomycin (Iono, 1.5 µM) for 40 min. PGE$_2$ release was stimulated significantly by EGF + ionomycin in the cells expressing M1 GFP-iPLA$_2^\gamma$ WT and all mutants.*P<0.05, **P<0.01 vs corresponding untreated groups, 7 experiments. In these experiments, basal PGE$_2$ release (vector + COX1-transfected, untreated cells) was 827 pg/ml. B. Cell lysates were immunoblotted with antibodies to GFP or tubulin. The blot shows comparable levels of expression.
Figure 10
**Figure 10.** The S511A/S515A double mutation inhibits stimulated iPLA$_2$γ activity. COS-1 cells were transiently transfected with M1-GFP-iPLA$_2$γ WT, GFP-iPLA$_2$γ S511A/S515A mutant or empty vector, together with COX1. A. After 24 h, cells were untreated, or were incubated with EGF (100 ng/ml) + ionomycin (Iono, 1.5 µM) for 40 min. PGE$_2$ release was stimulated significantly by EGF + ionomycin in the cells expressing M1 GFP-iPLA$_2$γ WT, *P<0.0001 vs corresponding untreated group. The increase in PGE$_2$ release was smaller in cells expressing GFP-iPLA$_2$γ S511A/S515A mutant, **P<0.01 vs cells expressing M1 GFP-iPLA$_2$γ WT and treated with EGF + Iono, 4 experiments. In these experiments, basal PGE$_2$ release (vector + COX1-transfected, untreated cells) was 191 pg/ml. B. Cell lysates were immunoblotted with antibodies to GFP or tubulin. The blot shows comparable levels of expression. C. Cell lysates were immunoprecipitated with anti-GFP antibody (+), and were immunoblotted with anti-R/KXXpS/T or anti-GFP antibodies. The blot shows enhanced phosphorylation of S511 (pS511) in the M1-GFP-iPLA$_2$γ WT in EGF + Iono-stimulated cells. Phosphorylation of S511 is faint in unstimulated WT-expressing cells, and is absent in the mutant. D. Total lysates of the above immunoprecipitation experiments blotted with anti-GFP antibody.
Figure 11
**Figure 11.** Constitutively active MNK1 activates and phosphorylates iPLA$_2$$\gamma$. COS-1 cells were transiently co-transfected with M1 GFP-iPLA$_2$$\gamma$ WT, COX1 and GST-MNK1 WT, GST-MNK1 T332D, GST-MNK1 T2A2, or with empty vector. A. PGE$_2$ release was measured 48 hours after transfection. *P<0.05 MNK1 WT vs vector, *P<0.01 MNK1 T332D vs vector and **P<0.05 MNK1 T332D vs MNK1 T2A2, 5 experiments. In these experiments, basal PGE$_2$ release (vector + M1 GFP-iPLA$_2$$\gamma$ WT + COX1-transfected cells) was 164 pg/ml. B. Lysates were immunoblotted with antibodies to GFP, GST or actin. The blot shows comparable levels of expression. C. COS-1 cells were transiently co-transfected with M1 GFP-iPLA$_2$$\gamma$ WT and GST-MNK1 T332D or vector. After 48 h, cells were treated with ionomycin (10 µM, 40 min). (Ionomycin was included in these experiments to enhance the phosphorylation signal, although ionomycin did not independently induce phosphorylation; see Fig. 12, below). Cell lysates were immunoprecipitated with anti-GFP antibody (+), or nonimmune IgG in controls (-), and were immunoblotted with anti-R/KXXpS/T or anti-GFP antibodies. The blot shows enhanced phosphorylation of iPLA$_2$$\gamma$ S511 (pS511) in MNK1 T332D transfected cells. D. Total lysates of the above immunoprecipitation experiments blotted with anti-GFP or anti-GST (MNK1) antibodies.
Figure 12

A

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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pS511</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>GFP-iPLA2γ</td>
<td>+</td>
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B

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C

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<th>WT</th>
<th>S511A/S515A</th>
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</tr>
<tr>
<td>Actin -</td>
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**Figure 12.** Complement induces phosphorylation of iPLA$_{2\gamma}$ on S511. GEC were transiently transfected with M1 GFP-iPLA$_{2\gamma}$ WT. After 24 h, cells were incubated with ionomycin (Iono, 5 µM) for 30 min (+), and then incubated with antibody and NS (3%, 40 min), or HIS in controls, A. Cell lysates were immunoprecipitated with anti-GFP antibody (+), and were immunoblotted with anti-R/KXXpS/T or anti-GFP antibodies. The blot shows phosphorylation of S511 (pS511) in NS-stimulated cells (with or without ionomycin). Phosphorylation is absent in the HIS-stimulated cells. B. GEC neo and GEC that express M1 GFP-iPLA$_{2\gamma}$ WT or GFP-iPLA$_{2\gamma}$ S511A/S515A (transient transfection) were incubated with antibody and complement, as above. PGE$_2$ production was amplified in NS-stimulated M1 GFP-iPLA$_{2\gamma}$ WT expressing cells, whereas the amplification was smaller in GEC expressing the double mutant. *$P<0.001$ M1 GFP-iPLA$_{2\gamma}$ WT (NS) vs GEC-Neo (NS) and **$P<0.05$ M1 GFP-iPLA$_{2\gamma}$ WT (NS) vs GFP-iPLA$_{2\gamma}$ S511A/S515A (NS), 3 experiments. C. Cell lysates were immunoblotted with antibodies to GFP or actin. The blot shows comparable levels of GFP-iPLA$_{2\gamma}$ expression.
**Tables:**

**Table I.** PCR primers employed to construct iPLA$_{2\gamma}$ mutants.

Bold letters denote base substitutions encoding for mutated amino acids.

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<thead>
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<th>GFP-iPLA$_{2\gamma}$</th>
<th>Primer</th>
<th>Primer sequence (5' to 3')</th>
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<td>F2</td>
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<td>M4-R1</td>
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Table II. Effect of the MNK1 inhibitor, CGP57380, on PGE$_2$ production.

COS-1 cells were co-transfected with M1 GFP-iPLA$_{2\gamma}$ WT and COX1. After 24 h, some cells were treated with CGP57380 (20 µM) for 16 h. Then, cells were incubated with EGF + ionomycin (see legend to Fig. 8) for 40 min. Stimulated PGE$_2$ production was inhibited by CGP57380. *$P<0.001$ EGF + ionomycin + CGP57380 vs EGF + ionomycin, 3 experiments. Basal PGE$_2$ release (untransfected, untreated cells) was 51 pg/ml.

<table>
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<td>GFP-iPLA$_{2\gamma}$ + COX, untreated</td>
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* $p<0.001$ EGF + ionomycin + CGP57380 vs. EGF + ionomycin, three experiments.
Preface to Chapter 3
Membranous nephropathy and anti-GBM nephritis are complement-mediated diseases, which are associated with ER stress (9). Overexpression of iPLA$_2\gamma$ induced cytoprotection against complement-dependent GEC injury (1). We showed that complement-mediated activation of iPLA$_2\gamma$ is mediated via ERK and p38 pathways, and phosphorylation of S511 and/or S515 plays a key role in the catalytic activity and signaling of iPLA$_2\gamma$ (Chapter 2). iPLA$_2\gamma$ is reported to localize at the ER (86). Therefore, the second aim was to address the role of iPLA$_2\gamma$ in ER stress, specifically, if the cytoprotective effect of iPLA$_2\gamma$ involves the UPR (Chapter 3). Modulation of ER stress is potentially a novel target for therapy of glomerular diseases.
CHAPTER 3: SUBMITTED MANUSCRIPT
ROLE OF CALCIUM-INDEPENDENT PHOSPHOLIPASE A$_2$$\gamma$ IN ENDOPLASMIC RETICULUM STRESS: ACTIVATION OF ATF6

Hanan Elimam, Joan Papillon, Tomoko Takano, and Andrey V. Cybulsky

Department of Medicine, McGill University Health Centre, McGill University, Montreal, Quebec, Canada

Running title: Activation of ATF6 by calcium-independent phospholipase A$_2$$\gamma$

Address for correspondence: Andrey V. Cybulsky, MD, Division of Nephrology, Royal Victoria Hospital, 687 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1. Tel: 1-514-398-8148. Fax: 1-514-843-2815. E-mail: andrey.cybulsky@mcgill.ca; Tomoko Takano, MD, PhD, Division of Nephrology, McGill University, 3775 University Street, Rm 236, Montreal, Quebec, Canada H3A2B4. Tel: 1-514-398-2171, Fax: 1-514-843-2815. E-mail: tomoko.takano@mcgill.ca
ABSTRACT

Injury of visceral glomerular epithelial cells (GECs) causes proteinuria in many glomerular diseases. We reported previously that calcium-independent phospholipase A2γ (iPLA2γ) is cytoprotective against complement-mediated GEC injury. Since iPLA2γ is localized at the endoplasmic reticulum (ER), the present study addressed whether the cytoprotective effect of iPLA2γ involves the ER stress unfolded protein response (UPR). In cultured rat GECs, overexpression of the full-length iPLA2γ, but not a mutant iPLA2γ that fails to associate with the ER, augmented tunicamycin-induced activation of activating transcription factor-6 (ATF6) and induction of the ER chaperones, glucose regulated protein 94 (grp94) and grp78. Augmented responses were inhibited by the iPLA2γ inhibitor, R-bromoenol lactone (R-BEL), but not by the cyclooxygenase inhibitor, indomethacin. Tunicamycin-induced cytotoxicity was reduced in GECs expressing iPLA2γ, and the cytoprotection was reversed by dominant-negative ATF6. GECs from iPLA2γ knockout mice showed blunted ATF6 activation and chaperone upregulation in response to tunicamycin. Unlike ATF6, the two other UPR pathways i.e. inositol-requiring enzyme 1α and protein kinase RNA-like ER kinase pathways were not affected by iPLA2γ. Thus, in GECs, iPLA2γ amplified activation of the ATF6 pathway of the UPR, resulting in upregulation of ER chaperones and cytoprotection. These effects were dependent on iPLA2γ catalytic activity and association with the ER, but not on prostanoids. Modulating iPLA2γ activity may provide opportunities for pharmacological intervention in glomerular diseases associated with ER stress.
INTRODUCTION

Phospholipases A₂ (PLA₂) play an important role in numerous cellular processes. PLA₂ comprises a family of enzymes that hydrolyze the acyl bond at the sn-2 position of phospholipids to yield free fatty acids, such as arachidonic acid (AA), and lysophospholipids (67,68). Both products represent precursors for signaling molecules that can exert multiple biological functions. Glomerular visceral epithelial cells (GECs) or podocytes are intrinsic components of the kidney glomerulus and play a key role in the maintenance of glomerular permselectivity. Various forms of glomerulonephritis are associated with podocyte injury, which may lead to impaired glomerular function or permselectivity (proteinuria), apoptosis, and glomerulosclerosis. In experimental membranous nephropathy, GEC injury is associated with activation of diverse signaling pathways, which include phospholipases, as well as protein kinases, proteinases, COX₂, endoplasmic reticulum (ER) stress, reactive oxygen species, and others. These pathways contribute to changes in GEC lipid structure and function, actin cytoskeleton re-organization, and displacement of filtration slit diaphragm proteins, ultimately resulting in proteinuria (1,9,53). The effects of C5b-9 in GECs are mediated via signaling pathways, including calcium-independent PLA₂ (iPLA₂) and mitogen-activated protein kinases (MAPKs), that is extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. Expression of iPLA₂γ induces cytoprotection against complement-dependent GEC injury (1).

iPLA₂γ is a membrane-bound enzyme that is reported to localize at the endoplasmic reticulum (ER), peroxisomes, and mitochondria (86). These distinct sites of localization may be a result of specific domains in the structure of the enzyme (87). Recently, we characterized the mechanisms of iPLA₂γ activation by complement in GEC injury. Specifically, we addressed the role of various kinases known to be activated by complement. In GECs, we demonstrated the subcellular localization of iPLA₂γ at the ER and mitochondria, which was dependent on the
N-terminal region of iPLA$_2$$^\gamma$. Complement-induced activation of iPLA$_2$$^\gamma$ was mediated via ERK and p38 pathways. Stimulation of iPLA$_2$$^\gamma$ was dependent on phosphorylation of Ser-511 and/or Ser-515 via MAPK-interacting kinase 1 (MNK1) (114).

The ER is an organelle where secretory, luminal and membrane proteins, are processed after translation. Nascent proteins are folded in the ER with the assistance of molecular chaperones and folding enzymes, and only correctly folded proteins are transported to the Golgi apparatus (106), while misfolded proteins may undergo ERAD. When the amount of misfolded protein in the ER exceeds the capacity of the folding apparatus and ERAD machinery, the misfolded proteins lead to ER stress and activation of the unfolded protein response (UPR) (109-111,145). The UPR is activated to rescue misfolded proteins and improve protein folding. There are three major UPR pathways activated via protein sensors located in the ER membrane, including activating transcription factor-6 (ATF6), inositol-requiring enzyme 1$\alpha$ (IRE1$\alpha$), and protein kinase RNA-like ER kinase (PERK) (108,109,111). In resting cells, the three sensors are inactive, and are believed to be in association with the ER chaperone, glucose regulated protein 78 (grp78; bip). Accumulation of misfolded proteins leads to release of activating transcription factor 6 (ATF6) from bip and translocation to the Golgi, where it is cleaved by site 1 and 2 proteases. The cleaved cytosolic fragment of ATF6, which has a DNA-binding domain, migrates to the nucleus to activate transcription of ER chaperones. Upregulation of ER chaperones, including grp94 and bip, enhances ER protein folding capacity and may limit cytotoxicity. The IRE1$\alpha$ endoribonuclease cleaves X box–binding protein-1 (xbp1) mRNA and changes the reading frame to yield a potent transcriptional activator, which works in parallel with ATF6 to activate transcription of ER chaperones genes. The third aspect of the UPR involves PERK, whose activation phosphorylates the eukaryotic translation initiation factor-2 $\alpha$ subunit (eIF2$\alpha$), which reduces the general rate of translation and aims at decreasing the protein load on a damaged ER. The three UPR pathways are often activated together, but
selective activation of some pathways together with suppression of others can occur (111,112).

A previous study performed in our lab showed that expression of iPLA$_2$$^\gamma$ induces cytoprotection against complement-dependent GEC injury (1). We also showed that iPLA$_2$$^\gamma$ is localized at the ER and activation of iPLA$_2$$^\gamma$ occurs in GECs (114). Taken together, these findings support an important role of iPLA$_2$$^\gamma$ in ER function. One of the protective mechanisms from complement attack is exposure of the cells to mild stress, which induces ER stress proteins that may be protective to additional insults. The physiological functions of iPLA$_2$$^\gamma$ at the ER have not been fully delineated. Determining whether the cytoprotective effect of iPLA$_2$$^\gamma$ involves the UPR is intriguing, as it will potentially link membrane phospholipid hydrolysis with ER stress.

The goal of the present study was to address the role of iPLA$_2$$^\gamma$ in ER stress, specifically, if the cytoprotective effect of iPLA$_2$$^\gamma$ involves the UPR. In GECs, we demonstrate that iPLA$_2$$^\gamma$ amplifies tunicamycin-induced ATF6 activation and upregulates ER chaperones which enhance protein-folding capacity of the ER. Induction of ATF6 by iPLA$_2$$^\gamma$ in GECs is responsible for limiting cell injury.

**EXPERIMENTAL PROCEDURES**

**Materials.**
Tissue culture media and Lipofectamine 2000 were from Invitrogen-Life Technologies (Burlington, ON). Electrophoresis reagents were from Bio-Rad Laboratories (Mississauga, ON). Mouse monoclonal anti-green fluorescent protein (GFP) and rat anti-grp94 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-grp78 (bip) antibody was from Enzo Life Sciences, Inc. (Farmingdale, NY). Rabbit monoclonal anti-actin, tunicamycin, and thapsigargin were from Sigma-Aldrich Canada (Mississauga, ON). Enhanced chemiluminescence (ECL) reagents were from GE Healthcare (Baie d’Urfé, QC).
R-BEL, S-BEL, and prostaglandin E\(_2\) (PGE\(_2\)) enzyme immunoassay kits were from Cayman Chemical (Ann Arbor, MI). Wild type (WT) and M4 GFP-iPLA\(_{2}\gamma\) cDNAs were described previously (114). Plasmids 5xATF6-GL3 and pCGN-ATF6(1-373)m1 were from Addgene (Cambridge, MA) (146). tsA58 was kindly provided by Prof. Parmjit Jat (Ludwig Institute for Cancer Research, London, UK) (147).

*Cell Culture and Transfection.*

Rat GECs were derived from glomerular extracts, and the methods for culture and characterization have been described previously (130). GECs were maintained in K1 medium on plastic substratum. Rat GECs stably transfected with GFP-iPLA\(_{2}\gamma\) and neo-control GECs were described previously (114). 293T and COS-1 cells were cultured on plastic substratum in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum. Transient transfection of GECs or COS-1 cells with GFP-iPLA\(_{2}\gamma\), vector or other plasmids was performed using Lipofectamine 2000.

iPLA\(_{2}\gamma\) knockout (KO) mice were kindly provided by Dr. Richard Gross (Washington University, St. Louis, MO) (148). Mouse GECs were derived from iPLA\(_{2}\gamma\) KO mice and WT control. Briefly, glomeruli were isolated from both KO and WT mice by differential sieving, as described previously (149). The purity of glomeruli obtained from the isolation was consistently high (70-80%). Isolated glomeruli were seeded on type I collagen-coated culture dishes for 5 days in DMEM/F12 (1:1) medium containing 10% NuSerum and 1% hormone mix. A week after the first epithelial cell outgrowth, cells were immortalized by retroviral infection with tsSV40A (147,150), and selection with puromycin. Glomeruli were removed from the culture using cloning rings. Then, the cells were allowed to grow for two additional weeks under permissive conditions (33°C). More than 98% of cells showed morphological characteristics of GECs. By immunofluorescence microscopy (see below), both cell lines displayed positive staining for synaptopodin (Fig. 1A-D) and nephrin (Fig. 1E-H) at both permissive
(33°C) and nonpermissive temperatures (37°C). These results confirm that the cells are of podocyte origin, although they are distinct from certain other GEC lines, where expression of synaptopodin was reported only at nonpermissive temperatures (149,151).

Quantitative reverse transcriptase polymerase chain reaction (qPCR; see below) was performed to measure expression of iPLA$_2^γ$ mRNA isoforms in cultured GEC lines (Table 1). We were not able to compare the level of iPLA$_2^γ$ protein expression, since we could not identify an antibody that reacted with endogenous iPLA$_2^γ$ protein reliably.

**Immunofluorescence microscopy.**

Cells were cultured on glass coverslips for 24 h. All incubations were carried out at 22°C. To examine the expression of nephrin and synaptopodin, cells were fixed with 3% paraformaldehyde in PBS for 15 min and were permeabilized with 0.1% Triton-X-100 in PBS for 30 min. After washing with PBS, cells were incubated with rabbit anti-nephrin antiserum, rabbit anti-synaptopodin antibody, or nonimmune rabbit serum (negative control) diluted in 3% BSA for 2 h. Cells were washed and incubated with FITC-conjugated goat anti-rabbit IgG in 3% BSA for 30 min. Nuclei were counter-stained with 4′,6-diamidino 2-phenylindole (DAPI, 30 nM) in PBS for 4-5 min just before mounting the coverslips onto glass slides. Staining was visualized with a Zeiss AxioObserver fluorescence microscope with visual output connected to an AxioCam digital camera.

**Reverse transcriptase polymerase chain reaction (RT-PCR).**

For the analysis of iPLA$_2^γ$ mRNA expression, total RNA was extracted from iPLA$_2^γ$ KO and WT immortalized mouse GECs using the RNeasy mini kit (Qiagen). cDNA synthesis was performed by QuantiTect® reverse transcriptase (Invitrogen). The RT step was omitted in negative controls. PCR for iPLA$_2^γ$ was done using the following mouse iPLA$_2^γ$ sequence-specific primers: forward 5′-ATTGATGGTGAGGAGCAAGA-3′; reverse 5′-
ATGGCCTGCCACATTTTATAC-3’. PCR consisted of 23 cycles [95°C for 30 s; 62°C for 30 s; and 72 °C for 1:45 min (but 10 min in the final cycle)] and was carried out with Taq DNA polymerase. PCR products were analyzed on 2% agarose gels after staining with ethidium bromide. We detected mRNA expression of iPLA2γ in WT mouse GECs but not in two clones of KO cells (Fig. 2).

For analysis of xbp1 mRNA splicing, total RNA was extracted from GECs and reverse transcribed as above. To amplify xbp1 mRNA, the following primers were used: forward 5’-AACCTCCAGCTAGAAAATCAGC-3’ and reverse 5’-CCATGGGAAGATGTCTGGG-3’. PCR consisted of 25 cycles [95°C for 30 s; 58°C for 30 s; and 72 °C for 1 min (but 10 min in the final cycle)] and was carried out with Taq DNA polymerase. Xbp1 products were visualized on agarose gels, as 556 and 530 bp fragments representing spliced (xbp1S) and unspliced xbp1 (xbp1U).

**Quantitative Real-Time PCR**

Total RNA was prepared from GECs and glomeruli using Trizol reagent (Invitrogen). cDNA synthesis was performed using the QuantiTect® Reverse Transcription kit (Qiagen, Mississauga, ON). The PCR for iPLA2γ was done using the following mouse iPLA2γ sequence-specific primers: forward 5’-CCTGAAGGAAAAGTGG-3’; reverse 5’-CTTGTTCCTCCACCACATCAAT-3’. Real-time PCR reactions were performed on an ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA), and amplified DNA was detected by SYBR green incorporation. Values were normalized to hypoxanthine-guanine phosphoribosyltransferase or glyceraldehyde-3-phosphate dehydrogenase mRNA levels in the same sample.

**Dual luciferase reporter assay.**

Cells were transiently transfected with empty vector or iPLA2γ, together with a firefly luciferase reporter cDNA and a cDNA-encoding renilla luciferase, using Lipofectamine 2000. Cells were cultured for 24 h then stimulated with...
tunicamycin (0.1 µg/ml, 18 h). Lysates were assayed for firefly and renilla luciferase activities using a kit (Dual Luciferase Reporter Assay System, Promega, Madison, WI), according to manufacturer's instructions. Measurements were performed in a Lumat LB 9507 luminometer (Berthold, Oak Ridge, TN), and the ratios between firefly and renilla luciferase activities (the latter a marker of transfection efficiency) were calculated.

**Immunoblotting.**
Cells were lysed in ice-cold buffer containing 1% Triton X-100, 125 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EGTA, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM sodium pyrophosphate, 25 mM NaF, and protease inhibitor cocktail (Roche Diagnostics). Equal amounts of lysate proteins were dissolved in Laemmli buffer and were subjected to SDS-PAGE under reducing conditions. Proteins were then electrophoretically transferred onto a nitrocellulose membrane and blocked at room temperature for 1 h with 5% dry milk in buffer containing 10 mM Tris, pH 7.5, 50 mM NaCl, 2.5 mM EDTA, and 0.05% Tween 20. The membrane was then incubated with primary and secondary antibodies, and developed with ECL.

**Assay for tunicamycin-induced cell death.**
Tunicamycin-induced cell death was determined by measuring lactate dehydrogenase (LDH) release, as described previously (105). Briefly, cells were cultured in a 24 well plate for 48 h. DNA transfection (when required) was performed 24 h after plating. Cells were then treated with tunicamycin 1-10 µg/ml for 24 h. At the end of the incubation, cell supernatants were collected, and the cells adherent to the culture dishes were lysed with 1% Triton X-100. Then, both cell supernatants and Triton lysates were analysed for LDH activity by adding NAD in glycine/lactate buffer. LDH converts lactate and NAD to pyruvate and NADH. The rate of increase in the absorbance of the reaction at 340 nm, due to formation of NADH, represents LDH activity. Specific release of LDH release was calculated as supernatant / (supernatant + lysate) x 100.
**PGE$_2$ Assay.**

iPLA$_2$ enzymatic activity was monitored by measuring PGE$_2$ release into cell supernatants by ELISA, as described previously (132).

**Statistics.**

Data are presented as mean ± SE. One-way ANOVA was used to determine significant differences among groups. Where significant differences were found, individual comparisons were made between groups using the Student’s t-test and adjusting the critical value according to Bonferroni’s method.

**RESULTS**

**iPLA$_{2\gamma}$ amplifies the activation of ATF6.**

Previous studies demonstrated that complement activates iPLA$_{2\gamma}$ and that the iPLA$_{2\gamma}$ pathway restricts complement-induced cytotoxicity in GECs (1,114). Given that iPLA$_{2\gamma}$ is, in part, localized at the ER, we hypothesized that the cytoprotective actions of iPLA$_{2\gamma}$ may involve pathways in the ER. We employed GECs overexpressing GFP-iPLA$_{2\gamma}$ (referred to as “iPLA$_{2\gamma}$”) to examine whether the cytoprotective effect of iPLA$_{2\gamma}$ is mediated by the UPR. In a previous study, we showed that the enzymatic activity of GFP-iPLA$_{2\gamma}$ was comparable to untagged iPLA$_{2\gamma}$ (114). First, we addressed the activation of ATF6, by employing a reporter construct containing five tandem copies of an ATF6 response element fused to firefly luciferase (p5xATF6-GL3) (146). A preliminary study in GECs demonstrated that, the N-linked glycosylation inhibitor, tunicamycin, induces ER stress after 18 h and stimulates ATF6 reporter activity. GECs were co-transfected with iPLA$_{2\gamma}$ (or empty vector in control) and the ATF6 luciferase reporter. Cells were then treated (for 18 h) with tunicamycin, which causes accumulation of misfolded proteins in the ER, and is a potent inducer of ER stress (145). In these experiments, tunicamycin stimulated ATF6 reporter activity, and overexpression of iPLA$_{2\gamma}$ amplified the effect of tunicamycin.
M4 iPLA₂γ is a N-terminally truncated form of iPLA₂γ, which is not associated with the ER and localizes mainly in the cytosol (114). Overexpression of M4 iPLA₂γ in GECs did not amplify the effect of tunicamycin on ATF6 reporter activity, suggesting that the association of the full length (WT) iPLA₂γ with the ER is required for ATF6 activation (Fig. 3A). Both the tunicamycin-stimulated and the amplified ATF6 activities were inhibited by the iPLA₂γ-directed inhibitor, R-bromoenol lactone (R-BEL), but not by indomethacin, an inhibitor of cyclooxygenases (Fig. 3D). These results suggest that the tunicamycin-induced activation of the ATF6 reporter is dependent on endogenous and overexpressed iPLA₂γ catalytic activity, but not prostanoids. An analogous experiment was performed using different ER stressors. We examined the effects of thapsigargin, a potent inhibitor of the endoplasmic reticulum Ca²⁺-ATPase (152), and DTT, a reducing agent, which prevents formation of disulfide bonds between cysteine residues of proteins, thereby inducing protein misfolding and ER stress (153). Similar to tunicamycin, thapsigargin and DTT stimulated ATF6 reporter activity, and iPLA₂γ amplified their effects (Fig. 3B). Taken together, these results indicate that overexpression iPLA₂γ in GECs modulates the ATF6 pathway.

To substantiate the conclusion that endogenous iPLA₂γ can also modulate the ATF6 pathway, we employed GECs derived from WT and iPLA₂γ KO mice. The tunicamycin-induced activation of the ATF6 reporter was reduced in iPLA₂γ KO GECs, compared to WT (Fig. 3E), indicating that endogenous iPLA₂γ in GECs modulates the ATF6 pathway.

*iPLA₂γ amplifies the induction of ER chaperones.*

In mammalian cells, the upregulation of ER chaperones, including grp94 and grp78 (bip), is believed to be due mainly to the activation of ATF6. Since iPLA₂γ amplified the effect of tunicamycin on ATF6 activation, we proceeded to investigate whether this effect on ATF6 activation was associated with
upregulation of ER chaperones. GECs overexpressing iPLA$_2$$\gamma$ and incubated with tunicamycin for 6 h did not show significant increases in grp94 and grp78 expression versus empty vector-transfected cells (results not shown). However, incubation of untransfected GECs for 18 h with tunicamycin resulted in modest increases in the expression of grp94 and grp78. This effect was amplified by overexpression of iPLA$_2$$\gamma$, compared to control cells transfected with empty vector (Fig. 4A, C and D). Therefore, the effect of iPLA$_2$$\gamma$ on amplifying tunicamycin-induced ATF6 activation is indeed associated with upregulation of grp94 and grp78. In additional experiments, cells were pretreated with R-BEL, or indomethacin, prior to the incubation with tunicamycin. By analogy to the ATF6 luciferase-reporter assay, the amplified grp94 and grp78 expression was reduced by R-BEL, but not by indomethacin (Fig. 4B, E and F), implying that the tunicamycin-induced upregulation of ER chaperones is dependent on iPLA$_2$$\gamma$ catalytic activity, but not on prostanoids.

The experiments described above demonstrated that overexpression of iPLA$_2$$\gamma$ amplified the induction of ER chaperones by ER stressors in GECs. The next set of experiments was designed to determine if comparable effects of iPLA$_2$$\gamma$ can occur in other cell lines. Thus, we overexpressed iPLA$_2$$\gamma$ in COS-1 cells. Similar to the result in GECs, iPLA$_2$$\gamma$ was shown to amplify tunicamycin-induced upregulation of grp94 and grp78 in COS-1 cells (Fig. 5 A, C and D). Transfection of iPLA$_2$$\gamma$ in COS-1 cells independently resulted in a modest increase in ATF6-luciferase reporter activity (Table IIA), and in addition, amplified the tunicamycin-induced stimulation of reporter activity (Table IIB). These results in COS-1 cells are consistent with the overexpression of iPLA$_2$$\gamma$ in GECs.

To verify that endogenous iPLA$_2$$\gamma$ can also modulate ER chaperone induction, we employed GECs derived from WT and iPLA$_2$$\gamma$ KO mice. Mouse GECs were incubated with tunicamycin 0.5 or 5 $\mu$g for 6 h. iPLA$_2$$\gamma$ KO cells showed significantly reduced induction of grp94 and grp78, compared with WT (Fig. 6), supporting a role for endogenous iPLA$_2$$\gamma$ in tunicamycin-induced upregulation of
ER chaperones. After 18 h of incubation with tunicamycin, there were large increases in grp94 and grp78 expression, such that there were no significant differences between WT and KO GECs (data not shown).

We then addressed the induction of ER chaperones in 293T cells, which were reported to express a significant amount of endogenous iPLA$_{2\gamma}$ (121). Incubation of 293T cells with tunicamycin resulted in robust increases in grp94 and grp78. The effects were reduced substantially when endogenous iPLA$_{2\gamma}$ was inhibited in the 293T cells with R-BEL (Fig. 5B, E and F). These results are in keeping with the deletion of iPLA$_{2\gamma}$ in GECs.

*iPLA$_{2\gamma}$ does not amplify tunicamycin-induced PERK and IRE1$\alpha$ pathway activation.*

A second aspect of the UPR involves activation of PERK and phosphorylation of eIF2$\alpha$. This phosphorylation is associated with a global decrease in translation, but preferential translation of ATF4, which leads to the induction of several genes, including CHOP, a gene closely associated with apoptosis and/or growth arrest (154). Further studies were performed to address the effect of iPLA$_{2\gamma}$ on the PERK pathway. Tunicamycin stimulated induction of CHOP in control (vector-transfected) cells after 6 (Fig. 7C) and 18 h (Fig. 7A and B), but unlike ER chaperones, the stimulation of CHOP was not affected by overexpression of iPLA$_{2\gamma}$, suggesting that the PERK pathway is not modulated by iPLA$_{2\gamma}$. In a third UPR pathway, IRE1$\alpha$ endonuclease activity splices xbp1 mRNA to yield a potent transcriptional activator. To determine if IRE1$\alpha$ is modulated by iPLA$_{2\gamma}$, we monitored xbp1 splicing by RT-PCR. Tunicamycin (6 h incubation) induced nearly complete splicing of xbp1 in both vector (control)- and iPLA$_{2\gamma}$-transfected cells (Fig. 7D). Complete splicing of xbp1 was evident in control GECs incubated with tunicamycin for 18 h (data not shown). Overexpression of iPLA$_{2\gamma}$ did not, however, enhance the xbp1 splicing. These results indicate that, similar to PERK, the activation of IRE1$\alpha$ was not modulated by iPLA$_{2\gamma}$.
iPLA\textsubscript{2}\gamma reduces tunicamycin-induced cell death.

The above experiments showed that iPLA\textsubscript{2}\gamma amplified tunicamycin-induced expression of ER chaperones in GECs. This aspect of the UPR is typically cytoprotective, since upregulation of chaperones enhances the protein-folding capacity of the ER. Based on these results, we hypothesized that the amplification of chaperone induction by iPLA\textsubscript{2}\gamma is responsible for limiting cell injury. Prolonged incubation of cells with tunicamycin can induce a cytotoxic ER stress response, the latter being, at least in part, associated with the induction of CHOP (155). Therefore, we examined the effect of iPLA\textsubscript{2}\gamma on cytotoxicity during chronic incubation with tunicamycin. Cytotoxicity was monitored by release of LDH (105). Tunicamycin (5-10 µg/ml, 24 h incubation) significantly induced LDH release in control cells. This effect was abolished almost completely in GECs overexpressing iPLA\textsubscript{2}\gamma (Fig. 8A), indicating that iPLA\textsubscript{2}\gamma is cytoprotective in tunicamycin-induced cell death.

The cytoprotective effect of iPLA\textsubscript{2}\gamma is dependent upon ATF6 pathway.

To further investigate whether the cytoprotective effect of iPLA\textsubscript{2}\gamma is dependent on ATF6, we employed a cDNA construct that blocks the activity of endogenous ATF6, ATF6(1-373)m\textsubscript{1} (ATF6m\textsubscript{1}). This construct has point mutations (KNR to TAA at amino acids 315-317) in the basic region of ATF6, which is predicted to disrupt the DNA binding activity of the cytoplasmic domain of ATF6. ATF6m\textsubscript{1} acts as a dominant negative, as it dimerizes with endogenous ATF6 and prevents its binding to ATF6 DNA binding sites (146). After transfection of GECs with HA-ATF6m\textsubscript{1}, expression of HA-ATF6m\textsubscript{1} protein was readily detectable (Fig 9A). As expected, ATF6m\textsubscript{1} did not activate the 5xATF6 reporter, and completely inhibited induction of the reporter by tunicamycin (Fig. 9B), in keeping with an earlier study (146). Next, we monitored cell death in GECs co-transfected with iPLA\textsubscript{2}\gamma and/or ATF6m\textsubscript{1}. Tunicamycin significantly induced LDH release in
control cells by 1.4-fold, compared with untreated cells (Fig. 9C). Consistent with the previous results (Fig. 8A and B), iPLA$_2$$\gamma$ protected against tunicamycin-induced cell death (Fig. 9C). ATF6m1 alone did not affect tunicamycin-induced cell death. However, in cells co-transfected with iPLA$_2$$\gamma$ and ATF6m1, the tunicamycin-induced LDH release was increased by ~2-fold compared with untreated (Fig. 9C). This result indicates that the cytoprotective effect iPLA$_2$$\gamma$ was reversed by ATF6m1. Taken together, these results demonstrate that the cytoprotective effect of iPLA$_2$$\gamma$ during ER stress is dependent on the ATF6 pathway.

*iPLA$_2$$\gamma$ enhanced tunicamycin- and thapsigargin-stimulated production of PGE$_2$.*

In the experiments presented above, we demonstrated that expression of iPLA$_2$$\gamma$ can modulate the activation of ATF6. In the next set of experiments, we examined if activation of the UPR is associated with stimulation of iPLA$_2$$\gamma$ catalytic activity. Similar to a previous study, we monitored the activation of iPLA$_2$$\gamma$ by measuring production of PGE$_2$. Incubation of rat GECs with tunicamycin or thapsigargin induced modest, but significant increases in PGE$_2$ production (Fig. 10A). In rat GECs, stable overexpression of iPLA$_2$$\gamma$ amplified tunicamycin- and thapsigargin-stimulated production of PGE$_2$, compared with control cells (Fig. 10A). Therefore, induction of ER stress was associated with production of PGE$_2$, and furthermore, ER stress stimulated PGE$_2$ production via iPLA$_2$$\gamma$. The enhanced production of PGE$_2$ in cells overexpressing iPLA$_2$$\gamma$ was blocked by R-BEL (Fig. 10A), confirming that iPLA$_2$$\gamma$ is activated during induction of ER stress. Tunicamycin and thapsigargin also stimulated PGE$_2$ production in WT mouse GECs and stimulated PGE$_2$ production was attenuated in iPLA$_2$$\gamma$ KO GECs, compared with WT podocytes (Fig. 10B).
DISCUSSION

In a previous study it was demonstrated that overexpression of iPLA₂γ induced cytoprotection against complement-dependent GEC injury (1). The goal of the present study was to address the mechanism of iPLA₂γ cytoprotection. We focused on ER stress as a potential mechanism of cytoprotection, because iPLA₂γ WT is localized in the ER (114). The present study demonstrates that in GECs, iPLA₂γ amplified the activation of ATF6 (Fig. 3), and the upregulation of the ER chaperones, grp94 and grp78 (Fig. 4 and 5). These effects were dependent on iPLA₂γ catalytic activity, but not prostanoids (Fig. 3 and 4). Furthermore, ATF6 amplification occurred only when the full length iPLA₂γ was expressed, but not a N-terminally truncated mutant, which does not associate with the ER membrane (Fig. 3). Unlike the ATF6 pathway, the activation of the other two UPR pathways, PERK and IRE1α, was not modulated by iPLA₂γ (Fig. 6). To our knowledge, this is the first demonstration that activation of ATF6 of the UPR pathway can occur via iPLA₂γ catalytic activity. Overexpression of iPLA₂γ in GECs abolished tunicamycin-induced cytotoxicity. Moreover, the cytoprotective effect iPLA₂γ was reversed by dominant negative ATF6 (ATF6m1). Taken together, the cytoprotective effect of iPLA₂γ during ER stress is dependent on the ATF6 pathway.

The overexpression of GFP-iPLA₂γ mRNA in our experiments involving rat GECs was ~18-fold (Table I). However, the resulting overexpression of iPLA₂γ protein and enzymatic activity was likely substantially lower. Overexpression of GFP-iPLA₂γ in COS-1 cells increased iPLA₂γ activity ~2-3-fold, suggesting that iPLA₂γ mRNA is not translated efficiently into catalytically-active enzyme. Therefore, the total amount of iPLA₂γ activity in GECs was most likely within the physiological range. Furthermore, iPLA₂γ KO and WT immortalized mouse GECs were examined for iPLA₂γ mRNA levels. As shown in Table I, iPLA₂γ mRNA in WT mouse GECs was approximately half of the level in rat GECs, and as expected, iPLA₂γ mRNA was not detected in two clones of KO mouse GECs.
By analogy to the upregulation of ER chaperones (grp94 and grp78) in rat GEC, tunicamycin also induced upregulation of the same ER chaperones in WT mouse GEC. However, in iPLA2γ KO mouse GEC, less upregulation of ER chaperones was detected compared with WT control (Fig. 6). The results strengthen the conclusion that iPLA2γ amplifies the induction of ER chaperones.

By analogy to GECs, the role of iPLA2γ in the induction of ER stress was also confirmed in COS-1 cells, i.e. overexpression of iPLA2γ amplified the upregulation of ER chaperones (grp94 and grp78). Furthermore, 293T cells, which express ample endogenous iPLA2γ (121), showed upregulation of ER chaperones during ER stress, which was attenuated by an inhibitor of iPLA2γ. Taken together, these results indicate that the role of iPLA2γ in ER stress was not restricted to a single cell type.

We and others have shown functional coupling of iPLA2γ with COX1. Activation of iPLA2γ resulted in release of AA and production of prostanoids (1,121). In the present study, we demonstrated that the effect of iPLA2γ on tunicamycin-mediated activation of ATF6 and upregulation of ER chaperones was independent of prostanoids. Therefore, the cytoprotective effect of iPLA2γ in this model of cell death would be prostanoid-independent. In contrast, Cohen et al. demonstrated that overexpression of iPLA2γ attenuated complement-induced GEC injury, and this effect was partially reversed by indomethacin, suggesting that the cytoprotective effect of iPLA2γ was at least in part mediated by generation of prostanoids (1). Most likely, prostanoids play a role in one of the protective pathways in complement-mediated injury, which may be distinct from the ER stress protective pathway.

As discussed above, overexpression of iPLA2γ amplified the ATF6 pathway of the UPR, resulting in upregulation of ER chaperones and cytoprotection. The mechanism by which iPLA2γ stimulates ATF6 could potentially involve ER membrane lipids. Perturbation of cellular lipid composition can activate the UPR.
in several cells. UPR signaling has been enhanced in cholesterol-loaded macrophages (156), in liver of mice fed a high fat diet (157), and in pancreatic beta cells exposed to saturated fatty acids (158). The precise biochemical mechanisms by which perturbation of the cellular lipid activates UPR pathways in these cells remain unknown. Altered membrane lipid composition can lead to Ca\textsuperscript{2+} depletion from ER, which is proposed to activate the UPR by interfering with Ca\textsuperscript{2+}-dependent chaperones and enzymes required for protein folding (159). Induction of ER stress by the sarco/endoplasmic reticulum calcium ATPase (SERCA) inhibitor, thapsigargin, increases the association of iPLA\textsubscript{2}\beta and ER protein calnexin, which might, for example, influence the conformation of the iPLA\textsubscript{2}\beta protein in such a way to activate it, possibly by phosphorylation (160). Perturbing membrane lipids, by iPLA\textsubscript{2}\gamma, may potentially alter Ca\textsuperscript{2+} homeostasis by inhibition of SERCA, causing ER stress (157). Alternatively, iPLA\textsubscript{2}\gamma may facilitate ATF6 translocation to Golgi by facilitating release of ATF6 from the ER membrane. Perhaps, direct sensing of lipid perturbation contributes to activation of ATF6. Indeed, a recent study by Volmer et al. has demonstrated that perturbation of the membrane lipid composition of the ER promoted IRE1\alpha and PERK activation by enhanced dimerization via their transmembrane domains (159).

Thuerauf et al. showed that p38 can efficiently phosphorylate ATF6, which augments serum response element (SRE)-mediated transcription (161). We previously showed that complement-induced activation of iPLA\textsubscript{2}\gamma was mediated via the p38 pathway, and stimulation of iPLA\textsubscript{2}\gamma was dependent on phosphorylation of Ser-511 and/or Ser-515 via MNK1 (114). Therefore, p38 can potentially phosphorylate both ATF6 and iPLA\textsubscript{2}\gamma. In the present study, activation of ATF6 of the UPR pathway occurred via iPLA\textsubscript{2}\gamma. Products of iPLA\textsubscript{2}\gamma, including AA, have been shown to enhance p38 activity (162). Thus, we propose that p38-dependent phosphorylation and activation of ATF6 may be enhanced by AA. Defining the precise mechanism of ATF6 activation by iPLA\textsubscript{2}\gamma will require further investigation.
In addition to amplifying the activation of the ATF6 pathway and thereby enhancing ER chaperoning capacity, there are other reported mechanisms of cytoprotection involving iPLA₂γ. For example, during oxidative stress, an ER-associated iPLA₂ in renal proximal tubular cells recognizes, cleaves, and removes oxidized phospholipids from the ER membrane (86); thus, iPLA₂γ may act to repair or prevent lipid peroxidation during oxidative stress (86). Another study demonstrated that expression of iPLA₂γ-directed shRNA increased lipid peroxidation and induced apoptosis in renal cells (94). In GECs, a portion of iPLA₂γ was localized at the mitochondria (114). Thus, by analogy to its action in kidney cortical preparations, iPLA₂γ may protect GEC mitochondria from oxidant-induced lipid peroxidation and dysfunction (134).

In conclusion, the results of the present study demonstrate a key relationship between iPLA₂γ and ATF6 activation. Additionally, we have shown that iPLA₂γ plays a cytoprotective role involving the UPR. Further investigation is required to define the precise mechanism of ATF6 activation by iPLA₂γ. Our observations provide a rationale for developing non-toxic methods to induce expression of ER stress proteins, which may eventually have applications for therapy in glomerular disease. Stimulation of iPLA₂γ enzymatic activity represents a potential novel approach to limit GEC injury in complement-mediated diseases.
**FIGURES:**

**Figure 1.** Immunofluorescence staining for podocyte-specific proteins in mouse GECs. Cells were cultured on glass coverslips for 24 h then stained for synaptopodin and nephrin. WT and KO iPLA$_2$γ mouse GECs displayed positive staining for synaptopodin at permissive (33°C) temperature and changed to strong expression at nonpermissive temperatures (37°C), particularly in the cytoplasm and extending into cell processes (A-D). Expression of nephrin was visualized as punctuated cell surface staining and cytoplasmic distribution. Nephrin was expressed at both permissive (33°C) and nonpermissive temperatures (37°C) in both cell lines (E-H).
Figure 2. Reverse transcriptase polymerase chain reaction (RT-PCR). Endogenous expression of iPLA$_{2\gamma}$ mRNA in mouse GEC was assessed by RT-PCR. A. WT mouse GECs express iPLA$_{2\gamma}$ mRNA. iPLA$_{2\gamma}$ KO #1 and #2, two different clones of mouse GECs, are missing iPLA$_{2\gamma}$ mRNA. Control lanes (−) were devoid of reverse transcriptase. B. The 28S and 18 S ribosomal RNAs were visualised by staining with ethidium bromide, and used as the loading control.
Figure 3
**Figure 3.** iPLA$_{2\gamma}$ amplifies tunicamycin-induced ATF6 luciferase-reporter activity. A. Rat GECs were transiently co-transfected with GFP-iPLA$_{2\gamma}$ or M4-iPLA$_{2\gamma}$, or vector (in control), plus renilla-luciferase, and ATF6 firefly luciferase-reporter. After 24 h, cells were untreated (Untr), incubated with tunicamycin (Tm, 0.1 µg/ml, 18 h). Tunicamycin stimulated ATF6-reporter activity (expressed in RLU), and iPLA$_{2\gamma}$ amplified the effect of tunicamycin. *$P<0.001$ iPLA$_{2\gamma}$ + Tm vs vector + Tm. **$P<0.001$ M4-iPLA$_{2\gamma}$ + Tm vs iPLA$_{2\gamma}$ + Tm. 3 experiments. B. GECs were co-transfected as in panel A. Cells were untreated, were incubated with thapsigargin (Tg, 0.5 µM, 18 h), or DTT (0.5 µM, 18 h). Thapsigargin and DTT stimulated ATF6 reporter activity, and iPLA$_{2\gamma}$ amplified the effect of thapsigargin and DTT. *$P<0.05$ iPLA$_{2\gamma}$ + Tg vs vector + Tg. **$P<0.05$ iPLA$_{2\gamma}$ + DTT vs vector + DTT, 4 experiments. C. Representative immunoblots. D. Rat GECs were transiently co-transfected as in panel A. After 24 h, cells were untreated (Untr), incubated with tunicamycin (Tm, 0.1 µg/ml, 18 h), or pre-incubated with R-BEL (5 µM, 30 min) or indomethacin (10 µM, 30 min) before treatment with tunicamycin. Tunicamycin stimulated ATF6-reporter activity, and iPLA$_{2\gamma}$ amplified the effect of tunicamycin. The amplified ATF6 activity was inhibited by R-BEL (5 µM), but not by indomethacin (10 µM). *$P<0.05$ iPLA$_{2\gamma}$ + Tm vs vector + Tm. **$P<0.05$ vector + R-BEL + Tm vs vector + Tm. *$P<0.001$ iPLA$_{2\gamma}$ + R-BEL + Tm vs iPLA$_{2\gamma}$ + Tm, 5 experiments. F. WT and iPLA$_{2\gamma}$ KO mouse GECs were co-transfected with renilla-luciferase, and ATF6 firefly luciferase-reporter. Cells were untreated or incubated with tunicamycin (0.1 µg/ml, 18 h). In iPLA$_{2\gamma}$ KO cells tunicamycin-induced activation of ATF6 reporter was lower, compared with WT. *$P<0.001$ WT + Tm vs KO + Tm, 4 experiments.
Figure 4
**Figure 4.** iPLA$_2^\gamma$ amplifies the tunicamycin-induced upregulation of ER chaperones in GECs. Rat GECs were untransfected (Untrans), or were transfected with GFP-iPLA$_2^\gamma$, or empty vector in control. After 24 h, cells were then incubated with tunicamycin (5 or 10 µg/ml, 18 h). Lysates were immunoblotted with antibodies for grp94, grp78, GFP and actin. Tunicamycin (5 and 10 µg/ml) increased grp94 and grp78 expression levels in control cells. This effect was amplified by overexpression of iPLA$_2^\gamma$ (A, C and D). (A, representative immunoblots; C and D, densitometric quantification). C. grp94: *P<0.05 iPLA$_2^\gamma$ + Tm 5 vs vector + Tm 5. *P<0.05 iPLA$_2^\gamma$ + Tm 10 vs vector + Tm 10. D. grp78: *P<0.05 iPLA$_2^\gamma$ + Tm vs vector + Tm 5. *P<0.05 iPLA$_2^\gamma$ + Tm 10 vs vector + Tm 10, 3 experiments. Upregulation of ER chaperones is dependent on iPLA$_2^\gamma$ catalytic activity, but not prostanoids. GECs were untransfected (Untrans), or were transfected with GFP-iPLA$_2^\gamma$. After 24 h, cells were preincubated with R-BEL (5 µM) or indomethacin (Indo, 10 µM) for 30 min. Cells were then treated with tunicamycin (Tm, 5 µg/ml, 18 h). Lysates were immunoblotted with antibodies for grp94, grp78, GFP and actin. Tunicamycin increased grp94 and grp78 expression levels in PLA$_2^\gamma$-transfected cells. This effect was reduced by R-BEL but not by indomethacin (B, E and F). (B, representative immunoblots; E and F, densitometric quantification). E. grp94: *P<0.001 Tm vs Untr. **P<0.001 R-BEL + Tm vs Tm. F. grp78: *P<0.001 Tm vs Untr. **P<0.01 R-BEL + Tm vs Tm, 3 experiments.
Figure 5

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**Figure 5.** iPLA$_2^\gamma$ amplifies the tunicamycin-induced upregulation of ER chaperones in different cell lines. COS-1 cells were transfected then incubated with tunicamycin (10 µg/ml, 18 h) as above. Lysates were immunoblotted with antibodies for grp94, grp78, GFP and actin. Tunicamycin (10 µg/ml) increased grp94 and grp78 expression levels in control cells. This effect was amplified by overexpression of iPLA$_2^\gamma$ (A, C and D). (A, representative immunoblots; C and D, densitometric quantification). C. grp94: *$P=0.0015$ iPLA$_2^\gamma$ + Tm vs iPLA$_2^\gamma$ Untr. D. grp78: *$P<0.0001$ iPLA$_2^\gamma$ + Tm vs iPLA$_2^\gamma$ Untr. **$P=0.0015$ iPLA$_2^\gamma$ + Tm vs vector + Tm, 3 experiments. 293T cells were incubated with tunicamycin and/or BEL. Lysates were immunoblotted with antibodies for grp94, grp78, and actin. Tunicamycin increased grp94 and grp78 expression levels. This effect was reduced by BEL. (B, E and F). (B, representative immunoblots; E and F, densitometric quantification). E. grp94: *$P<0.0001$ Tm vs Untr. **$P<0.0001$ BEL + Tm vs Tm. F. grp78: *$P<0.0001$ Tm vs Untr. **$P<0.0001$ BEL + Tm vs Tm. +$P<0.001$ BEL + Tm vs BEL, 3 experiments.
Figure 6. Endogenous iPLA$_{2}$$^\gamma$ upregulates ER chaperones. WT and iPLA$_{2}$$^\gamma$ KO mouse GECs were incubated with tunicamycin (Tm, 0.5 or 5 µg for 6 h). Lysates were immunoblotted with antibodies for grp94, grp78, and actin. Tunicamycin increased grp94 and grp78 expression levels in WT podocytes more than in iPLA$_{2}$$^\gamma$ KO podocytes. (A, representative immunoblots; B and C, densitometric quantification). B. grp94: *P<0.01 KO + Tm 5 vs WT + Tm 5, 6 experiments. C. grp78: *P<0.05 KO + Tm 5 vs WT + Tm 5, 5 experiments.
Figure 7
Figure 7. PERK and IRE1α pathways are not modulated by iPLA$_2$γ during ER stress. GECs were untransfected, or were transfected with GFP-iPLA$_2$γ, or empty vector in control. After 24 h, cells were treated with tunicamycin (5 or 10 µg/ml, 18 h). A and B. Lysates were immunoblotted with antibodies for CHOP, GFP and actin. Tunicamycin (5 and 10 µg/ml) increased CHOP expression levels in untransfected and vector-transfected control cells. The stimulation of CHOP was not affected by iPLA$_2$γ overexpression. (A, representative immunoblots; B, densitometric quantification) *$P<0.001$ vector + Tm 5 vs vector Untr, *$P<0.001$ vector + Tm 10 vs vector Untr, **$P<0.01$ iPLA$_2$γ + Tm 5 vs iPLA$_2$γ Untr, **$P<0.01$ iPLA$_2$γ + Tm 10 vs iPLA$_2$γ Untr, 3 experiments. C. As in panel 7B, except incubation with tunicamycin was 6 h. *$P=0.001$ vector + Tm 10 vs vector Untr. **$P<0.01$ iPLA$_2$γ + Tm vs iPLA$_2$γ Untr, 3 experiments. D. mRNA was reverse transcribed and PCR was conducted to amplify xbp1 variants. Tunicamycin induced the splicing of 26 bp from xbp1 in control cells, which reflects activation of IRE1α. Activation of IRE1α was not affected by overexpression of iPLA$_2$γ.
Figure 8. iPLA$_2$$\gamma$ reduced tunicamycin-induced cell death. GECs were transfected with GFP-iPLA$_2$$\gamma$, or empty vector in control. After 24 h, cells were treated with tunicamycin (1, 5, or 10 µg/ml, for 24h). Tunicamycin-induced cytotoxicity was monitored by LDH release. Tunicamycin (5 and 10 µg/ml) significantly induced LDH release in control cells. This effect was reduced in GECs overexpressing iPLA$_2$$\gamma$. *$P<0.05$ vector + Tm 5 vs vector + untreated. **$P<0.01$ vector + Tm 10 vs vector + untreated. +$P<0.01$ iPLA$_2$$\gamma$ + Tm 10 vs vector + Tm 10, 5 experiments.
Figure 9. The cytoprotective effect of iPLA$_{2\gamma}$ during ER stress is dependent on the ATF6 pathway. GECs were transiently transfected with HA-ATF6m1 (dominant negative ATF6), or empty vector. A. Lysates were immunoblotted with antibody for HA. ATF6m1 expression is shown. B. ATF6m1 inhibits ATF6 reporter activation (ATF6 luciferase-reporter assay). GECs were transiently co-transfected with renilla luciferase, ATF6 firefly luciferase-reporter and ATF6m1 or vector in control. Cells were incubated with tunicamycin (0.1 µg/ml, 18 h). ATF6m1 completely inhibited stimulated ATF6 reporter activity. *$P<0.001$ ATF6m1 + Tm vs Vector + Tm, 2 experiments performed in duplicate. C. Tunicamycin-induced cytotoxicity was monitored by LDH release. GECs were transfected with vector, iPLA$_{2\gamma}$, ATF6m1, or iPLA$_{2\gamma}$ + ATF6m1. Tunicamycin (10 µg/ml) induced significant LDH release in control cells. Overexpression of iPLA$_{2\gamma}$ attenuated the tunicamycin-induced release of LDH. The cytoprotective effect iPLA$_{2\gamma}$ was reversed by ATF6m1. *$P<0.05$ iPLA$_{2\gamma}$ + Tm vs vector + Tm. **$P<0.01$ iPLA$_{2\gamma}$ + ATF6m1 + Tm vs iPLA$_{2\gamma}$ + Tm, 5 experiments.
Figure 10

A. PGE$_2$ (pg/ml)

B. PGE$_2$ Release (Fraction)

Vector | iPLA$_2$$^\gamma$

Untr  | Tm  | Tg  | Untr  | Tm  | Tg  | R-BEL + Tm | R-BEL + Tg

WT    | KO  

$^*$ Significance

** Strong significance
**Figure 10.** Activation of iPLA$_2^\gamma$ during ER stress. A. Rat GECs were stably transfected with GFP-iPLA$_2^\gamma$, or empty vector (control). Cells were incubated with tunicamycin (Tm, 5 µg/ml) or thapsigargin (Tg, 1 µM) for 18 h. Certain groups of cells were co-incubated with R-BEL (5 µM), following 30 min preincubation. PGE$_2$ production was measured in cell supernatants. Overexpression of iPLA$_2^\gamma$ amplified tunicamycin- and thapsigargin-stimulated production of PGE$_2$ compared with untreated (Untr) cells. *$P<0.05$ iPLA$_2^\gamma$ + Tm vs vector + Tm, and $P<0.05$ iPLA$_2^\gamma$ + Tg vs vector + Tg. Stimulated increases in PGE$_2$ production were inhibited by R-BEL. **$P<0.001$ iPLA$_2^\gamma$ + R-BEL + Tm vs iPLA$_2^\gamma$ + Tm, and $P<0.001$ iPLA$_2^\gamma$ + R-BEL + Tg vs iPLA$_2^\gamma$ + Tg, 3 experiments. B. WT and iPLA$_2^\gamma$ KO mouse GECs were incubated with tunicamycin or thapsigargin, as above. Tunicamycin and thapsigargin stimulated production of PGE$_2$, compared with untreated cells. *$P<0.01$ Tm vs untreated and $P<0.01$ Tg vs untreated (WT cells). **$P<0.001$ WT + Tm vs KO + Tm, and $P<0.01$ WT + Tg vs KO + Tg, 5 experiments. In these experiments basal PGE$_2$ release (WT, untreated cells) was 184.5 pg/ml.
TABLES:

**Table I.** Expression of iPLA$_2\gamma$ in GECs and glomeruli.

Total RNA was extracted from GECs and glomeruli. RNA was reverse transcribed to cDNA, and amplified using qPCR. In the GECs transfected with GFP-iPLA$_2\gamma$ WT or M4 GFP-iPLA$_2\gamma$, RNA was extracted 24 h after transfection. RQ is presented as an average of (3-6) values.

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**Table II.** Effect of iPLA$_2\gamma$ on ATF6 luciferase activity in COS-1 cells.

COS-1 cells were untransfected, or were transiently co-transfected with GFP-iPLA$_2\gamma$ or vector (in control), plus renilla-luciferase, and ATF6 firefly luciferase-reporter. After 24 h, cells were untreated (Untr) or incubated with tunicamycin (Tm, 0.1 µg/ml, 24 h). ATF6-reporter activity is expressed in RLU. *p<0.001 vs vector (4 experiments), **p<0.015 vs vector+tunicamycin and vs untransfected+tunicamycin (4 experiments).

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Preface to Chapter 4

We and others have provided considerable insight into the functions of iPLA$_2\gamma$ in cultured kidney cell lines (Chapters 2 and 3); however, information on the functional role of iPLA$_2\gamma$ in the kidney is lacking. The third aim was to employ iPLA$_2\gamma$ knockout (KO) mice to better understand the role of iPLA$_2\gamma$ in normal podocyte function, and in podocyte injury in vivo (Chapter 4).
CHAPTER 4: MANUSCRIPT IN PREPARATION
GENETIC ABLATION OF CALCIUM-INDEPENDENT PHOSPHOLIPASE A$_{2\gamma}$ EXACERBATES GLOMERULAR INJURY IN MICE

Hanan Elimam, Lamine Aoudjit, Tomoko Takano, and Andrey V. Cybulsky

Department of Medicine, McGill University Health Centre, McGill University, Montreal, Quebec, Canada

Running title: Increased podocyte injury in calcium-independent phospholipase A$_{2\gamma}$ KO mice

Address for correspondence: Andrey V. Cybulsky, MD, Division of Nephrology, Royal Victoria Hospital, 687 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1. Tel: 1-514-398-8148. Fax: 1-514-843-2815. E-mail: andrey.cybulsky@mcgill.ca; Tomoko Takano, MD, PhD, Division of Nephrology, McGill University, 3775 University Street, Rm 236, Montreal, Quebec, Canada H3A2B4. Tel: 1-514-398-2171, Fax: 1-514-843-2815. E-mail: tomoko.takano@mcgill.ca
ABSTRACT

Injury of visceral glomerular epithelial cells (GECs) causes proteinuria in many glomerular diseases. We reported previously that calcium-independent phospholipase A2γ (iPLA2γ) is cytoprotective against complement-mediated GEC injury. In addition, iPLA2γ amplified activation of the ATF6 pathway of the UPR, resulting in upregulation of ER chaperones and cytoprotection during ER stress. The pathophysiological roles of iPLA2γ in kidney are poorly understood. The present study addressed the protective role of iPLA2γ in glomerulonephritis. Deletion of iPLA2γ in mice did not result in podocyte injury or albuminuria up to 15 months age. However, in the context of glomerular injury induced by anti-GBM antibody, iPLA2γ KO mice exhibited higher albuminuria compared to wild type (WT) mice. Deletion of iPLA2γ in mice with anti-GBM nephritis resulted in a ~60% decrease of podocyte number, and iPLA2γ KO mice showed more severe abnormalities in podocyte ultrastructure, compared to WT mice. Thus, expression of iPLA2γ is cytoprotective. Defining the role of iPLA2γ in glomerulonephritis provides opportunities for development of novel therapeutic approaches to GEC injury and proteinuria.
INTRODUCTION

Phospholipase A\textsubscript{2} (PLA\textsubscript{2}) enzymes catalyze the cleavage of the sn-2 ester bond of phospholipids to yield free fatty acids, such as arachidonic acid (AA), and lysophospholipids (67,68). Both products represent precursors for signaling molecules that can exert multiple biological functions. Calcium independent PLA\textsubscript{2}s (iPLA\textsubscript{2}s) are members of the group VI family of PLA\textsubscript{2} enzymes (69). Group VIB (iPLA\textsubscript{2γ}), is homologous to iPLA\textsubscript{2β} in the C-terminal catalytic domain but shows no similarities in N-terminal region; accordingly, iPLA\textsubscript{2γ} may have distinct regulatory properties from those of iPLA\textsubscript{2β} (82,83).

Genetic ablation of iPLA\textsubscript{2γ} in mice resulted in the generation of viable progeny that demonstrated reduced growth rate, cold intolerance due to impaired fat burning in brown adipose tissue, and a defect in mitochondrial cytochrome oxidase (Complex IV) function when stimulated with ascorbate (148). Several studies showed that iPLA\textsubscript{2γ} knockout (KO) mice display multiple bioenergetic dysfunctional phenotypes. For example, iPLA\textsubscript{2γ} deletion induced profound alterations in hippocampal phospholipid metabolism and mitochondrial phospholipid homeostasis resulting in enlarged and degenerating mitochondria, leading to autophagy and cognitive dysfunction (97). Another recent study showed that genetic ablation of iPLA\textsubscript{2γ} prevented obesity and insulin resistance during high fat feeding by mitochondrial uncoupling and increased adipocyte fatty acid oxidation (100). These reports identified an obligatory role for iPLA\textsubscript{2γ} in mitochondrial lipid metabolism and membrane structure, perturbation of which may profoundly influence fatty acid β-oxidation, oxygen consumption, energy expenditure, and thus, tissue homeostasis. Despite the recent advance in studying the pathophysiological role of iPLA\textsubscript{2γ} in the brain, liver (98), heart and skeletal muscles (99), the pathophysiological roles of iPLA\textsubscript{2γ} in kidney are poorly understood.
Visceral glomerular epithelial cells (GECs), commonly known as podocytes, are highly differentiated cells of the kidney glomerulus, which have a critical role in the maintenance of glomerular permselectivity. Podocytes have complex morphology that is characterized by foot processes. The unique shape of podocytes is supported by the actin cytoskeleton, which allows the podocyte to continuously, and dynamically alter shape. Podocytes are attached to the GBM by integrins (predominantly the α3β1 integrin) and α- and β-dystroglycans (7). Changes in size and charge properties of podocytes occurring in kidney diseases contribute to the development of proteinuria. Specifically, proteinuria is associated with the disruption of the foot processes with the concomitant transformation of the actin cytoskeleton (22). Thus, the intricate structure of foot processes is central to their function in maintaining glomerular permselectivity, i.e. filtration of plasma but restriction of proteins. Various forms of glomerulonephritis are associated with podocyte injury, which may lead to foot process effacement, apoptosis, impaired glomerular function and permselectivity (proteinuria), and glomerulosclerosis (9).

Previously, we demonstrated expression and activity of iPLA2γ in cultured GECs, and we characterized the mechanisms of iPLA2γ activation by complement C5b-9 in GEC injury. In GECs, we demonstrated that iPLA2γ is localized at the ER and mitochondria, and this localization was dependent on the N-terminal region of iPLA2γ. Complement stimulated the activation of iPLA2γ, which was mediated via the extracellular signal-regulated kinase (ERK) and p38 pathways. Stimulation of iPLA2γ was dependent on phosphorylation of Ser-511 and/or Ser-515 via MAPK-interacting kinase 1 (MNK1) (114). In addition to characterizing these signaling pathways, we showed that overexpression of iPLA2γ in cultured GECs reduced complement-mediated GEC injury (1). In primary cultures of rabbit renal proximal tubules cells, knockdown of iPLA2γ expression increased lipid peroxidation and decreased mitochondrial function (94). Likewise, knockdown of iPLA2γ in C2C12 myoblast cells also caused elevation of lipid peroxidation and reduction of ATP synthesis (99). Thus, iPLA2γ may play a role in removing
peroxidized phospholipid from the mitochondrial membrane, thereby preserving membrane integrity. Furthermore, there is evidence supporting a cytoprotective role of iPLA$_2$$\gamma$ in vivo (99).

So far, we and others have provided considerable insight into the functions of iPLA$_2$$\gamma$ in cultured cell lines, but information on the functional role of iPLA$_2$$\gamma$ in the kidney is lacking. The aim of the present study was to employ iPLA$_2$$\gamma$ KO mice to better understand the role of iPLA$_2$$\gamma$ in normal podocyte function, and in podocyte injury. We show that deletion of iPLA$_2$$\gamma$ caused neither albuminuria nor morphological changes in the glomerulus. However, after induction of anti-glomerular basement membrane (GBM) nephritis, iPLA$_2$$\gamma$ KO mice exhibited significantly increased levels of proteinuria and a marked loss of podocytes, compared to WT mice. Our results indicate that iPLA$_2$$\gamma$ has a protective role in glomerulonephritis.

**EXPERIMENTAL PROCEDURES**

*Materials*

Mouse Albumin ELISA Quantification Kit was purchased from Bethyl Laboratories (Montgomery, TX). Creatinine Assay Kit was from Cayman Chemical Company (Ann Arbor, MI). Rabbit anti-Wilm’s tumor (WT)-1 antibody was purchased from Santa Cruz Biotechnology (Santa, CA). Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-sheep IgG was from Zymed (South San Francisco, CA). FITC-conjugated goat anti-mouse complement C3 was from MP Biomedical (Santa Ana, CA). Production of rabbit anti-rat GEC antiserum and sheep anti-rat GBM antiserum was described previously (102,163). HistoPrep was purchased from Fisher Scientific (Ottawa, ON).
**Mice**

iPLA$_{2\gamma}$ KO mice were kindly provided by Drs. Richard Gross and David Mancuso (Washington University School of Medicine, St. Louis, MO) (148). Animal protocols were reviewed and approved by the McGill University Animal Care Committee. Conscious animals fed *ad libitum* were weighed and urine samples were collected at various time points after weaning up to 12-15 months of age.

**Assay for albuminuria**

Mouse urine albumin concentration was quantified using an enzyme-linked immunosorbent assay kit according to the manufacturer’s instructions. The assay utilizes urine samples diluted 1:5000 or 1:150000, analyzed in plate reader set at 450 nm. Urine creatinine concentration was measured using a colorimetric assay kit according to the manufacturer’s instructions. The assay measures creatinine in urine samples diluted 1:10, using a picric acid-based method and analyzed on a plate reader set at 490-500 nm. Excretion of albumin is expressed as albumin to creatinine ratio (ACR).

**Induction of anti-GBM nephritis in mice**

Anti-GBM nephritis was induced in 3-4 month old C57BL/6 mice that were either iPLA$_{2\gamma}$ KO or WT, by a single tail-intravenous injection of 5-10 µl of sheep anti-GBM antiserum diluted in 0.1 ml of sterile PBS. After 24 h, mice were sacrificed by CO$_2$ asphyxiation, and kidneys were dissected. Kidney sections were collected for immunofluorescence, light, and electron microscopy (see below). Glomeruli were isolated utilizing a differential sieving technique (163).
Kidney poles were snap-frozen using HistoPrep in isopentane (−80°C) for immunofluorescence (IF) microscopy. Four-µm sections were cut in a cryostat, followed by air-drying for 15-30 min, and stored at −80°C. Sections were fixed in 4% paraformaldehyde for 5 min. Sections were then blocked with 5% normal rabbit serum (for anti-GBM antibodies) or goat serum (for complement C3) in 5% BSA for 15 min. The blocking solution was replaced with FITC-conjugated rabbit anti-sheep IgG or FITC-conjugated goat anti-mouse complement C3 diluted in 5% BSA for 30 min. Sections were examined with a Zeiss AxioObserver fluorescence microscope with visual output connected to an AxioCam digital camera. Fluorescence intensity was quantified using Image J software. Results are expressed in arbitrary units.

For Wilm’s tumor-1 (WT-1) immunofluorescence, frozen sections were fixed with 4% paraformaldehyde for 5 min. Then, sections were immersed in 10 mmol/L sodium citrate, pH 6.0 for 8 min at 100°C. After cooling to 22°C, sections were blocked with 10% normal goat serum, and were incubated with rabbit anti-WT-1 antibody (4°C, overnight), followed by rhodamine-goat anti-rabbit IgG (22°C, 1 hour). WT-1 positive cells were quantified in 30 to 40 glomeruli of KO and control mice by visual counting.

For light microscopy, kidney tissue was fixed in formalin, and was processed and embedded in paraffin according to conventional techniques. Sections were stained with periodic acid Schiff (PAS) at the Goodman Cancer Research Center Histology Facility. Electron microscopy was carried out at the McGill University Facility for Electron Microscopy Research. Briefly, kidney tissue was fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer containing 0.1% CaCl₂, pH 7.4. Samples were washed and postfixed with 1% aqueous OsO₄ and 1.5% aqueous potassium ferrocyanide. Then, samples were dehydrated with
serially increasing concentrations of acetone (30 to 100%), and samples were infiltrated with epon/acetone. Sections of 90 to 100 nm were placed onto grids, and stained with uranyl acetate and Reynold’s lead. Sections were viewed with a FEI Tecnai 12 transmission electron microscope operating at an accelerating voltage of 120 kV, and equipped with a Gatan Bioscan CCD camera, model 792.

Statistics

Data are presented as mean ± SE. One-way ANOVA was used to determine significant differences among groups. Where significant differences were found, individual comparisons were made between groups using the Student’s t-test and adjusting the critical value according to Bonferroni’s method. The Student’s t-test was used to determine significant differences between two groups.

RESULTS

Baseline Urinary Albumin Excretion

General phenotypic features of mice null for iPLA₂γ were described previously (148). In keeping with the earlier report, growth retardation was visible in KO mice after 2 months of age. At 3-4 months, KO animals (males and females) had a mean weight of 22.09 ± 2.37 g versus 26.02 ± 2.39 g for WT (N=10 per group). There is no significant difference in weight between males and females in the same group. We quantified the urine albumin-to-creatinine ratio (ACR). Mice at 3-5 months of age showed a low ACR, and there was no significant difference between KO and WT mice (Fig. 1). The urine ACR tended to be slightly higher in male mice, compared with females. ACR between WT and age/gender matched KO mice did not differ significantly up to 15 months of age (Fig. 1). Light microscopy (PAS staining) showed normal glomerular and tubular morphology in both WT and KO mice 10-12 months of age (results not shown).
Studies in cultured GEC have shown a protective role for iPLA$_2$ in the context of injury induced by complement (1) and tunicamycin (unpublished data). To address the role of iPLA$_2$ in podocyte injury in vivo, we induced anti-GBM nephritis in WT and iPLA$_2$ KO mice. In preliminary studies, we determined that administration of 5-10 µl of anti-GBM antisera induces mild to moderate albuminuria. Therefore, 7 KO mice (4 females and 3 males) and 7 WT mice (2 females and 5 males) (age 3-4 months) were injected with sheep anti-GBM antisera and 4 mice each were injected with PBS (control). Urine samples were collected just before administration of antibody and after 24 h. Both WT and iPLA$_2$ KO mice developed albuminuria 24 h after induction of anti-GBM nephritis. However, iPLA$_2$ KO mice exhibited significantly increased levels of albuminuria, compared to WT (Fig. 1).

Deposition of anti-GBM antibody and activation of complement was assessed by immunofluorescence microscopy. There was prominent linear deposition of sheep IgG along the glomerular capillary walls in both WT and iPLA$_2$ KO mice (Fig. 2 A-B and E). Glomerular C3 staining was fainter, compared with IgG, but was clearly present in both groups of mice (Fig. 2 C-E). Quantification of IgG and C3 fluorescence intensity did not demonstrate significant differences between the two groups. Therefore, the increased albuminuria in iPLA$_2$ KO mice occurred despite equal glomerular anti-GBM antibody and C3 deposition in WT and iPLA$_2$ KO mice (Fig. 2).

Effect of iPLA$_2$ deletion on podocyte number

To further address potential mechanisms for the cytoprotective effect of iPLA$_2$ in vivo, we quantified the number of podocytes in glomeruli of KO and WT mice by WT-1 immunostaining. In the glomerulus, WT-1 is expressed specifically in
podocytes. In saline-treated mice, deletion of iPLA$_{2\gamma}$ did not alter the number of WT-1 positive cells, as compared with WT. Furthermore, in WT mice, anti-GBM antiserum did not alter the number of WT-1-positive cells. In contrast, there was a $\sim$60% decrease in the number of WT-1 positive cells in iPLA$_{2\gamma}$ KO glomeruli after anti-GBM antiserum injection (Fig. 2, F–J). Therefore, genetic ablation of iPLA$_{2\gamma}$ induced a marked loss of podocytes when these cells were subjected to injury.

**Effects of iPLA$_{2\gamma}$ deletion on podocyte ultrastructure**

After injection with anti-GBM antibody, glomerular and tubular morphology of WT and iPLA$_{2\gamma}$ KO mice (N=6-7) appeared normal by light microscopy. Kidneys of four albuminuric mice (two in each group) and three control mice (saline treated groups) were examined by electron microscopy. The control mice (WT or KO) showed no glomerular abnormalities, and podocyte ultrastructure appeared entirely normal (Fig. 3A and B). In contrast, anti-GBM injected WT mice showed focal abnormalities in podocyte ultrastructure. Podocyte cell bodies appeared swollen, and in some podocytes there was villous transformation and microvesiculation of the plasma membranes. In some areas of the glomerulus, foot processes appeared normal, but in other areas, there were marked abnormalities. These ranged from short, and widened foot processes to patchy effacement (Fig. 3C). iPLA$_{2\gamma}$ KO mice showed substantially more severe abnormalities in podocyte ultrastructure. Podocyte cell bodies were swollen with fewer intact organelles, and there was extensive villous transformation and microvesiculation of the plasma membrane. Foot processes were severely malformed and were almost completely effaced (Fig. 3D and E).

**Discussion**

In this study, we demonstrated that deletion of iPLA$_{2\gamma}$ in mice did not result in podocyte injury or albuminuria up to 15 months age (Fig. 1). However, in the
context of glomerular injury induced by anti-GBM antibody, iPLA$_2$$\gamma$ KO mice exhibited higher albuminuria with a remarkable reduction in podocytes, compared to WT mice (Fig. 1-2). iPLA$_2$$\gamma$ KO mice showed substantially more severe abnormalities in podocyte ultrastructure (Fig. 3).

The podocyte plays an important role in maintaining glomerular permselectivity. Actin filaments are the core structural components of podocyte foot processes. Condensation of the actin cytoskeleton at the base of effaced podocyte foot processes together with alterations in filtration slits, and displacement and disruption of slit diaphragms are features of proteinuric glomerular diseases (9). We addressed the effect of iPLA$_2$$\gamma$ on podocyte injury in heterologous anti-GBM nephritis. The heterologous anti-GBM antiserum binds to the GBM, and in part, to podocyte surface antigens and activates complement. The anti-GBM model features prominent podocyte injury, which is at least in part mediated by complement C5b-9 (164). Using relatively low doses of anti-GBM antibody, we demonstrated that iPLA$_2$$\gamma$ KO mice with anti-GBM nephritis have increased proteinuria compared to WT mice (Fig. 1), implying that expression of iPLA$_2$$\gamma$ is cytoprotective. This in vivo result is in keeping with earlier studies, which showed that overexpression of iPLA$_2$$\gamma$ in cultured GECs reduced complement-mediated GEC injury (1).

Ablation of iPLA$_2$$\gamma$ in mice with anti-GBM nephritis resulted in a ~60% decrease of podocyte number (Fig. 2). There is both experimental and clinical evidence, which supports the view that in certain glomerular diseases, apoptosis is a major contributor to reduced podocyte numbers, and resulting proteinuria (20). In anti-GBM nephritis, loss of podocytes may be reflecting increased apoptosis, and the presence of iPLA$_2$$\gamma$ may provide an anti-apoptotic effect. Our preliminary studies indicate that iPLA$_2$$\gamma$ (which is localized at the ER) may enhance the adaptive/cytoprotective unfolded protein response, thereby reducing podocyte injury (unpublished data). A second possibility is that ablation of iPLA$_2$$\gamma$ may induce podocyte detachment from the GBM, thus accounting for the reduction in
podocyte number in anti-GBM nephritis. There is substantial evidence documenting podocyte detachment and subsequent appearance in the urine (podocyturia) (165,166). Moreover, many of these urinary podocytes were viable suggesting a primary defect in attachment to the underlying GBM. iPLA2γ ablation may induce changes in the expression or function of proteins involved in mediating podocyte adhesion to the GBM. Decisive proof of these mechanisms will require further study.

Future considerations:

Further studies will be directed at defining the mediatory role of iPLA2γ in podocyte injury in vivo. To address a potential role in apoptosis, glomeruli of WT and KO mice will be examined for the presence of apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nick-end staining or by immunoblotting with anti-activated caspase-3 antibody. In addition, glomeruli will be analyzed for changes in the expression/localization of podocyte proteins, which are believed to play key roles in maintaining ultrastructure and slit diaphragms. These proteins include nephrin (9) and podocin (3,20,167). Since iPLA2γ is localized at the ER, the expression of ER stress markers (e.g. grp94, grp78, and CHOP) will be compared in glomeruli of WT and KO mice by immunoblotting and immunofluorescence microscopy. Given that iPLA2γ is also localized at mitochondria (114), future studies will be required to address whether there are mitochondrial ultrastructural or functional abnormalities in podocytes, and if abnormalities are worsened by deletion of iPLA2γ. These mitochondrial abnormalities may consist of enlargement and abnormal shape, variations in the number of cristae and particular patterns of cristae. Other analyses may include expression of various mitochondrial subunit components, as well as the presence of markers of oxidative injury to lipids and proteins in the glomeruli.
FIGURES:

Figure 1

![Graph showing ACR (µg/mg) over time and genotypes.](image)

- WT and KO genotypes for different age groups: 6-7 months, 8-11 months, 12-15 months.
- Comparison of ACR levels between Control and Anti-GBM conditions for WT and KO genotypes.

ACR (µg/mg)

- WT: 6-7 months, 8-11 months, 12-15 months
- KO: 6-7 months, 8-11 months, 12-15 months

*Significant difference indicated by *.
**Figure 1.** iPLA$_{2\gamma}$ deletion does not alter urinary albumin excretion in mice, but exacerbates albuminuria in anti-GBM nephritis. A. Urinary albumin/creatinine ratio. Each point represents a urine collection of a single mouse taken at monthly intervals between six and fifteen months of age. WT and iPLA$_{2\gamma}$ KO mice were divided into groups according to their gender. There are no significant differences among the groups. B. Anti-GBM nephritis was induced in WT and iPLA$_{2\gamma}$ KO mice of 3-4 months of age via single intravenous injection of sheep anti-rat GBM antiserum (control mice received saline). Urinary albumin/creatinine ratio was measured before and 24 h after the injection. After induction of anti-GBM nephritis, iPLA$_{2\gamma}$ KO mice had more proteinuria compared to WT mice. *P <0.05 iPLA$_{2\gamma}$ KO anti-GBM vs WT-anti-GBM. WT control, N = 4; KO control, N = 4; WT anti-GBM = 7; KO anti-GBM = 7.
Figure 2

 WT | KO
---|---
![Image of Anti-GBM](image1)

![Image of C3](image2)

![Graph showing FITIC intensity](image3)

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Figure 2 (continued)

F: WT Control
G: KO Control
H: WT Anti-GBM
I: KO Anti-GBM
J: WT-1 positive cells/glomerulus

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* indicates statistical significance.
**Figure 2.** Immunofluorescence staining for sheep IgG, complement C3, and WT-1 in anti-GBM nephritis. Kidney sections from WT and iPLA$_{2}\gamma$ KO mice with anti-GBM nephritis (at 24 h) were stained with FITC-conjugated rabbit anti-sheep IgG to visualize deposition of anti-GBM antibodies. There is bright glomerular fluorescence staining with sheep IgG in both WT and iPLA$_{2}\gamma$ KO mice (A, B and E). Sections were also stained with FITC-conjugated goat anti-mouse C3 to visualize deposition of complement C3. Fainter glomerular staining was present in both groups of mice (C-E). The C3 staining of Bowman’s capsule (surrounding the glomerulus) is observed in normal mouse kidneys, and is not due to the administration of anti-GBM antibody. Quantification of anti-GBM antibody and complement C3 deposition (fluorescence intensity), in arbitrary units, shows no significance differences between groups (E). iPLA$_{2}\gamma$ depletion reduced podocyte number in anti-GBM nephritis. WT-1 immunofluorescence was assessed in two WT mice without and with anti-GBM antibody (F and H) and four iPLA$_{2}\gamma$ KO mice without and with anti-GBM antibody (G and I). J: Quantification of WT-1-positive cells per glomerulus. *$P < 0.001$ KO, anti-GBM (N = 36) vs. WT, anti-GBM (N =38).
Figure 3 (continued)

WT-anti-GBM

KO-anti-GBM
Figure 3. iPLA2γ deletion induces abnormalities in podocyte ultrastructure. A: WT and B: iPLA2γ KO mice (age 3 months) show normal podocyte ultrastructure. Foot processes, cell bodies and intracellular organelles appear intact (Scale bar = 2 µm). C: WT mouse (age 3 months) injected with anti-GBM antibody. Podocyte foot processes appear widened with patchy effacement in some areas (arrows; Scale bar = 2 µm). The podocyte body (asterisk) is markedly swollen. D: iPLA2γ KO mouse (age 3 months) injected with anti-GBM antibody. Podocyte cell bodies are markedly swollen (asterisks) and the cell membranes show marked villous transformation and microvesiculation. Podocyte foot processes are completely effaced (Scale bar = 2 µm). E: iPLA2γ KO mouse (higher magnification). The podocyte cell body shows swelling, although many organelles appear intact. There is foot process effacement and villous transformation and microvesiculation of the plasma membrane (Scale bar = 500 nm).
CHAPTER 5: SUMMARY AND FINAL CONCLUSION
Summary and final conclusion

The work presented in this thesis characterizes the mechanism of iPLA$_2^\gamma$ activation in complement-mediated GEC injury, and shows that the cytoprotective effect of iPLA$_2^\gamma$ involves the ATF6 branch of the unfolded protein response, and that iPLA$_2^\gamma$ has a protective role in glomerulonephritis.

In the first manuscript, we demonstrate that complement C5b-9 activates endogenous and ectopically-expressed iPLA$_2^\gamma$. Activation of iPLA$_2^\gamma$ occurs via ERK and p38 pathways, and is dependent on an increase in cytosolic Ca$^{2+}$ concentration and phosphorylation of the enzyme on S511 and/or S515, most likely via MNK1 (114). To our knowledge, this is the first demonstration of phosphorylation-dependent activation of iPLA$_2^\gamma$. The S511 and S515 phosphorylation sites were proposed to be in the context of a phosphorylation motif for MK2 (91); however, we were not able to show activation of iPLA$_2^\gamma$ by MK2 overexpression. The protein kinase MNK1 is also activated by ERK and p38, and the amino acid sequence preceding S511 in iPLA$_2^\gamma$ (KLGS) resembles the MNK1 phosphorylation motif in eukaryotic translation initiation factor 4E (KSGS) (63). By mutating S511 and S515 to Ala, EGF + ionomycin-, as well as complement-stimulated iPLA$_2^\gamma$-dependent PGE$_2$ production were significantly attenuated. Moreover, phosphorylation of S511 was induced by EGF + ionomycin and by complement (Chapter 2, Figures 10 and 12) (114). Phosphorylation of S511 and/or S515 could induce a conformational change in the enzyme, leading to an increase in catalytic activity. Further studies will be required to define the mechanism more precisely.

In GECs, we demonstrated the subcellular localization of iPLA$_2^\gamma$ at the ER and mitochondria, which was dependent on the N-terminal region of iPLA$_2^\gamma$. Truncation of the N-terminal region of iPLA$_2^\gamma$ (220 amino acids) did not abolish iPLA$_2^\gamma$ enzymatic activity in vitro, consistent with the study by Tanaka et al. (90). Stimulation of full-length iPLA$_2^\gamma$ by complement was coupled with production of
PGE$_2$; however, the N-terminal truncated form of iPLA$_{2\gamma}$ was unable to induce significant PGE$_2$ production when expressed in cells (Chapter 2, Figure 3). The N-terminal region is involved in the membrane association of iPLA$_{2\gamma}$, thereby allowing the enzyme access to phospholipid substrate intracellularly. COX1 is localized in the ER membrane, and could couple with iPLA$_{2\gamma}$-mediated AA release. Thus mislocalization of the N-terminal truncated form of iPLA$_{2\gamma}$ from the membrane may be expected to reduce PGE$_2$ production. In addition, the N-terminal region may have positive regulatory elements that could enhance iPLA$_{2\gamma}$ activity.

Given that iPLA$_{2\gamma}$ WT localized in the ER, in the second manuscript we hypothesized that the cytoprotective actions of iPLA$_{2\gamma}$ (1,114) may involve pathways in the ER. The data presented in Chapter 3 (second manuscript) demonstrate that both endogenous and ectopic iPLA$_{2\gamma}$ in GECs amplified the activation of the ATF6 pathway of the UPR by stimulating the activation of ATF6, and upregulating the ER chaperones, grp94 and grp78 (Chapter 3, Figures 3-5). Upregulation of ER chaperones is required for protein folding and confers cytoprotection. To our knowledge, we are the first to identify that ATF6 activation pathway is dependent on iPLA$_{2\gamma}$ activity. In addition, we demonstrate that the effect of iPLA$_{2\gamma}$ on tunicamycin-mediated activation of ATF6 and upregulation of ER chaperones was independent of prostanoids (Chapter 3, Figures 3 and 4). In contrast to this result, Cohen et al. demonstrated that overexpression of iPLA$_{2\gamma}$ attenuated complement-induced GEC injury, and this effect was partially reversed by indomethacin, suggesting that the cytoprotective effect of iPLA$_{2\gamma}$ was at least in part mediated by generation of prostanoids (1). Most likely, the role of prostanoids as one of the protective pathways in complement-mediated injury is distinct from that of the ER stress pathway.

Experimental and human MN show injury to GEC mediated by subepithelial deposition of antibody, which activates complement and leads to C5b-9 assembly. The role of PLA$_2$s in complement-mediated GEC injury has been well-defined
One of the protective mechanisms from complement attack is exposure of the cells to mild stress, which induces ER stress proteins that may be protective to additional insults. Our data, in conjunction with previous characterization of cPLA$_2$’s role in complement-mediated GEC injury, indicate that both PLA$_2$s are involved in the pathogenesis of experimental MN. It is plausible that both cPLA$_2$ and iPLA$_2$$\gamma$ activate the ER stress response that protects GEC in initial stage of injury (102). At greater doses, C5b-9 insertion into the membrane may overwhelm the cytoprotective mechanism, and activate cytotoxic cascades.

In chapter 4, we employed iPLA$_2$$\gamma$ KO mice (148) to address the role of iPLA$_2$$\gamma$ in podocyte injury in heterologous anti-GBM nephritis, and we demonstrated that iPLA$_2$$\gamma$ KO mice with anti-GBM nephritis have increased proteinuria compared to WT mice (Chapter 4 Figure 1). In addition, ablation of iPLA$_2$$\gamma$ in mice with anti-GBM nephritis resulted in a decrease of podocyte number (Chapter 4, Figure 2). In anti-GBM nephritis, loss of podocytes may be reflecting increased apoptosis, and the presence of iPLA$_2$$\gamma$ may provide an anti-apoptotic effect. Apoptosis is a major contributor to reduced podocyte numbers, and resulting proteinuria (20). Alternatively, podocyte detachment from the GBM may account for the reduction in podocyte number in anti-GBM nephritis (165,166). Further study will be required to address the exact mechanism. Moreover, electron microscopy in iPLA$_2$$\gamma$ KO mice with anti-GBM nephritis showed severe abnormalities in podocyte ultrastructure. These in vivo results are in keeping with earlier studies, which showed that overexpression of iPLA$_2$$\gamma$ in cultured GECs reduced complement mediated GEC injury (114), suggesting that expression of iPLA$_2$$\gamma$ is cytoprotective.

Treatment approaches to idiopathic membranous nephropathy have often relied on nonspecific immunosuppressive agents and have not been necessarily based on the understanding of disease mechanisms. Sometimes the treatment relies on controlling the consequences of the disease rather than the cause. Although the
recent identification of PLA$_2$R as a major antigen in MN revolutionized the understanding of human MN, the pathogenic role of anti-PLA$_2$R antibodies is still unclear. Modulation of iPLA$_{2\gamma}$ enzymatic activity represents a potential novel approach to limiting GEC injury and maintaining a normal permselective barrier. Defining the role of iPLA$_{2\gamma}$ provides opportunities for development of novel therapeutic approaches to GEC injury and proteinuria.
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encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat Genet* **24**, 349-354


fatty acid release in the cell death pathway. Proteolytic fragment of type IVA cytosolic phospholipase A2alpha inhibits stimulus-induced arachidonate release, whereas that of type VI Ca2+-independent phospholipase A2 augments spontaneous fatty acid release. *J Biol Chem* **275**, 18248-18258


