Role of Calcium-independent Phospholipase $A_2\gamma$ in Glomerular Injury in Adriamycin Nephrosis in Mice

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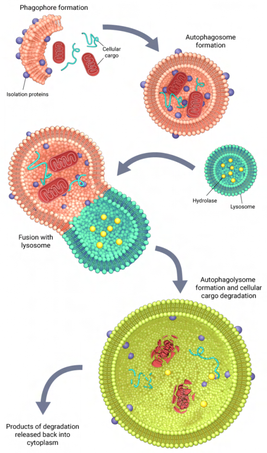
INTRODUCTION

Glomerular visceral epithelial cells (GECs) or **podocyts play a critical role in the maintenance of glomerular permselectivity.** These cells have a complex morphology characterized by cell bodies with projecting interdigitating foot processes that are bridged by filtration slit diaphragms. Various forms of glomerulonephritis are associated with podocyte injury, which may lead to impaired glomerular function (proteinuria), apoptosis, and glomerulosclerosis.



Autophagy is an essential "self-eating" process that begins with formation of a double membrane structure, the **phagophore**, which engulfs a portion of the cytoplasm. Numerous proteins are involved in assembly of **autophagosomes**. Among these, **microtubule-associated protein 1 light chain 3 (LC3-I &II). LC3-II is commonly used as a marker of autophagy.** Autophagosomes fuse with lysosomes to form autolysosomes. Malformed proteins or damaged organelles, are degraded by lysosomal hydrolases. **Autophagy recovers amino acids and fatty acids, thereby facilitating cell survival.**

We reported previously that calcium-independent phospholipase $A_2\gamma$ **(iPLA$_2\gamma$) mRNA and protein are expressed in the glomerulus in vivo. iPLA$_2\gamma$ is cytoprotective** in complement-mediated GEC injury. Moreover, genetic ablation of iPLA$_2\gamma$ in mice results in striking mitochondrial ultrastructural abnormalities and enhances the number of autophagosomes in podocytes, and leads to loss of podocytes in aging mice, without detectable albuminuria (Elimam, H. et al. J Biol Chem 291, 14468-82 (2016)). **In anti-GBM nephritis, deletion of iPLA$_2\gamma$ exacerbated albuminuria. Thus, iPLA$_2\gamma$ has a protective functional role in the normal glomerulus and in glomerulonephritis. Our studies in cultured GECs verified that deletion of iPLA$_2\gamma$ is associated with mitochondrial dysfunction and enhanced autophagy.**

RATIONALE & OBJECTIVES

Given the importance of mitochondrial function and autophagy in the maintenance of homeostasis in podocytes, and to further address the role of iPLA$_2\gamma$ in glomerular injury, our focus has been on the interaction of iPLA$_2\gamma$ with these two processes. The physiological functions of iPLA$_2\gamma$ in the mitochondria of podocytes have not been fully delineated.

The aim of the present study is to address the role of iPLAγ in glomerular injury, focusing on mitochondrial function and autophagy.

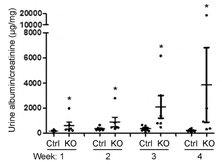
METHODS

Adriamycin nephrosis was induced in wild type (WT) or iPLA γ KO mice (age 3.5-4.5 months) by a single intravenous injection of adriamycin (12 mg/kg). Urine was collected at weekly intervals in the morning. After 4 weeks, kidneys were collected for IF microscopy, and glomeruli were isolated utilizing a differential sieving technique.

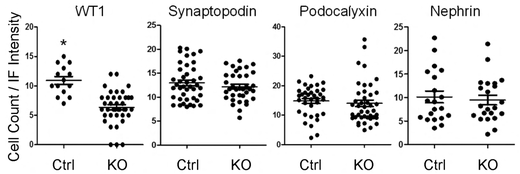
Cultured WT or iPLA$_2\gamma$ KO GECs were transfected with mito-YFP (to label mitochondria), RFP-LC3 (to label autophagosomes) and RFP-LAMP1 (to label lysosomes). Colocalization of fluorescent signals was measured by the Pearson correlation coefficient.

RESULTS

Deletion of iPLA$_2\gamma$ exacerbates albuminuria and reduces podocyte number in adriamycin nephrosis

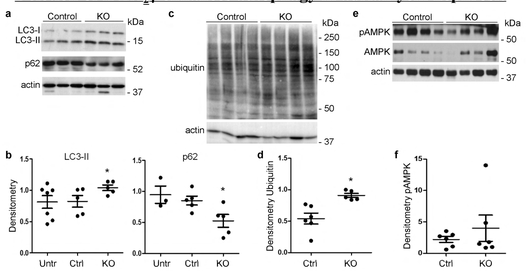


Control (Ctrl) and iPLA$_2\gamma$ KO mice were injected with adriamycin (12 mg/kg). Urine was collected at weekly intervals for up to 4 weeks. *P<0.001 KO vs control; 6 mice per group.



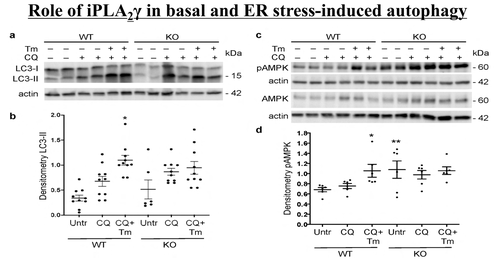
Kidney sections were stained with antibodies to WT1, synaptopodin, podocalyxin and nephrin. The number of WT1 positive nuclei (reflecting number of podocytes) was lower in KO mice. *P<0.0001 KO vs control, 14 measurements in control group (4 mice) and 39 in KO group (5 mice). There are no significant differences between control and KO mice in IF staining intensity of synaptopodin, podocalyxin, and nephrin. 20-40 measurements in control group (4-6 mice) and 22-41 in KO group (4-6 mice).

Deletion of iPLA$_2\gamma$ enhances autophagy in adriamycin nephrosis

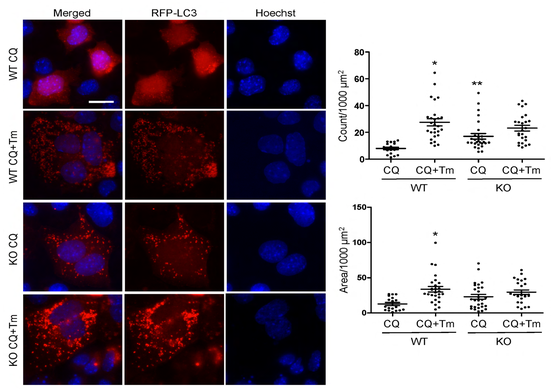


Glomeruli were isolated from control (Ctrl) and iPLA$_2\gamma$ KO mice 4 weeks after adriamycin administration. a and b) LC3-II/actin was increased and p62/actin was decreased in iPLA$_2\gamma$ KO mice. *P<0.035 KO vs control (adriamycin); 5 mice per group. In panel b, glomerular LC3-II levels in 6 untreated (Untr) mice (2 control and 4 KO) and p62 levels in 3 untreated mice (1 control and 2 KO) are shown for comparison. c and d) Glomerular lysates were immunoblotted with anti-ubiquitin antibody. *P=0.005 KO vs control, 6 control mice and 5 KO mice. e and f) Levels of AMPK were highly variable among mice, and while there was an upward trend, there was not a significant difference in pAMPK/AMPK between control and KO mice (6 mice per group).

Role of iPLA$_2\gamma$ in basal and ER stress-induced autophagy

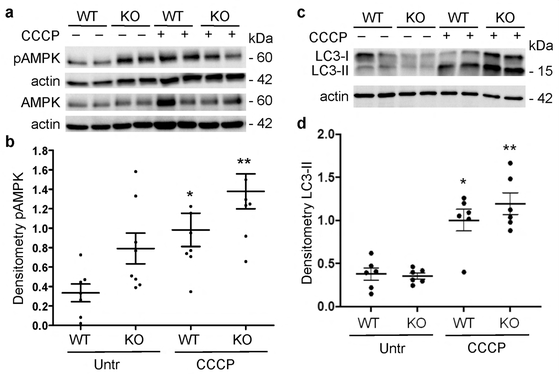


WT and iPLA$_2\gamma$ KO GECs were untreated (Untr), or incubated with or without chloroquine (CQ, 25 µM) and tunicamycin (Tm, 5 µg/ml) for 18 h. Lysates were immunoblotted with antibodies to LC3 (a) or AMPK and pAMPK (c). b and d) Densitometric quantification. b) LC3-II/actin *P<0.0001 KO vs WT (CQ+Tm), P=0.08 KO vs WT (CQ). 5 experiments performed in duplicate. d) pAMPK/AMPK *P<0.05 CQ+Tm vs Untr (WT), **P<0.05 KO vs WT (Untr). 3 experiments performed in duplicate.



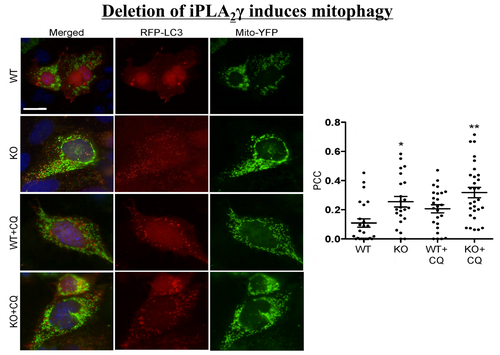
ER stress induce changes in LC3-II puncta. WT and iPLA$_2\gamma$ KO GECs were transfected with RFP-LC3, and were incubated with or without chloroquine (CQ, 25 µM) and tunicamycin (Tm, 5 µg/ml) for 18 h. Representative photomicrographs and quantification of LC3-II puncta are presented. Bar = 25 µm. Puncta count: *P<0.001 CQ+Tm vs CQ (WT), **P<0.05 KO vs WT (CQ). Puncta area: *P<0.001 CQ+Tm vs CQ (WT). 18-28 cells per group in 2 experiments.

Effect of iPLA$_2\gamma$, carbonyl cyanide m-chloro-phenylhydrazone (CCCP) on pAMPK and LC3-II

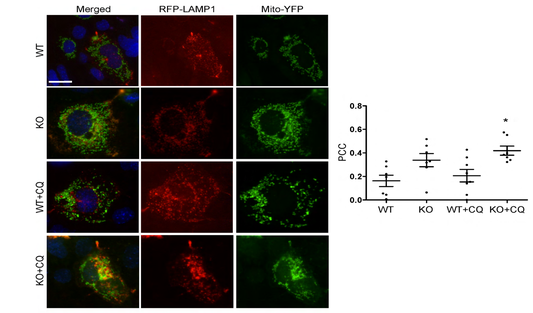


WT and iPLA$_2\gamma$ KO GECs were untreated (Untr), or incubated with CCCP (10 µM) for 18 h. Lysates were immunoblotted with antibodies to AMPK and pAMPK (a) or LC3 (b). a and c) Representative immunoblots. b and d) Densitometric quantification. B) pAMPK/AMPK *P<0.05 CCCP vs Untr (WT), **P<0.05 CCCP vs Untr (KO). 3 experiments performed in duplicate. d) LC3-II/actin *P=0.001 CCCP vs Untr (WT), **P<0.0001 CCCP vs Untr (KO). 3 experiments performed in duplicate.

Deletion of iPLA$_2\gamma$ induces mitophagy

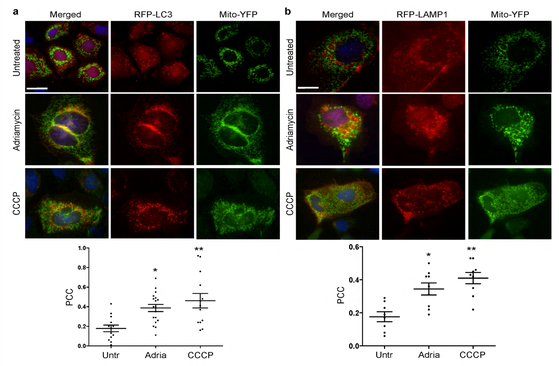


WT and iPLA$_2\gamma$ KO GECs were co-transfected with RFP-LC3 and mito-YFP cDNAs. Cells were then incubated with or without chloroquine (CQ) for 6 h. Representative photomicrographs and the Pearson correlation coefficient (PCC) for the colocalization of RFP-LC3 and mito-YFP are presented. Bar = 25 µm. *P<0.01 KO vs WT, **P<0.05 KO+CQ vs WT+CQ. 23-29 cells per group in 4 experiments.



WT and iPLA$_2\gamma$ KO GECs were co-transfected with RFP-LAMP1 and mito-YFP cDNAs. Cells were then incubated with or without chloroquine (CQ) for 6 h. Representative photomicrographs and the Pearson correlation coefficient (PCC) for the colocalization of RFP-LAMP1 and mito-YFP are presented. Bar = 25 µm. *P<0.05 KO+CQ vs WT+CQ. 7-8 cells per group in 2 experiments.

Induction of mitophagy by adriamycin and CCCP



WT GECs were co-transfected with RFP-LC3 and mito-YFP (a) or RFP-LAMP1 and mito-YFP cDNAs (b). Cells were then untreated (Untr), or incubated with CCCP (10 µM) or adriamycin (Adria; 1 µM) in the presence of chloroquine (CQ) for 24 h. Representative photomicrographs and the Pearson correlation coefficient (PCC) for colocalization are presented. Bars = 25 µm. a) *P<0.05 Adriamycin vs Untreated, **P<0.01 CCCP vs Untreated. 13-18 cells per group in 3 experiments. b) *P<0.01 Adriamycin vs Untreated, **P<0.001 CCCP vs Untreated. 7-8 cells per group in 2 experiments.

SUMMARY

1- **Deletion of iPLA$_2\gamma$ exacerbated albuminuria and reduced podocyte number.**

2- Glomerular **LC3-II increased and p62 decreased in adriamycin-treated iPLA$_2\gamma$ knockout (KO) mice**, compared with treated control, in keeping with increased autophagy in KO.

3- **iPLA$_2\gamma$ KO GECs in culture demonstrated increased autophagy,** compared with control GECs.

4- iPLA$_2\gamma$ KO GECs showed increased phosphorylation of AMP kinase **(pAMPK), consistent with mitochondrial dysfunction.** Adriamycin further stimulated pAMPK and autophagy.

5- After co-transfection of GECs with mito-YFP (to label mitochondria) and RFP-LC3 (to label autophagosomes), or RFP-LAMP1 (to label lysosomes), there was **greater colocalization of mito-YFP with RFP-LC3-II and with RFP-LAMP1 in iPLA$_2\gamma$ KO GECs, compared with WT, indicating enhanced mitophagy in KO.**

CONCLUSION

iPLA$_2\gamma$ plays an important role in the maintenance of podocyte integrity both in health and disease. Understanding the mechanisms by which iPLA$_2\gamma$ maintains mitochondrial structure and function, and how damaged mitochondria may be repaired in the glomerulus is essential for **development of novel therapies for glomerular disease and proteinuria.**

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