Stony Brook University

Effects of Phospholipase D2 on Cardiovascular Function and Disease

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by

Rochelle K. Nelson

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in Partial Fulfillment of the

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Abstract of the Dissertation

Effects of Phospholipase D2 on Cardiovascular Function and Disease

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Cardiovascular disease is a major health concern for adults and youths in the United States. Over the past several decades many drug therapies have been designed to help combat this disease, however, the prevalence of risk factors (e.g., diabetes, obesity, hypercholesterolemia, and hypertension) is still high. According to the CDC, about 75 million American adults suffer from hypertension and 1,100 deaths each day are due to hypertension. Therefore, effectively controlling, and or preventing the occurrence of hypertension will lead to a further increase in life expectancy along with a better quality of life. To accomplish this, interventions that target specific genes associated with hypertension is imperative.

The phospholipase D (PLD) enzyme superfamily is known for its role in signal transduction leading to endocytosis, intracellular vesicle trafficking, cell migration, and survival. Novel physiological activities for PLD have been reported, including the role of isoform PLD2 in the development of hypertension and atherosclerosis because of its interaction with angiotensin II type 1 receptor and its regulation of peroxisome proliferator-activated receptor-γ.

In part, the work presented in this dissertation reveals a novel role for PLD2 in the development of hypertension through the endothelial nitric oxide synthase (eNOS) pathway. Using a $PLD2^{-/-}$ mouse model and shRNA gene silencing in endothelial cells, we show that $PLD2^{-/-}$ results in a decrease in eNOS abundance and subsequent nitric oxide (NO) production, ultimately causing a significant increase in mice systemic blood pressure.

Finally, this dissertation also elucidates PLD2's role in cholesterol internalization and the development of atherosclerosis. Using $PLD2^{-/-}$ mice and primary macrophage isolation, we that $PLD2^{-/-}$ mice may be more susceptible to arterial plaque deposition and development of atherosclerosis, due to a decrease in reverse cholesterol transport exhibited by a significant decrease in liver fatty accumulation and ATP-binding cassette transporter ABCA1 protein and mRNA levels.

Dedication Page

To my Sébastien.

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List of Abbreviations

ABCA1……ATP-binding cassette transporter A1

Ac-LDL……Acetylated LDL

ADMA……...Asymmetric dimethylarginine

Ang I……Angiotensin I

Ang II……..Angiotensin II

ACE……Angiotensin converting enzyme

AT1R…….Angiotensin II type 1 receptor

BP……...Blood pressure

BH4……...tetrahydrobioterin

Cav-1……Caveolin-1

CNS……..Central nervous system

cGMP……...Cyclic guanosine monophosphate

ER……Endoplasmic reticulum

eNOS……Endothelial nitric oxide synthase

EF……...Ejection Fraction

FIPI…….5-Fluoro-2-Indolyl des-Chlorohalopemide

FS……Fractional shortening

GWAS……..Genome-wide analysis study

GTP……..Guanosine triphosphate

HDL……High density lipoprotein

HFD……...High fat diet

HMG-CoA……..Hydroxymethylglutaryl-coenzyme A

iNOS…….inducible nitric oxide synthase

LCAT……Lecithin–cholesterol acyltransferase

L-NAME……L-N^G-Nitroarginine methyl ester

LDL……Low density lipoprotein

mRNA……Messenger RNA

miR……...microRNA

MSR1……Macrophage Scavenger Receptor 1

nNOS……Neuronal nitric oxide synthase

NFOT……N-[2-[1-(3-Fluorophenyl)-4-oxo-1,3,-8-triazaspiro[4.5]dec-8-yl]ethyl]-2-naphthalenecarboxamide

NO……...Nitric oxide

ox-LDL……Oxidized-LDL

PLD……Phospholipase D

PLD1……Phospholipase D1

PLD2……Phospholipase D2

PLD3……Phospholipase D3

PLD4……Phospholipase D4

PLD5……Phospholipase D5

PLD6/mitoPLD……Phospholipase D6

PA……..Phosphatidic acid

PC……..Phosphatidylcholine

PtdEtOH……Phosphatidylethanol

piRNAs……..Piwi-interacting RNAs

PX……Phox consensus sequence

PH……Pleckstrin homology domain

PM……Plasma membrane

RAAS……Renin-angiotensin-aldosterone system

RNAi…….RNA interference

VSMC……..Vascular smooth muscle cell

VLDL……Very low density lipoprotein

WT…….Wild-type

Preface

This dissertation has been developed from a review article published in The Journal of Lipid Research (Chapter 1) and a research article under review (Chapter 3). Chapter 2 and, Appendix A provide additional background and context for the genes, and mechanisms explored in Chapter 3. Chapter 4 presents conclusions and future directions for the work introduced in Chapter 3, and describes an additional project that has not yet been completed (which is supported by Appendix B).

Acknowledgments

Thank you, to everyone who has been instrumental in my growth and success as a doctoral student. Without your kindness, patience, guidance and support, I would not be the Scientist I am today.

Special thank you to Dr. M. Raafat El-Maghrabi. You have witnessed and guided my growth since my start as a doctoral student and have been a major source of support to me. There are not enough words to express my appreciation and gratitude for your never-ending belief in me.

Chapter 1

Physiological and Pathophysiological roles for Phospholipase D

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ABSTRACT

Individual members of the mammalian Phospholipase D (PLD) superfamily undertake roles that extend from generating the second messenger signaling lipid phosphatidic acid through hydrolysis of the membrane phospholipid phosphatidylcholine, to functioning as an endonuclease to generate small RNAs and facilitating membrane vesicle trafficking through seemingly non-enzymatic mechanisms. With recent advances in genome-wide association studies and RNAi screens, next-generation sequencing approaches, and phenotypic analyses of knockout mice, roles for PLD family members are being uncovered in autoimmune, infectious neurodegenerative, and cardiovascular disease, as well as in cancer. Some of these disease settings pose opportunities for small molecule inhibitory therapeutics, development of which is underway.

Phospholipase D overview

The mammalian Phospholipase D superfamily is best known for the catalytic action of its classical family members to hydrolyze phosphatidylcholine (PC), the most abundant membrane phospholipid, into choline and the second messenger signaling lipid phosphatidic acid (PA) [1]. As transphosphatidylases, classical PLD enzymes more formally conduct headgroup exchange at the terminal phosphodiester bond on PA [2] (Fig. 1). In the most common cellular setting, water is used as the nucleophile to exchange an –OH group for the choline headgroup [3]. However, because of a 1000-fold higher preference for primary alcohols [2], production of phosphatidylalcohol [4] is the primary outcome when even relatively modest amounts (1-3%) of ethanol or 1-butanol are present [5]. Phosphatidylethanol (PtdEtOH) has a long half-life relative to ethanol and can be detected in serum for up to a month subsequent to alcohol consumption [6], making it an increasingly popular biomarker for assessment of acute and even chronic drinking. PtdEtOH can be found at low levels even in non-drinking individuals though since intestinal bacteria generate small amounts of ethanol through fermentation. PtdEtOH is generally thought to be physiologically inert; however, there is some evidence to suggest that it may play a significant role in ethanol tolerance [7] and colon cancer [8]. Primary alcohols have historically been used to block production of PA by the classic mammalian enzymes PLD1 and PLD2 to assess their cellular signaling roles. However, alcohols have many other effects on cells, preventing definitive results from being obtainable with this approach [9, 10]. PLD2 can also use glycerol as the nucleophile to generate phosphatidylglycerol, which has been proposed to play roles in wound healing [11, 12]. PLD1 and PLD2 have been proposed to undertake roles in many cell biological and physiological settings as will be subsequently described.

Enzymatic activities have not been discovered for other members of the PLD family (PLD3, PLD4, and PLD5); PLD5 in fact has non-conservative substitutions in its putative catalytic site that make it very unlikely to be enzymatically active. PLD6 (MitoPLD) has been reported to hydrolyze cardiolipin on the outer surface of the mitochondria to generate PA [13] as well as to function as an endonuclease (via phosphodiesterase action) to generate specialized micro-RNAs known as piwi-interacting RNAs (piRNAs) [14] which are critical during spermatogenesis [15]. Despite the lack of evidence for PLD3 and PLD4 catalytic activity, they nonetheless have important functions, loss of which creates pathology as discussed below. Definitive cellular and physiological roles for PLD5 have not yet been identified.

Structure and regulation

PLD1 [16] and PLD2 [17], which are ~50% identical in primary sequence and have almost the same protein domain organization (Fig. 2), are widely expressed in different tissues and cell types, and are activated by a variety of signaling molecules including protein kinase C and the small GTPases RhoA and ARF [1, 18-20]. The PLD catalytic site is defined by the presence of two highly-conserved His-x-Lys-x-x-x-x-Asp sequences (x is any amino acid) termed the HKD motif [16], or more broadly, the PLD-c domain, each of which creates half of the split-catalytic site [21]. The HKD motifs are essential for PLD enzymatic activity [2]. A phox consensus sequence (PX), a pleckstrin homology (PH) domain, and an acidic $PI4,5P_2$ binding motif are also found and are highly conserved in PLD1 and PLD2. These regions function in regulating subcellular localization [22, 23] through protein-protein interactions [24] and binding to phosphatidylinositol phosphates [23, 25, 26]. PLD1 sequence uniquely contains an internal loop region that negatively regulates its activity [27] and thus may constitute the mechanism underlying the observation that PLD1's level of basal activity is lower than that of PLD2 [28].

PLD3 (Hu-K4, SAM-9) encodes an abundant and widely-expressed type 2 transmembrane protein that localizes to the endoplasmic reticulum (ER) and is anchored there by an N-terminal transmembrane domain and short cytoplasmic sequence, with the putative catalytic domain localizing to the lumen of the ER [29] (Fig. 3). Similar to PLD1 and PLD2, PLD3 encodes two HKD motifs; however, it lacks both the PX and PH domains [29, 30]. PLD3 has been linked to cellular differentiation and survival [29, 31-33]. PLD4 is also an ER

transmembrane glycoprotein that contains the canonical pair of HKD motifs and lacks PX and PH domains [34].

PLD6 / MitoPLD [13] is most closely related to the bacterial protein Nuc [35], which is an endonuclease. PLD6, however, contains an N-terminal extension that localizes it to mitochondria as well as anchoring it into the outer leaflet of the mitochondrial surface [13] (Fig. 4). PLD6 encodes only one HKD motif and dimerizes to exhibit catalytic activity. PLD6 promotes mitochondrial fusion, and through its ability to recruit Lipin-1, a PA phosphatase [36], PLD6 indirectly facilitates mitochondrial fission [15]. PLD6 also encodes an endonuclease activity that is required to generate piRNAs during spermatogenesis [14].

Physiological and pathophysiological roles

Phospholipase D1

Roles for PLD1 in thrombotic disease [28, 37, 38], cancer [39], and auto-immunity [40] based on animal model studies have recently been identified [5]. Many other possible roles are suggested by cellular studies that have not yet been addressed *in vivo*, some of which will be reviewed here.

PLD1 and cancer

PLD1 expression and activity are increased in many types of cancer [20, 41-43]. However, the significance of this observation is uncertain since PLD1's chromosomal location, 3q26 is adjacent to that of PI3Kinase-α, which is strongly amplified in numerous cancers. Most groups have studied the role of PLD1 in tumor cell viability, proliferation, and invasion, whereas our group has also shown a role for PLD1 in the tumor environment; in the context of facilitating tumor neoangiogenesis and subsequent metastasis [39]. As an example of the types of studies that have been reported, we will review the association of PLD1 with gliomas here.

Gliomas are the most common primary tumors of the central nervous system (CNS), diagnosed at a rate of 17,000 new cases per year in the United States. Despite clinical management, survival time after diagnosis is dismal, averaging between 12 to 15 months. Current treatment modalities consist of concomitant radiotherapy and chemotherapy, but are suboptimal in slowing disease progression. Patients incur frequent clinical complications including seizures, fluctuating neurological symptoms, and adverse effects of chemotherapy. PLD1 has been proposed to play important roles in the invasive migration of glioma cells [44],

glioma cell proliferation [45], cell adhesion [45], and viability [46]. The tumor signaling pathways and mechanisms relevant to PLD1 function are complex and have been proposed to include activation of AKT [46], upregulation of HIF1-α [47], and increased VEGF [47] and MMP-2 secretion [48]. Overlapping roles have been proposed for PLD2 [49]. Small molecule inhibitors of PLD1 and PLD2 such as FIPI [50] or isoform-selective analogs [20] have been shown to have dramatic effects on human glioma cell lines in tissue culture studies in the context of the PLD-driven roles above. How useful suppression of PLD activity will be for management of gliomas *in vivo* though remains to be determined.

PLD1 and Fibrosis

PLD1 has been speculated to participate in the process of fibrogenesis in multiple tissue types including liver [51, 52], lung [53], and the heart [54, 55]. PLD1 is known to be directly connected to autophagy [56, 57], the self-degradative process required for cellular homeostasis that is linked to several forms of liver disease [58-60]. Based on recent reports, cardiac fibrosis is of particular interest. PLD mRNA, protein, and activity levels decrease during congestive heart failure, subsequent to myocardial infraction in the scar tissue [54]. The importance of this observation is corroborated by a report that inhibition of PLD activity markedly attenuates left ventricular fibrosis, resulting in subsequent improvement in cardiac function [61]. PLD would thus seem to be an attractive therapeutic target for scar remodeling and reducing left ventricular fibrosis. On the other hand, PLD1 deficiency, which blunts immune responses [62], hinders immune-driven elements of the repair process after myocardial infarction [38]. Thus, there may be a balance between too little and too much PLD1 activity in this setting or specific sites at

which PLD1 expression is harmful or beneficial. Similarly, there may be specific times during the repair process when PLD1 elevation is either helpful or harmful.

Phospholipase D2

Roles for PLD2 in thrombotic disease [37, 63], cancer [64, 65], Alzheimer's disease and immune function [62] based on animal model studies have recently been summarized [5]. Other potential functions have been raised by tissue culture studies, some of which will be reviewed here.

PLD2 and Influenza

Influenza epidemics and reccurring pandemics continue to pose a great threat to public health worldwide, in part due to the viruses' high mutation and replication rates [66, 67]. As a consequence, treatment and prevention measures for influenza virus infections remain challenging. For example, in this current flu season, for which the immunization cocktail was largely ineffective due to being directed at the incorrect strains, the anti-influenza therapeutic amantadine was also found to be of relatively little benefit due to extensive acquired viral resistance to it. Thus, new therapeutic approaches are needed. A genome-wide RNA interference (RNAi) screen identified 287 human host cell genes that influence the viruses' ability to replicate, 29 of which were required for all the viral strains tested. Among these, PLD2 was identified as a targetable candidate [68]. A subsequent study, using an isoform-selective PLD2 inhibitor, further supported a critical role in the viral replication process: PLD2 was found to mediate rapid endocytosis of the virus, facilitating its escape from innate immune detection [69]. Given that mice lacking PLD2 appear grossly normal to inspection, PLD2 would appear to fit the category of a "temporarily dispensable host gene" that could be acutely targeted to suppress viral replication. One caution for this approach would entail potential effects of PLD2 inhibition on the immune system, which were previously reported to decrease macrophage phagocytosis and

neutrophil migration [62]. However, this might not be a substantive issue if the effects on the immune response to influenza were limited, whereas the effects on viral replication are profound.

PLD2 and Cancer

PLD2 polymorphisms, as well as up-regulated protein activity levels have been observed in several types of cancer including gastric, colorectal, kidney and breast [65, 70-72]. In a particularly interesting recent report, it was observed that expression of microRNA (miR)-203 in high WHO grade glioma tissues was significantly lower than in low WHO grade gliomas and normal brain tissue. Transfection of a miR-203 mimic into human glioma cells strongly and directly downregulated PLD2 expression, and simultaneously suppressed proliferation and invasion of the glioma cells, whereas PLD2 overexpression rescued the effects induced by the miR-203 mimic. Taken together, these observations suggest important causal roles for PLD2 in glioma proliferation and invasive capacity [49]. In a human breast cancer xenograph model, it was shown that increased PLD2 expression in tumor cells suppresses apoptosis, ultimately facilitating tumor growth and chemoresistance [65]. PLD2 may also play roles in the tumor environment similar to those previously reported for PLD1 [39], since PLD2 ablation from endothelial cells suppresses their hypoxia-induced Hif1-α expression and VEGF secretion, reducing proximal tumor neovascularization and growth [64]. Although the overall expression levels of PLD2 may vary in tumors, there is a significant correlation between PLD2 expression level and tumor size ($p<0.05$) as well as with survival of patients with colorectal carcinoma ($p<0.05$) [73]. Immunohistochemical staining of 30 human colon cancer samples revealed a high level of correlation between Hif1-α and PLD2 [74]. Moreover, Hif1-α and PLD2 expression

levels are much higher in colon cancer tissues than in normal colon tissues ($p<0.01$) [74], and under hypoxic conditions, Hif1-α upregulates PLD2 expression in colon cancer cells [74]. Like PLD1, PLD2 should also be viewed as a major therapeutic target in the treatment of several forms of cancer.

Phospholipase D3

PLD3 and Alzheimer's disease

PLD1, PLD2 and PLD3 have all been implicated in Alzheimer's disease (AD) [75-77]. PLD3 is highly expressed in the brain, including, but not limited to, mature neurons of the forebrain, the hippocampus and cortex [77-79]. Rare coding variants in PLD3 have been associated with up to 9% of late-onset AD in 14 families of European ancestry [77] (Fig. 5). More specifically, Val232Met, a putative loss-of-function polymorphism, is proposed to increase pathogenic amyloid peptide (Aβ) secretion and hence increase the risk for late-onset AD [77]. This increased risk is independent of the APOE genotype [77]. Similarly, PLD3 putative loss-of-function polymorphisms correlate with increased risk of AD in African-Americans [77]. Independent of the coding variants, PLD3 protein expression is down-regulated in AD brains [80] and in cortical membrane lipid rafts prepared from the 3xTgAD murine model of AD [81].

The mechanism of action of PLD3, as well as whether it encodes any type of catalytic activity, is still unknown, but its placement in the ER and secretory system raises suggestions as to how it might suppress Aβ secretion. AßPP, the precursor protein to Aβ, is proteolytically processed to generate Aβ in early endosomes, and the extent of this processing depends on how rapidly it is trafficked from the early endosome to late endosomes and lysosomes. Key in this process is the phosphatidylinositol-3-phosphate effector Hrs, an early endosome-associated ubiquitin-interacting motif (UIM)-containing protein that plays a central role in directing trafficking of membrane cargo proteins from early endosomes to luminal vesicles of multivesicular bodies for eventual degradation in the lysosome. Knock-down of Hrs or other

proteins required for the transport of AßPP from early endosomes to luminal vesicles of MVBs, results in increased amyloidogenic processing [82], supporting the general hypothesis that any defect that keeps AßPP and its processing enzyme BACE1 in endosomes will increase Aβ production and drive pathology [83]. Intriguingly, a recent screen for ubiquitinated proteins, specifically recognized by Hrs, identified 48 targets, among which were AßPP and PLD3 [84]. PLD3 has been reported in secretory granules in an insulin-producing pancreatic β-cell line [85] and in a pattern partially overlapping with lysosomes in HeLa cells [86], suggesting that PLD3 protein may traffic through endosomal pathways, even if the most abundantly observed steady-state location is in the ER in cultured cell lines [29, 32]. PLD3 has been identified in multiple screens for proteins that become ubiquitinated (e.g. [84, 87-89]). A key finding is that one of the two sites subject to ubiquitination is K11 in the short cytoplasmic N-terminal domain [87, 89] (Fig. 5); PLD3 thus undergoes cytoplasmic ubiquitination and could be recognized and sorted by Hrs to co-traffic with AßPP from endosomes to luminal vesicles of MVBs. Supporting this hypothesis, M6R, one of the highest scoring PLD3 alleles that associates with late-onset AD, lies close to K11 and could potentially affect ubiquitination, providing a basis for its disease linkage. Taken together, if ubiquitinated, Hrs-trafficked PLD3 plays a role in moving AßPP from early endosomes to luminal vesicles of MVBs for eventual lysosomal degradation, then decreased or non-ubiquitinated or non-functional PLD3 could cause AßPP retention in early endosomes and increased Aß production.

Potentially related, a screen performed for targets of the FBOX6 ubiquitin ligase complex, which triggers endoplasmic reticulum-associated degradation (ERAD) by mediating glycoprotein ubiquitination, identified 29 targets including PLD3 [88]. The ERAD system functions by

recognizing improperly folded glycoproteins and poly-ubiquitinating and transferring them to the cytosol to be degraded by proteasomes. The second PLD3 site that becomes ubiquitinated is in the C-terminal ER-luminal portion of the protein (K309, Fig. 5) [87] and would be a candidate target site for this mechanism. The PLD3 late-onset AD report [77] identified six disease-associated alleles that are predicted by Polyphen-2 [90] to be possibly or probably damaging and are located in or near putative glycosylation sites (Fig. 5). If these mis-sense mutations cause altered glycosylation or misfolding, then the ERAD system might target the PLD3 protein for degradation, causing a significant decrease in protein expression levels.

It is worth noting that none of the alleles identified encoded nonsense mutations (premature stop codons), suggesting that full or even heterozygous PLD3 loss might be deleterious. PLD3$^{-/-}$ mice have not been generated yet. A *drosophila* line with a P element insertion into its PLD3 homolog does exist and was found to be embryonically lethal when homozygous (unpublished). However, this line could have other genetic abnormalities or the P element could be affecting expression of other nearby genes, so additional studies would need to be performed to conclude that PLD3 loss creates lethality.

Phospholipase D4

PLD4 and Autoimmune Diseases

As is the case for PLD3, it is not known whether PLD4 has a bona fide enzymatic function. Nonetheless, PLD4 clearly has important functional roles. Initial reports described PLD4 expression in microglia, the macrophage-like innate immune cells of the CNS, as well as in splenic cells, presumably macrophages. PLD4 expression increases with microglial activation, which is also characterized by increased phagocytic capacity [34, 91]. siRNA knockdown of PLD4 suppressed phagocytosis, suggesting a role for PLD4 in the setting of CNS injury and infection [34, 91]. A nonsense mutation in PLD4 (W215X) in Fleckvieh cattle causes severe skin lesions, generally poor health, and decreased survival [92]. PLD4 deficiency in humans has been linked through genome-wide association studies to syndromes such as rheumatoid arthritis (RA) [93] and the autoimmune disease systemic sclerosis [94]. Taken together, these findings suggest that PLD4 deficiency results in hyper-activation of the immune system, causing a variety of autoimmune-like syndromes.

Phospholipase D5

PLD5 and Uterine Fibroids

Despite being very unlikely to manifest catalytic activity, PLD5 has been linked to a number of diseases, including a profibrotic uterine phenotype that occurs during childbearing years, and PLD5 polymorphisms may be associated with an increased risk of multiple cutaneous and uterine leiomyomatosis tumor progression [95].

PLD5 is most widely known for its correlation with neuropsychiatric disorders. Autism, the neurological disorder associated with impaired social relationships and communication as well as repetitive behavior, is predominantly linked to *de novo* and inherited copy number variants of genes important for neuronal development [96-98]. High-resolution genotyping of 1558 families on the autism spectrum uncovered a PLD5 gene polymorphism as possibly being connected with an autism phenotype [99]. Although the association signal of this SNP was borderline significant, further investigation is warranted, since autism has been proposed to be caused primarily by multigene interactions rather than solely by single rare mutations.

Phospholipase D6 (MitoPLD)

PLD6-deficient mice, which cannot generate piRNAs to suppress transposon mobilization during spermatogenesis, are completely sterile [15], but are otherwise grossly normal to inspection. PLD6 would not appear to be a major cause of human infertility; sequencing of PLD6 in 400 azoospermic European men did not uncover any PLD6 polymorphisms (unpublished observation). Nonetheless, PLD6 may have other, less obvious roles.

PLD6 and Cervical Cancer

Even with the current advances in the diagnosis and characterization of cervical intraepithelial neoplasia (CIN), highly discriminating biomarkers are still needed [100, 101]. Cervical cancer is the second most common cancer in women worldwide. In 2008, there were 529,800 cases of cervical cancer, with 14.5% occurring in developed countries and 85.5% occurring in developing countries, approximately 275,000 of which resulted in mortality [102]. Cervical cancer is caused by infection with the human papilloma virus (HPV) [103, 104]. Infection leads to the development of noninvasive neoplastic lesions, CIN [105]. CIN is premalignant transformation and dysplasia of the cervix, and is categorized into three major groups by the World Health Organization: CIN1, CIN2 and CIN3, where CIN1 is the least likely to progress into cervical cancer [106]. Without proper diagnosis or medical intervention 5-30% of CIN2/CIN3 (collectively CIN2+) patients develop cervical cancer; however, 10-40% of women diagnosed with CIN2+ exhibit spontaneous regression of the lesion [107]. This past year, PLD6 was identified as a predictive biomarker for regression of CIN2+ to CIN1 [101]. PLD6 was expressed in 12 out of 20 cervical punch biopsy samples taken from women 25-40 years old

who experienced spontaneous regression, whereas no PLD6 expression was found in any of the biopsy samples from women whose CIN2+ progressed to cervical cancer [101]. piRNAs can be recovered from the human HeLa cervical cancer cell line, suggesting that the machinery to generate piRNAs is functional in cervical tissue [108]. Adding measurement of PLD6 to current biomarkers for CIN2+ regression or persistence should further increase sensitivity in determining whether a patient's cervical intraepithelial neoplasia will spontaneously regress or persist and develop into cervical cancer.

Concluding Remarks

With many of the PLD-deficiency animal models only recently generated, the field is in an explosive period of discovery for roles undertaken by this fascinating superfamily of enzymes. Some of the associated pathophysiological roles reflect undesirable PLD activity, whereas others occur as a consequence of inadequate activity. With the on-going development of PLD small molecule inhibitors for several of the superfamily members, the former represent excellent therapeutic opportunities and it is likely that PLD1 and PLD2 inhibition will find their roles in several disease settings.

FIGURES

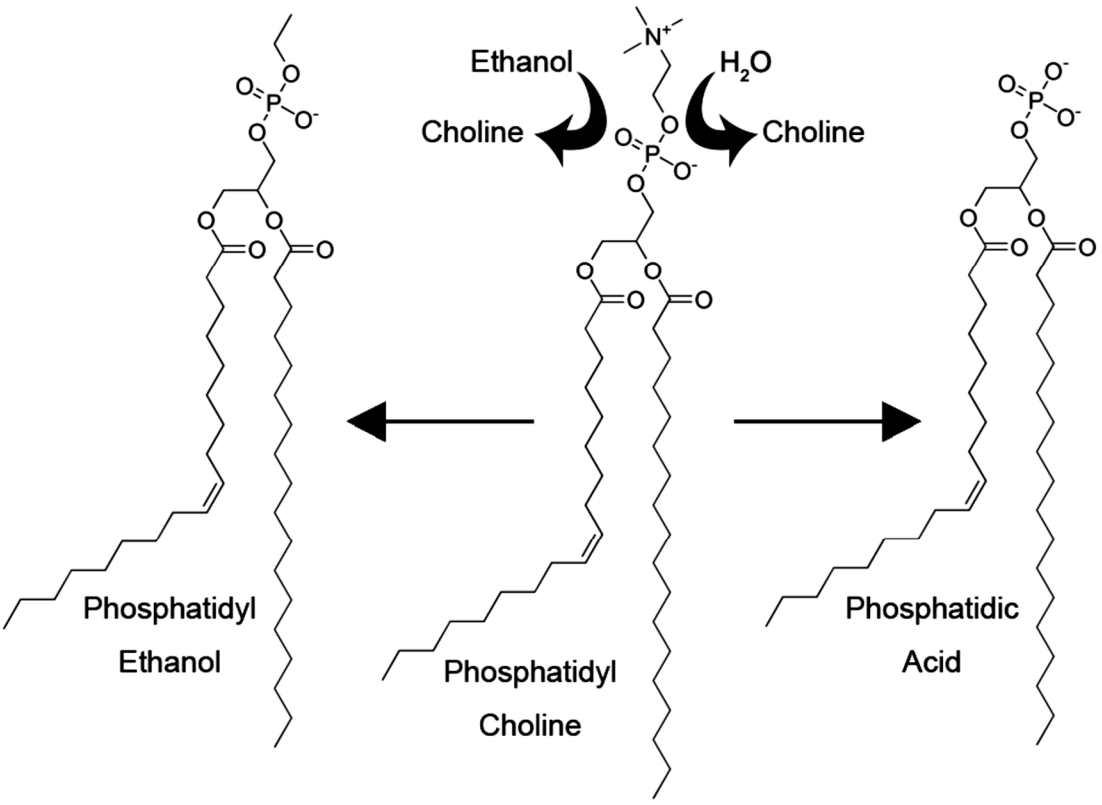

Figure 1-1. Schematic depiction of phospholipase D (PLD) generation of phosphatidic acid (PA) and phosphatidylethanol.

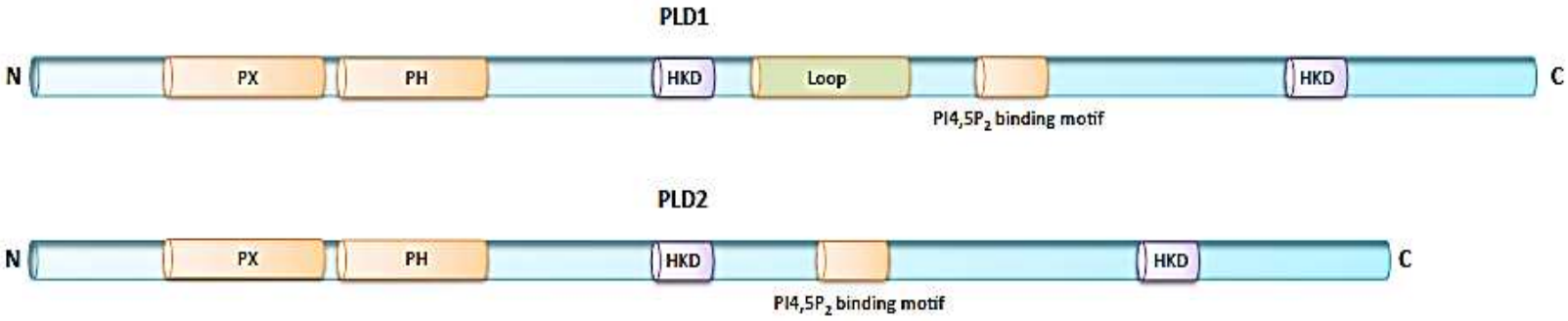

Figure 1-2. Schematic depiction of PLD1 and PLD2. PX, phox consensus sequence; PH, pleckstrin homology domain; HKD, PLD superfamily catalytic motif; loop, region found uniquely in PLD1.

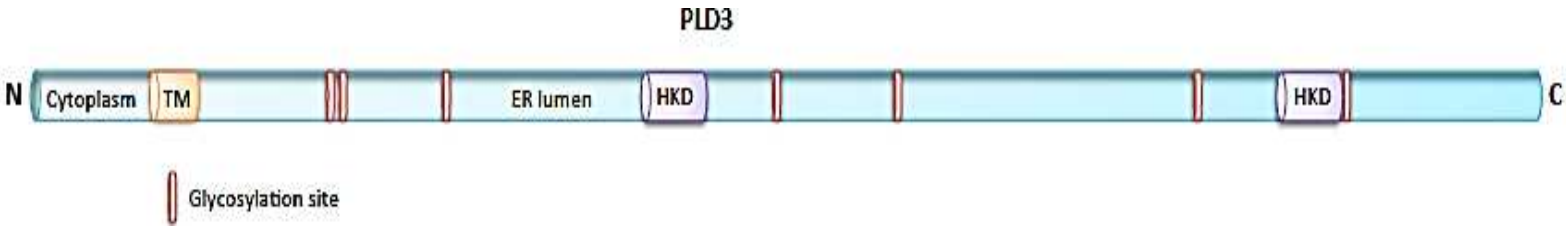

Figure 1-3. Schematic depiction of PLD3. Cytoplasm, localization of the N-terminal region; TM, transmembrane domain; ER lumen, localization of remainder of protein; pink vertical bars, glycosylation sites (29); HKD, PLD superfamily catalytic motif.

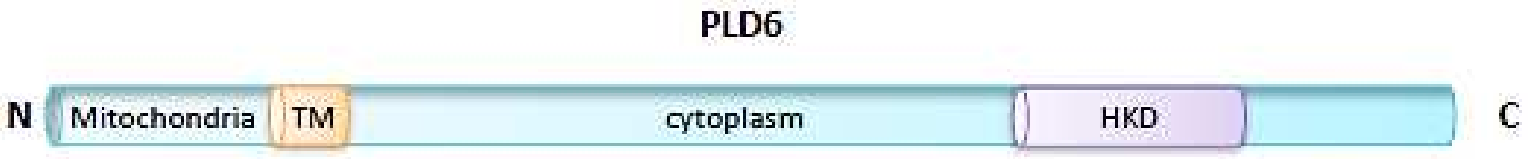

Figure 1-4. Schematic depiction of PLD6. TM, transmembrane domain that anchors PLD6 into the outer leaflet of the outer mitochondrial membrane; cytoplasm, localization of remainder of protein; HKD, PLD superfamily catalytic motif.

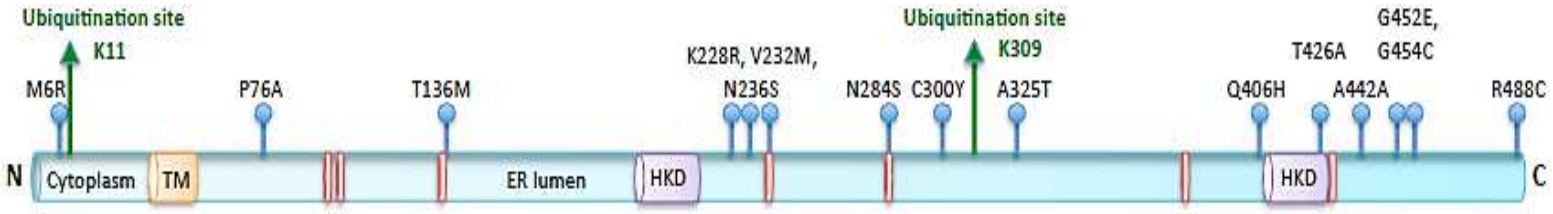

Figure 1-5. Key features of PLD3 protein sequence. Blue lollypops, alleles associated with late-onset AD (77); Green arrows, ubiquitinated lysines (87, 89).

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Chapter 2

Synopsis of Endothelial Nitric Oxide Synthase (eNOS) and Blood Pressure Regulation

ABSTRACT

High blood pressure is the chronic elevation of pressure in the arteries. 1 in 3 American adults, or 32% of Americans suffers, from high blood pressure. This disease is linked to an increased occurrence of cardiovascular and renal diseases. The regulation of blood pressure is highly modulated by concurrent activities of multiple physiological systems including the sympathetic nervous system, the renal system, and the cardiovascular system. Endothelial nitric oxide synthase (eNOS) is known to play a major role in the maintenance of cardiovascular health due to its activities in endothelial cells. eNOS assists in the regulation of blood pressure through its metabolism of L-arginine and the subsequent synthesis of free radical, nitric oxide (NO). This dissertation further elucidates eNOS' role in cardiovascular homeostasis through its production of NO following a decrease in phospholipase D2 (PLD2). To fully understand PLD2's effect on blood pressure regulation, an in depth look at the systems which regulate blood pressure is taken, as well as a summary of eNOS' activation and inactivation, and activity is provided.

Blood Pressure Overview

General look at Blood Pressure

Blood pressure is defined as the force of blood pushing against the arteries. Blood pressure readings provide two distinct and equally important values, the first of which being systolic blood pressure. Systolic blood pressure is a measure of the force created by the flow of blood within the arteries following the heart's contraction. Secondly, diastolic blood pressure is the decrease in blood pressure experienced by the arteries when the heart relaxes following a contraction. These two values together provide strong insight into the health and well-being of an individual's cardiovascular system. According to The National Heart, Lung and Blood Institutes, an individual who is classified as having a healthy/normal blood pressure generally has a systolic value of 120 mmHg or less and a diastolic value of 80 mmHg or less.

Individuals who do not maintain a healthy lifestyle, including inadequate exercise and weight management and excessive drinking and smoking are at a higher risk of developing high blood pressure. Along with lifestyle, one's genetics may also play a crucial role in the development of high blood pressure [1]. Genetic factors have been shown to account for approximately 30% of the blood pressure variation in different populations [2]. High blood pressure (clinically termed, hypertension) is the chronic elevation of pressure in the arteries. Hypertension has two distinct classifications: stage 1 hypertension and stage 2 hypertension. A stage 1 hypertensive individual is one who has a systolic blood pressure value of 140-159 mmHg and a diastolic blood pressure value of 90-99 mmHg. A stage 2 hypertensive individual is one

who presents with a systolic blood pressure value greater than 160 mmHg and a diastolic blood pressure value greater than 100 mmHg.

According to the Centers for Disease Control (CDC), 1 in 3 American adults or 32% of Americans suffers from hypertension [3]. More alarming, the morgue reports of 410,000 (1,100 American deaths per day) had hypertension listed as their primary cause of death in 2014 [4]. Hypertension is linked to an increased occurrence of cardiovascular and renal diseases. With every 20 mmHg increase in blood pressure, the risk of developing cardiovascular and renal diseases doubles [5].

According to the Behavioral Risk Factor Surveillance System, CDC, in the United States, the prevalence of hypertension varies geographically. The prevalence of hypertension is also linked to age, gender and race and ethnicity. In adults 45 years and younger, hypertension primarily affects men, and in adults 65 years and older, the disease primarily affects women [6]. Similarly, African Americans are noted to develop hypertension earlier in life and more often than their white, and Hispanic counterparts [6]. Luckily, hypertension can be controlled through various efforts including taking and complying with prescribed medications, significantly reducing the consumption of sodium and alcohol, increasing daily activity and quitting smoking.

Control of Blood Pressure: Sympathetic Nervous System

Short-term Response:

Chronically elevated blood pressure is sometimes due to an elevated sympathetic tone. The sympathetic system is one of two sub-parts of the autonomic nervous system. The sympathetic system is best known for providing the body's fight-or-flight response and includes noradrenergic ganglionic neurons which control the cardiovascular system [7]. Activation of the sympathetic nervous system stimulates the heart to pump blood throughout the circulatory system with increased force, where activation of the parasympathetic nervous system leads to a decrease in heart rate. For acute blood pressure regulation following changes in arterial pressure, the sympathetic system activates the baroreceptor reflex mechanism. The baroreceptors in the walls of blood vessels, once activated, send signals to the cardio regulatory center of the medulla oblongata in the central nervous system for processing. Inhibition of the baroreceptor system in canines showed that the normal 24 hour regulation of blood was lost [8]. Canines' blood pressure within the 24 hour period ranged from being hypotensive, to normal, to hypertensive [8]. This study was the first to provide an understanding of the short-term regulation of the sympathetic system on blood pressure regulation. In summary, the role of the baroreceptor reflex in regard to the cardiovascular system is now known to control a narrow blood pressure range throughout the 24 hour period [8]. Some ways in which this narrow blood pressure range is maintained by the baroreceptor reflex stems from the system's ability to respond to moment to moment changes in external influences (such as anxiety, excitation, fear etc.), changes in the organism's posture (e.g. moving from a lying to a standing position or from supine to the erect posture) and changes in diurnal rhythms (light and dark periods) [8].

The stretching of the blood vessels in response to increased blood pressure results in the activation of the baroreceptors. Once activated, the baroreceptors transmit this information to the medulla and two major modes of action take place to return the blood pressure back to normal. First, is a decrease in the systems sympathetic input and a subsequent increase in the parasympathetic (stimulates the body's rest-and-digest or feed and breed response) input to the heart. The result of this maneuver leads to a decrease in blood pressure due to a decrease in heart rate and stroke volume, and subsequent decrease in cardiac output and finally a decrease in blood pressure. Secondly, the medulla signals a decrease of sympathetic input to the blood vessels themselves, resulting in vasodilation which contributes to a decrease in blood pressure. In instances where baroreceptors detect a decrease in blood pressure, the opposite set actions are taken by the medulla resulting in an overall increase in blood pressure.

Moving from a supine to an erect posture is one of the body changes which activate baroreceptors. In individuals, whose autonomic nervous system is not functioning adequately this change in posture can lead to orthostatic hypotension. Orthostatic hypotension is best defined as low blood pressure classified as a 20 mmHg decrease in systolic blood pressure and or a 10 mmHg decrease in diastolic blood pressure within a couple minutes of moving to an erect position from that of a supine position. Individuals who suffer from this form of low blood pressure generally report symptoms of feeling dizzy or lightheaded upon standing. Orthostatic hypotension can also lead to a significant increase in peripheral venous blood pooling which then decreases stroke volume and cardiac output due to a decrease in the quantity of venous blood returning to the right atrium [9].

To adjust for orthostatic hypotension, the baroreceptors of the aortic arch, carotid sinuses and mechanoreceptors of both the heart and lungs are stimulated [9]. The baroreceptors of the carotid sinus are thought to be the main receptors responsible for the initial reaction to gravitational venous blood pooling [10-12]. In individuals' whose autonomic nervous system is impaired, the body's normal response to orthostatic hypotension is lessened and an increase in total peripheral resistance is seen [9, 13, 14].

Long-term Response:

The role of the sympathetic nervous system in long-term blood pressure regulation has been of great interest for some time now. Some studies have shown that this system may not significantly affect long term blood pressure [15] while other studies have shown that this system does indeed regulate long-term blood pressure, however, it does so through the renal sympathetic nerves (not via the aortic arch or carotid sinus) mediated by the release of aldosterone and regulation of the renin-angiotensin-aldosterone system [16]. Studies have shown that the sympathetic neural activity (which is used as a major index in the measurement of the sympathetic nervous system activity) is known to vary widely amongst hypotensive, normotensive and hypertensive individuals [15]. Meaning, a normotensive and a hypotensive individual can have the same amount of sympathetic neural activity despite the drastic differences in their cardiovascular health. Also, sympathetic neural activity is known to increase significantly with age [17, 18]. In aging individuals, the relationship between sympathetic neural activity and hypertension and heart failure become more closely linked [19].

Control of Blood Pressure: Renal System

The kidney is a major player in the maintenance of overall homeostasis of the body. Despite its significant importance, 10% of the entire world currently suffers from chronic kidney disease [20]. Arterial blood pressure is, in part, regulated by the kidneys' control of extracellular volume and renal arterial perfusion pressure. Pressure natriuresis is the process by which the kidneys control the urinary excretion of sodium in response to changes in renal arterial pressure. If blood pressure is too high, the renal arterial pressure will increase, thus causing the kidneys to increase the excretion of sodium (water follows) and reducing the extracellular fluid volume. Likewise, if blood pressure decreases, the renal arterial pressure will also decrease leading to a reduction in the excretion of sodium (water retention) and increasing the extracellular fluid volume.

Many molecules also affect the pressure natriuresis response. These include 20-hydroxyeicosatetraenoic acid (20-HETE), reactive oxygen species, NO, endothelin-1 and ATP [21-24]. The formation of 20-HETE from the metabolism of arachidonic acid is vital in the maintenance of renal function and subsequent arterial pressure. 20-HETE has been well documented to function as a vasoconstrictor, and has been correlated to an increase in oxidative stress as well as endothelial dysfunction. Deficiencies in 20-HETE formation are also known to contribute to the development of hypertension due to an inhibition in sodium transport resulting in sodium retention and hypertension.

In the kidneys, NO is also important for the overall regulation of blood pressure and the prevention of hypertension. In hypertensive rat models, a decrease in renal medullary NO is often observed in hypertensive rats [25]. An increase in renal arterial pressure is also known to result in an increase in medullary NO production [26]. Similarly, the inhibition of NO synthase within the renal medulla can cause a significant reduction in blood flow as well as reduce the excretion of sodium from the kidney, resulting in a retention of water and a subsequent maintenance of a hypertensive state [27]. NO also serves as a source of protection for the kidneys against chronic assault by vasoconstrictors, Ang II and vasopressin [28, 29]. NO's effect on the pressure natriuresis response is therefore a two-fold process; NO inhibits sodium transport in medullary cells and NO also causes an increase in renal interstitial hydrostatic pressure which ultimately results in a significant decrease in sodium reabsorption and water retention [30, 31].

Renal reactive oxygen species levels are also known to greatly affect the kidney's response to changes in arterial blood pressure. In contrast to the effects of NO, reactive oxygen species in large quantities are harmful to the blood pressure regulatory function of the kidney. Data has shown that individuals who suffer from hypertension generally also experience an imbalance in reactive oxygen species levels in the outer medulla of their kidney [32]. One source of harm from reactive oxygen species is their ability to counteract the activities of NO in the renal medulla [32]. Along with its opposing effects on NO, reactive oxygen species have also been shown to increase the transport of tubular sodium by the Na^+/K^+ ATPase as well as by the $Na^+/K^+/2Cl^-$ exchangers, which correlates with hypertension in several animal models [33-35].

In juxtaglomerular cells, ATP is known to stimulate the renin gene promotor through the $P2Y_{11}$ receptors as well as increase the release of renin - a key molecule in the renin-angiotensin-aldosterone system [36]. ATP's effects on blood pressure regulation also stem from its release from sympathetic nerves and its ability to produce an excitatory junction potential in juxtaglomerular cells as well as in smooth muscle cells [37]. This use of ATP as a cotransporter with noradrenaline ultimately leads to blood vessel vasoconstriction and a further release of renin from juxtaglomerular cells [37]. ATP also affects the microcirculation of the kidney. This control of microcirculation however, is a very complicated process, heavily dependent on circulating sodium levels, such that when animal models were sodium-restricted, ATP elicits an increase in medullary blood flow through vascular relaxation, while in salt-loaded animal models, ATP elicits vasoconstriction in the outer medulla [38].

The kidney's role in blood pressure regulation is also very dependent on one's genetics [1]. Previous studies have demonstrated that along with being born with the correct number of nephrons, an individual's likelihood of developing hypertension due to a mal-performing kidney is also critically dependent on that individual's genetic predisposition [39]. The number of nephrons and genetic predisposition both lead to an impaired pressure natriuresis response.

Most commonly studied is the effect of angiotensin II and its receptor angiotensin II receptor (AT1R) on the natriuretic response [40]. Excess activity of Ang II is significant enough to yield an increase in arterial blood pressure. One study where this relationship is evident is the infusion of Ang II in dogs which has been shown to produce salt-sensitive hypertension [41, 42]. A chronic infusion of Ang II elevates the intrarenal store of Ang II which then increases the rate

of sodium reabsorption, reducing the rate of renal sodium excretion, ultimately leading to an increase in arterial pressure [43, 44].

Some insights into the significance of Ang II's actions on AT1R and their effects on pressure-natriuresis stems from studies which genetically delete AT1R in the proximal tubule of kidneys. Such a deletion is shown to result in the loss of Ang II-induced hypertension [45]. Similarly, due to the loss of AT1R, overwhelming infusion of Ang II is not sufficient in inducing hypertension [46]. Together, these studies show that the loss of Ang II induced AT1R stimulation is protective against Ang II induced hypertension due to a disruption in the Ang II-NHE3 pathway which is harmful to the pressure-natriuresis response [45]. Similarly, genetic modifications, which result in single nucleotide polymorphisms (SNPs) are also significant enough to provide protection from renal mishandling of sodium and ultimate loss of blood pressure regulation. One such example is SNP rs13333226 found at the 5′ end of the *UMOD* gene which is associated with a significant decrease in blood pressure [47]. The *UMOD* gene is found on chromosome 16p12.3 and encodes the protein uromodulin. This protein is a glycosyl phosphatidylinositol anchored glycoprotein at the luminal face of tubular epithelia and is highly abundant in normal urine. Graham *et al.* showed that the deletion of *UMOD* in mice results in a lowered blood pressure as well as an increase in the kidney's ability to secrete salt [48].

However, not all gene mutations provide protection from the development of renal-mediated hypertension. One such example also considers the *UMOD* gene. Bleyer *et al.* identified four individual mutations in the *UMOD* gene that were found to be responsible for two major renal diseases, medullary cystic kidney disease 2 and familial juvenile hyperuricaemic

nephropathy, whose long-term complications include the development of severe hypertension [49].

Control of Blood Pressure: Vascular Tone

The circulatory system is closely linked to the function of the cardiac system. Blood vessels provide a conduit connecting the heart to the peripheral tissues allowing the movement of blood to these tissues. The vasculature is divided into three major vessel types based on their size and function (arteries, capillaries and veins) and are made up of three layers; the tunica intima (inner layer exposed to blood), the tunica media (middle layer) and the tunica externa (outer layer). The tunica intima is made up of a single layer (endothelium) of squamous cells known as vascular endothelial cells. These cells are in constant contact with the circulating blood and play a major role in the regulation of blood vessel function. The tunica media is made up of vascular smooth muscle cells and elastic fibers which work in tandem with the vascular smooth muscle cells to monitor vascular tone. The tunica externa is primarily made up of collagen fibers providing stability to the blood vessel.

Vascular tone is a major determinant of blood pressure based on the degree of constriction and maximal dilation experienced by a blood vessel. In an organism, the degree of constriction and dilation experienced by a vessel will vary depending on which organ the vessel is found in – larger organs such as the heart, skin and skeletal muscles have a large vasodilatory capacity and hence a higher vascular tone when compared to smaller organs, such as the kidneys that have low vasodilatory capacity and low vascular tone.

Many extrinsic (originating from outside the organ the vessel is found in) and intrinsic (originating from the vessel itself) factors determine the vascular tone of vessels. These factors

may either affect the degree of constriction experienced by the vessel and are therefore known as vasoconstrictors or may affect the degree of dilation experienced by the vessel and are known as vasodilators. Some extrinsic factors include Ang II, autonomic innervation of the vasculature and atrial natriuretic peptide. Extrinsic factors are known to either increase vascular tone through vasoconstriction while others may lead to a decrease in vascular tone. Intrinsic factors can also increase or decrease vascular tone through vasoconstriction and vasodilation, respectively. These factors stem from the vascular smooth muscle cells (myogenic) and endothelial cells (endothelin and nitric oxide). Hypoxia, metabolism of many substances (including arachidonic acid metabolites), histamine and bradykinin also serve as intrinsic factors.

Vascular tone – Extrinsic Factors (natriuretic peptides):

Natriuretic peptides participate in the long-term regulation of arterial pressure and blood volume. These peptides are released into circulation during moments of increased blood volume and blood pressure which lead to atrial and ventricular distension or mechanical stretching. There are two main forms of natriuretic peptides which are released from the heart. These include: atrial natriuretic peptide and brain-type natriuretic peptide. The mode of action (binding to the A type natriuretic peptide receptor leading to an increase in cyclic GMP and ultimate vasodilation and natriuresis) and synthesis of these peptides are similar and result in the same physiological effects.

Atrial natriuretic peptide is a 28-amino acid peptide hormone which is synthesized, stored and released from granules found in mammalian atria [50]. This peptide is released into circulation following an elevation in blood volume signaled by atrial distention, Ang II stimulation, endothelin release and sympathetic nervous system activation. In studies using $ANP^{-/-}$ mice models revealed that these mice experienced increased fluid retention and subsequently suffered from hypertension [51] while mice with increased levels of ANP suffered from long-term hypotension [52]. Therefore, atrial natriuretic peptide is an important component of circulatory homeostasis [53]. Upon release, atrial natriuretic peptide causes vasodilation of large arteries as well as natriuresis and diuresis via its effects on the kidneys to inhibit the renin-angiotensin-aldosterone system by the decrease in renin [53]. Taken together, the sum of atrial natriuretic peptide activity leads to a decrease in blood pressure (vasodilation) and a decrease in blood volume (natriuresis – sodium excretion, and diuresis – fluid excretion).

The creation of the biologically active peptide is a multi-step process stemming from the cleaving of a pre-prohormone which yields an active α-carboxyl terminal peptide [54, 55]. Atrial natriuretic peptide is first synthesized in cardiac myocytes as prepro-atrial natriuretic peptide, that is then cleaved into pro-atrial natriuretic peptide, that is finally cleaved into the biologically active form that is stored for later release.

Another type of natriuretic peptide is the brain-type natriuretic peptide. This 32-amino acid peptide is synthesized primarily by the ventricles but also in the brain. As with atrial natriuretic peptide, brain-type natriuretic peptide's activities also lead to vasodilation and a subsequent decrease in blood pressure as well as natriuresis and diuresis which lead to a decrease in blood volume. The synthesis of the active brain-type natriuretic peptide is like that of atrial natriuretic peptide. First a prepro-brain-type natriuretic peptide is created then cleaved into pro-brain-type natriuretic peptide and finally cleaved again into the active peptide. Unlike, atrial natriuretic peptide, brain-type natriuretic peptide has also been shown to be secreted from storage granules soon after its synthesis [56]. The actions of both natriuretic peptides are due to their interaction with the soluble guanylyl cyclase receptors. Both peptides very strongly antagonize the activities of the renin-angiotensin-aldosterone system making them key components in the regulation of blood pressure.

Vascular tone – Intrinsic Factors (endothelial cells – nitric oxide):

Endothelial cells are located on the inner lining (the intima) of the entire vascular system and are major regulators of vascular tone due to their ability to respond to many of the hormones, vasoactive factors and neurotransmitters which alter the vasculature [57]. This ability to respond to a large range of effectors makes endothelial cells extremely valuable in the maintenance of a homeostatic state in mammals. Endothelial cells are known to participate in the control of blood pressure, vasomotion, thrombosis and inflammation [57]. A properly functioning endothelium can protect the vasculature from the development and progression of many cardiovascular diseases like hypertension and atherosclerosis [58]. However, following damage, endothelial cells lose their ability to properly interpret these hormones and vasoactive factors, transforming the function of the endothelium to one which is harmful to the vasculature [58]. Such a change leads to endothelial dysfunction, an early indicator of the development of many cardiovascular diseases.

Endothelial cells are a primary source of the potent vasodilator, nitric oxide (NO). The work of Furchgott and Zawadzki was the first to identify NO as a potent vasodilator [59]. NO is a free radical which freely crosses the plasma membrane and has many important roles in human physiology as well in the development of many diseases. NO is synthesized from the metabolism of the amino acid L-arginine by a class of enzymes known as nitric oxide synthase (NOS) [60]. The major NOS enzyme which functions in endothelial cells is the endothelial nitric oxide synthase (eNOS) [61]. Once created, NO diffuses across the endothelial plasma membrane and into the layer of vascular smooth muscle cells below the endothelial cells. After traversing the

vascular smooth muscle cells, NO binds to and activates enzyme soluble guanylyl cyclase (sGC) which converts guanosine triphosphate (GTP) into cGMP [62, 63]. The newly synthesized cGMP promotes the sequestration of Ca^{2+} back into the sarcoplasmic reticulum, along with a subsequent decrease in myosin light chain kinase activity, and thus leads to the relaxation of vascular smooth muscle cells and a decrease in blood pressure [64].

Taken together, the activity of endothelium derived NO is important for its vasoactive abilities. Dysregulation in the production of NO by endothelial cells can be very detrimental the quality of life of mammals. In an $eNOS^{-/-}$ mouse model, the loss of eNOS activity was significant enough to produce a significant increase in leukocyte adhesion to the endothelium [65]. Such an increase can lead to an increased production of ox-LDL and foam cell formation, resulting in the development of atherosclerosis and hypertension. When produced at a normal physiological level, NO is shown to prevent these inflammatory responses and protect from atherosclerosis.

In conjunction with the vascular smooth muscle cells, endothelial cells are central to vascular homeostasis, and any deviation from the norm may lead to the development of endothelial dysfunction and subsequent cardiovascular disorders including atherosclerosis [66], hyperlipidemia and hypertension [67]. For this reason, the regulation of NO production via the enzymatic activity of eNOS is controlled by several factors.

PLD2 and Blood Pressure Regulation

The area of PLD2 and its role in blood pressure regulation is new and rapidly evolving. Researchers are now showing evidence that cancer is but one part of PLDs' major roles in disease development. Genetics plays a major role in the development of hypertension [68-70]. A genome-wide analysis study (GWAS) [71] and In Silico Bioinformatics [72] studies have all shown that several mutations in PLD2 greatly affects one's risk of developing hypertension. Using Chimera program 1.8, researchers are suggesting that this increased risk may in part be associated with a decrease in PLD2 protein stability [72].

There are two main physiological pathways which regulate systemic blood pressure; one system being the renin-angiotensin-aldosterone system (RAAS). PLD2 has been shown to facilitate the endocytosis of GPCR, Angiotensin II type 1 receptor (AT1R) [73]. Low blood volume triggers the release of renin, which then cleaves circulating angiotensin to yield angiotensin I (Ang I). Ang I is then further processed by angiotensin converting enzyme (ACE) in the lungs and kidneys to generate angiotensin II (Ang II). AT1R is the specific receptor for the now small polypeptide Ang II, which when bound to AT1R regulates the synthesis and release of aldosterone from adrenal cortex zona glomerulosa cells to increase renal Na^+ and water resorption [74].

Endothelial Nitric Oxide Synthase (eNOS) Overview:

The nitric oxide synthase or NOS enzyme family, consists of three major isoforms; neuronal, inducible, and endothelial NOS. Neuronal NOS (nNOS) whose enzymatic activity is most important in the nervous system. The inducible NOS (iNOS) that is stimulated by certain cytokines and endotoxins and therefore is most important in immune responses as part of the cell's defense mechanism, and finally, endothelial NOS (eNOS) which by its name is primarily found in endothelial cells and is vital to endothelial cells' function including the regulation of vascular tone. These isoforms are 50-60% homologous and share the presence of several domains including the heme-, L-arginine-, tetrahydrobiopterin (BH_4)-binding domains found in the N-terminal oxygenase domain, the calmodulin-binding domain found in the central region, as well as the NADPH, FAD and FMN binding sites found in the C-terminal reductase domain, all of which summarized by Stuehr [75].

eNOS requires the formation of multi-protein complexes with either calmodulin and heat shock protein 90 (Hsp90) or Cav-1 to either activate or inactivate its activities respectively [76]. The molecular chaperone Hsp90 assists in the folding and stability of eNOS as well as assists in disassociation of Cav-1 from eNOS by promoting the binding of the Ca^{2+}/calmodulin complex to eNOS, which confers positive effects on the enzyme's activity [77]. Protein-protein interactions occur between eNOS and Cav-1 creating an inhibitory eNOS/Cav-1 heteromeric complex and subsequent decrease in NO production [78, 79]. This interaction also serves to anchor inactive eNOS to its primary location on the plasma membrane in caveolae and requires the *N*–myristoylation and posttranslational cysteine palmitoylation of eNOS [80]. Endothelial cell activation by agonists bradykinin and acetylcholine [81] and mechanical hemodynamic stimuli

[82, 83] modulates transient increases in intracellular Ca^{2+} levels due to Ca^{2+} release from the endoplasmic reticulum. Influx of extracellular Ca^{2+} also leads to the activation of eNOS. Increased blood flow across the endothelium results in shear stress, this shear stress may lead to phosphorylation and activation of eNOS as well as allow the attachment of Ca^{2+} increasing agonists to receptors on endothelial cells [84]. The increased intracellular Ca^{2+} concentration leads to an escalation in the formation of Ca^{2+}/calmodulin complex in the cytoplasm which then binds to eNOS and assists in the allosteric activation of eNOS [85]. The Ca^{2+}/calmodulin complex instigates the dissociation of eNOS from Cav-1, resulting in an increase in NO production [86, 87]. When intracellular Ca^{2+} levels begin to decrease, the enzyme activating Ca^{2+}/calmodulin complex disassociates from eNOS allowing eNOS to re-bind with Cav-1 subsequently inactivating the enzyme.

Extensive loss of Cav-1 inhibition of eNOS has great implications in the development of cardiovascular diseases such as systemic hypertension. Multiple studies have shown that Cav-1 null mice experience hyperactivated eNOS which causes an increase in the development of a variety of cardiovascular pathologies [88-91]. In endothelial cells, $eNOS^{-/-}$ resulted in a classic hypertensive phenotype while over expressing eNOS protein in endothelial cells resulted in a marked decrease in blood pressure [92].

Regulation of eNOS also includes alterations of substrate L-arginine levels, as well as alterations in the levels of the endogenous eNOS inhibitor, asymmetric dimethylarginine (ADMA) [93]. Along with the fluctuation of calcium concentration, eNOS is also dependent on cofactors tetrahydrobiopterin (BH_4) and NADPH [94]. eNOS interactions are vital to the activity of eNOS. Loss of eNOS/BH_4 interaction can convert eNOS activity from one which is beneficial to the homeostasis of the vasculature through the production of NO, to one which is detrimental

to the vasculature due to the uncoupling of eNOS which results in the production of O_2^- (a situation which is termed "uncoupled NO synthase") [95]. eNOS uncoupling has been demonstrated in diseases like diabetes and hypertension.

Similarly, eNOS is highly susceptible to phosphorylation and de-phosphorylation at multiple serine and threonine sites by kinases and phosphatases. These kinases include protein kinase A, protein kinase B (Akt; following shear stress) and cyclic guanosine-3', 5-monophosphate (cGMP) protein kinase dependent II [96, 97]. In a coordinated manner, eNOS phosphorylation at Ser(1177) and dephosphorylation at Thr(495) is an activating signal where the phosphorylation of Thr(495) and the dephosphorylation of Ser(1177) is an inhibitory signal for the enzyme [98].

eNOS and the Plasma Membrane

The regulation of NO production in endothelial cells is also highly regulated via eNOS' subcellular localization [81, 99]. Much of a cell's free-cholesterol content is found in its plasma membrane and is necessary for maintaining the fluidity of the plasma membrane as well as assisting in several signal transduction pathways. eNOS is primarily localized to the specialized cholesterol and caveolin rich microdomain, caveolae [100]. The localization of eNOS to these cholesterol-enriched caveolae is a result of the *N*–myristoylation and posttranslational cysteine palmitoylation of eNOS (Cys-15 and Cys-26) [101-104]. *N*–myristoylation is thought to target eNOS to the plasma membrane, and once localized; palmitoylation stabilizes the enzyme's anchoring in the membrane. While at the plasma membrane, eNOS can receive several activating signals from vasoactive agonists such as bradykinin and acetylcholine. These agonists lead to an increase in intracellular calcium, which signals the binding of calcium/calmodulin to eNOS, thereby activating the enzyme [105]. Hence, eNOS' localization at the plasma membrane is important for its activation and production of NO.

Sessa *et al.* have shown that a loss of *N*-myristoylation of eNOS effectively abolishes the enzymes ability to localize to the plasma membrane, resulting in a cytosolic localization as well as a loss of eNOS activity [80]. Studies have shown that cholesterol in plasma membrane caveolae are crucial for eNOS localization and activation, and drugs like cyclodextrin [106], or the oxidation of membrane cholesterol [107], or the depletion of cholesterol from the plasma membrane [108] are all efficient in decreasing eNOS' activity and production of NO. Similarly, endothelial cells incubated with an external source of cholesterol exhibit an increase in caveolae content as well as an increase in NO release [109].

With atherosclerosis being one of the major risk factors for the development of endothelial dysfunction, it is not surprising that Statins, the class of drugs used to lower LDL cholesterol also provide several benefits to endothelial cells' health and function. Statins such as Simvastatin are used to directly inhibit the activity of hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the mevalonate pathway that produces cholesterol and isoprenoids. Several studies have shown that some of statins' benefits to endothelial cells and eNOS are due to strengthening eNOS' interactions with cofactors BH4 [110] and hsp90 [111]. Statins are also able to increase eNOS' activation via phosphorylation signals through the PI3K/Akt signaling pathway [112]. Most significantly, statins are known to stabilize eNOS mRNA through increased 3' polyadenylation [113], as well as increase eNOS gene transcription [114]. Direct inhibition of HMG-CoA reductase via statins independent of the drugs LDL lowering effects is sufficient to improve endothelial dysfunction experienced in humans suffering from atherosclerosis [110].

Caveolin-1

The fluid mosaic model explains the structure and overall function of the plasma membrane (PM) of animal cells. This model states that the PM is composed of several kinds of phospholipids, a vast number of proteins, and a large quantity of cholesterol [115]. Caveolin 1 is a membrane-bound structural protein which is associated with the flask-shaped invaginations known as caveolae [88, 116, 117]. Along with its link to several membrane-associated proteins, caveolae domains are also rich in sphingolipids and cholesterol [118, 119]. The cholesterol found in these microdomains allows for the association of the membrane-bound proteins with caveolae [120]. Smart et al. showed that Cav-1 migrates to the endoplasmic reticulum following the oxidation or damage of caveolae cholesterol. Once there, Cav-1 is then shown to transport newly synthesized cholesterol from the endoplasmic reticulum to caveolae on the plasma membrane of MA104 and normal human fibroblast cells [121]. Most significantly, Smart et al. also showed that caveolae fractions taken from systems lacking Cav-1 had a 3-4-fold decrease in cholesterol levels [121]. In this sense, a major part of Cav-1 activity is to regulate the necessary cholesterol found in caveolae [121].

Caveolae are linked to several disease states and are known to participate in many forms of cellular activity including reverse cholesterol trafficking and efflux [122] as well as acts as a chaperone for angiotensin type 1 receptor via the exocytic transport pathway to the plasma membrane [123]. Angiotensin II's proatherogenic activities are also shown to cause mislocalization and dysfunction of endothelial nitric oxide synthase (eNOS) in endothelial cells [124]. PLD2 activity is also implicated in many of the phenomena associated with caveolae and as a result was shown to be a regulator of many caveolae-dependent functions [73, 125-128]. The

expression levels and activity of PLD2 and Cav-1 in membrane microdomains are also known to be very closely linked [125, 129]. Czarny et al. found that PLD2 is highly localized in Cav-1 rich low density Triton-insoluble membrane domains. Other studies have also shown a direct relationship in PLD2 and Cav-1 mRNA levels, an upregulation in PLD2 mRNA levels was always associated with a correlated upregulation in Cav-1 mRNA in HT-29 MDR and MCF-7 AdrR cells [130]. In instances where PLD2 is knocked down, a similar decrease is observed in Cav-1 protein levels [125]. In coordination with these former experiments, PLD2 knockout mice and cell line used in the experiments presented in this dissertation also have a significant decrease in Cav-1 protein levels. This relationship between PLD2 and Cav-1 was maintained in our $PLD2^{-/-}$ mice (Figure 2-1) as well as in our Ea.hy926 endothelial cells, which were transduced with lentiviral particles to knockdown PLD2 as well as in Ea.hy926 endothelial cells, which were treated with PLD2 specific inhibitor NFOT (Figure 2-2).

With the growing knowledge on the roles of Cav-1 and caveolae; one can postulate some of the effects associated with a decrease in PLD2 protein levels may be due to PLD2's association and interaction with Cav-1. A decrease in PLD2 is correlated with a decrease in the caveolae-associated protein Cav-1 protein. With a subsequent decrease in Cav-1, the assembly of caveolae will be perturbed [88] causing significant effects on caveolae's role in lipid homeostasis, endocytosis and subsequent signal transduction events including the regulation of systemic blood pressure.

PLD2, Cholesterol, and eNOS

PLD is closely linked to the formation of triglyceride-rich lipoproteins known as very low-density lipoproteins (VLDL) [131, 132]. Activation of PLD by ADP-ribosylation Factor 1 in the liver is required for the production of VLDL [132]. Following secretion from the liver, VLDL particles are stripped of their triacylglycerol leading to the formation of the denser cholesterol-rich low-density lipoprotein (LDL). During circulation, LDL can be oxidatively modified yielding a lipoprotein particle which may have peroxides or the buildup of degradation products within the LDL particle. This modified LDL, now termed Oxidized-LDL (ox-LDL) is linked to several cardiovascular diseases [133-136]. Along with its roles in the formation of VLDL, PLD2 is also noted as a key member of cell signaling pathways related to cholesterol depletion in the plasma membrane [137]. Such modifications of the plasma membrane's cholesterol storage phenotypically lead to the perturbation of various signal transduction pathways [138] including that of eNOS [139]. Plasma membrane localized eNOS is more sensitive to activation by multiple agonists to produce NO and are also more sensitive to cholesterol changes than Golgi-bound eNOS [139].

High density lipoprotein (HDL) and LDL in serum act as regulators of the cholesterol content within caveolae. In the presence of ox-LDL and other agents which reduce the cholesterol levels of caveolae, the activity and release of NO by endothelial cells is significantly decreased [106, 140]. Ox-LDL disrupts caveolae formation and subsequently eNOS function by acting as a cholesterol acceptor [106], while, HDL is a source of cholesterol ester to caveolae allowing for the maintenance of eNOS activity. Subsequently, one of the major benefits of serum

HDL is its ability to prevent the ox-LDL induced uncoupling of eNOS which would lead to increased O_2^- production and systemic blood pressure.

The current dissertation provides novel insights in PLD2's role in blood pressure regulation through its effect on the endothelial nitric oxide synthase (eNOS) pathway. The research performed in this dissertation is the first to show that PLD2's activity is important in maintaining endothelial cells' eNOS protein abundance and activity, and consequently its importance in the production of nitric oxide (NO).

Hypothesis

Based on preliminary *in vivo* and *in vitro* studies used to characterize some of the major physiological changes in PLD2$^{-/-}$ mice compared to WT mice, the following hypothesis was developed: PLD2 has a central role in systemic blood pressure regulation through its effects on eNOS abundance due to a PLD2-mediated decrease in free cholesterol levels.

FIGURES

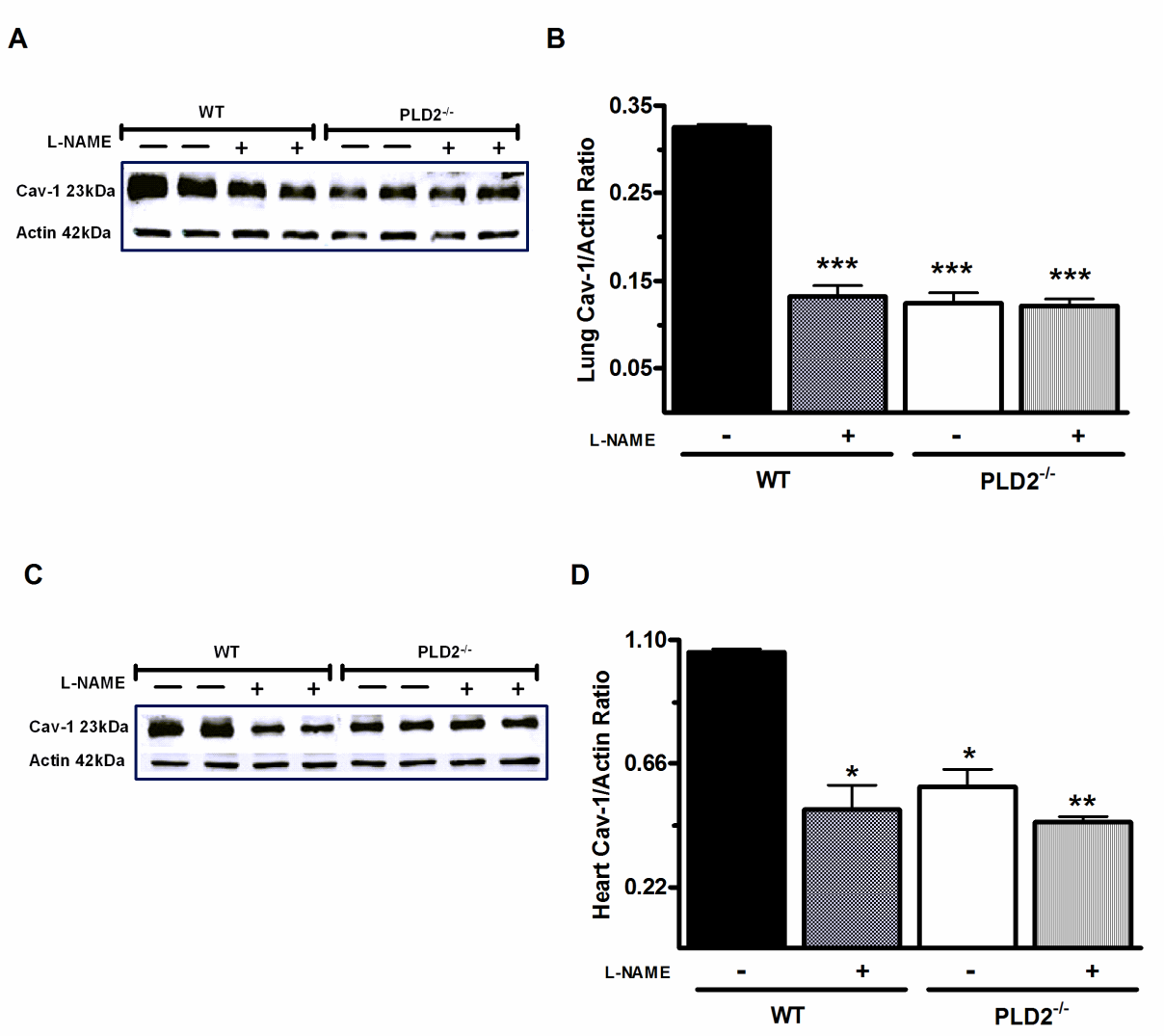

Figure 2-1. PLD2$^{-/-}$ mice lung and heart have a decrease in Caveolin-1 protein. Under normal conditions, PLD2$^{-/-}$ mice exhibit a decrease in lung (A and B) and heart (C and D) tissue caveolin-1 levels. Following treatment with eNOS inhibitor, L-NAME, WT mice caveolin-1 levels decreased, eliminating the significant difference between the groups. Western blot analyses of mice lung and heart extracts.

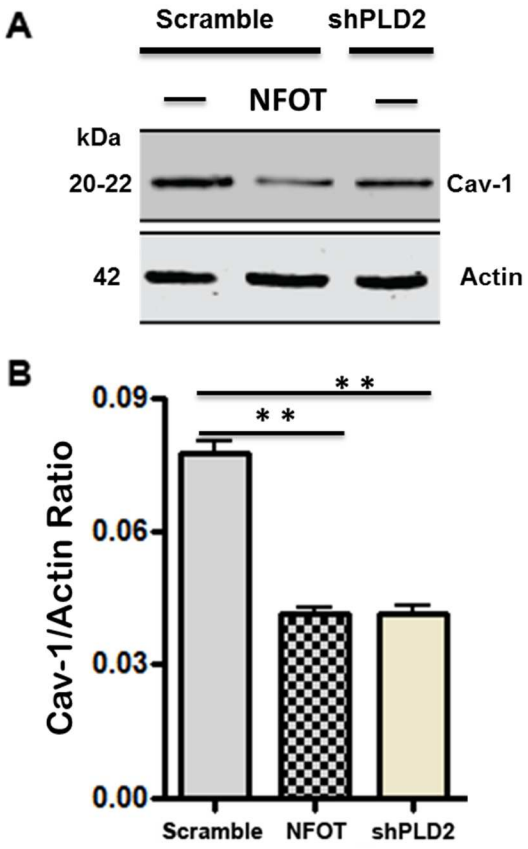

Figure 2-2. Loss of PLD2 activity leads to a decrease of caveolin-1 in Ea.hy926 endothelial cell line. Compared to WT Ea.hy926 endothelial cells (Scramble), PLD2 knockdown endothelial cells (shPLD2) have a significant decrease in caveolin-1 protein levels. Decrease in caveolin-1 is also observed in Scramble cells treated with PLD2 specific inhibitor NFOT. Western blot analysis of Wildtype and PLD2 knockdown endothelial cells.

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Chapter 3

Phospholipase D2 loss results in increased blood pressure via inhibition of the endothelial nitric oxide synthase pathway

ABSTRACT

The Phospholipase D (PLD) superfamily has been linked to neurological disease, cancer, and fertility. The recent report of a potential loss-of-function polymorphism in human *PLD2* that correlated with decreased blood pressure prompted us to examine this topic in mice genetically lacking *PLD2*. Surprisingly, *PLD2*$^{-/-}$ mice exhibit elevated blood pressure accompanied by associated changes in cardiac performance and molecular markers. *PLD2*$^{-/-}$ mice are slightly smaller than wild-type mice, have lower levels of total serum cholesterol and LDL, and have been reported to be resistant to high-fat induced glucose intolerance and weight gain, differentiating the current finding from the metabolic syndrome. Unexpectedly, we found decreased protein expression levels of endothelial nitric oxide synthase (eNOS), which generates the potent vasodilator nitric oxide (NO). An eNOS inhibitor phenocopied *PLD2* loss and had no further effect on *PLD2*$^{-/-}$ mice, confirming the functional relationship. Using the human umbilical vein endothelial cell line EA.hy926, we then delineated a pathway in which PLD2 loss of function lowers intracellular free cholesterol, causing upregulation of HMG Co-A reductase, the rate limiting enzyme in cholesterol biosynthesis. HMG Co-A reductase is known to function as a negative regulator of eNOS, and the PLD2-deficiency phenotype of decreased eNOS expression and activity could be rescued by cholesterol supplementation and HMG Co-A reductase inhibition. Together, these findings identify a novel pathway by which the lipid signaling enzyme PLD2 regulates blood pressure, which has implications for the on-going therapeutic development of PLD1 and PLD2 small molecule inhibitors. Finally, we show that the human *PLD2* polymorphism does not trigger eNOS loss, but rather creates another effect, suggesting a partial loss of function or possibly altered functioning for the allele.

INTRODUCTION

The classic Phospholipase D (PLD) isoforms, PLD1 and PLD2, hydrolyze phosphatidylcholine (PC), the most abundant membrane phospholipid, into choline and the second messenger signaling lipid phosphatidic acid (PA) [1]. PLD1 and PLD2 have partially distinct and partially overlapping / redundant roles *in vivo*, with current translational interest [2]; PLD1 being focused on thrombotic disease [3, 4], cancer [5], and the immune system [6-8], while for PLD2, Alzheimer's Disease [9], cancer [10], and influenza virus infection [11]. A recent study reported a negative correlation of a polymorphism in *PLD2,* R172C, with hypertension [12]. This polymorphism is in the lipid-binding regulatory phox consensus sequence (PX) domain that is conserved in PLD1 and PLD2. For PLD1, the PX domain binds phosphatidylinositol (3,4,5)-trisphosphate, which facilitates its localization to the plasma membrane and stimulates PLD1's enzymatic activity [13]. Both the PLD1 and PLD2 PX domains have also been reported to mediate interaction with signaling proteins such as epidermal growth factor receptor (EGFR) [14], facilitating its endocytosis. Arginine, as a basic amino acid, frequently found to bind to negatively-charged polyphosphoinositides and to protein targets via arginine fingers; thus, the substitution of cysteine for R172 could alter PLD2's cellular function.

PLD activity has been proposed to facilitate multiple pathways that function to increase blood pressure, including formation of very low-density lipoproteins (VLDL) [15, 16], which, following secretion from the liver, are metabolized to generate low-density lipoprotein (LDL) particles that can be pathogenically modified to form oxidized-LDL (ox-LDL), which oppose vascular relaxation and promote atherogenesis [17, 18]. PLD2 function has also been linked to endocytosis of the angiotensin II type 1 receptor (AT1R) [19], which promotes increased vascular tone and blood pressure via intracellular signaling, and to the production and secretion

of aldosterone [20, 21], which increases blood pressure by stimulating renal water and salt retention. Taken together, these reports provided multiple rationales for the observation that a polymorphism that could affect PLD2 function correlates with decreased blood pressure [12, 22].

Undertaking exploration of this topic, we uncovered, and report here, the unexpected observation that mice lacking PLD2 have *increased* blood pressure, and delineate the pathway through which PLD2 normally functions to lower blood pressure. Ultimately, we propose that the human R172C polymorphism creates a mutant protein with only partial loss of function or altered function, rather than PLD2 functional deficiency. Our findings have implications for therapeutic use of PLD2 inhibitors in other settings; the potential for increased blood pressure will need to be assessed and considered in terms of the risk and potential benefits.

MATERIALS AND METHODS

Materials. Simvastatin (Cayman Chemical) was used in culture for 24 hrs at 10 μM, N-[2-[1-(3-Fluorophenyl)-4-oxo-1,3,-8-triazaspiro[4.5]dec-8-yl]ethyl]-2-naphthalenecarboxamide (NFOT) (Tocris Bioscience) at 10 μM, and cholesterol (Sigma-Aldrich C8667) at 25 μM.

Animals. *PLD2*$^{-/-}$ mice (C57BL/6) [9], generously provided by Dr. Gilbert di Paolo (Columbia University), were bred in the Stony Brook University animal facilities. Mice were fed standard chow or high-fat diet chow and water *ad libitum* and kept on a 12:12-h dark-light cycle. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). Unless otherwise stated, male mice at 5 mos. of age were studied. Animals were injected with L-NAME (80210; Cayman Chemical) at a dose of 400 μg/g IP once a day for 15 days.

Non-Invasive Blood Pressure Measurements. Tail-cuff blood pressure (BP) readings were taken at days 0, 7, and 15 of L-NAME treatment using the CODA Multi-Channel, Computerized, and Non-invasive Blood Pressure System for Mice (Kent Scientific). BP measurements were taken twice during the two days leading to each acquisition date to acclimate the mice to the procedure. Blood pressure measurements were obtained by performing 5 acclimation cycles (a cycle = a BP reading) followed by 3 sets of 5 cycles per set.

Echocardiography. Echocardiogram measurements were taken after 15 days of L-NAME treatment using a Vevo 770 ultrasound device (VisualSonics) with a 30-MHz transducer. Mice were anesthetized with 1% isoflurane and transthoracic echocardiography performed as previously described [23].

Cell line creation and qRT-PCR. Human umbilical vein cell line EA.hy926 (CRL-2922; ATCC) was cultured in Dulbecco's modified Eagle's medium supplemented with L-glutamine (2 mM), 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were transduced with either Control shRNA Lentiviral Particles-A or with PC-PLD2 shRNA (h) Lentiviral Particles (Santa Cruz Biotechnology), which contain a puromycin-resistance gene to enable selection of stable cell lines or pools. The culture medium was changed 24 hrs following transduction and the cells cultured another day. Puromycin (0.5 μg/ml) was then added for positive selection. The resulting stably-transduced EA.hy926 cell pools were denoted "scramble" and shPLD2, respectively.

qRT-PCR. Total RNA was extracted with Trizol (Invitrogen). 200-300 ng of total cellular RNA was used with the qScript One-Step SYBR Green qRT-PCR Kit (Quanta Biosciences). Primers used for human HMG-CoA Reductase were: TGTGTGTGGGACCGTAATGG and ACCAAGTGGCTGTCTCAGTG; and for human GAPDH: ACAGTCAGCCGCATCTTCTT and GCATCGCCCCACTTGATTTT.

Western Blots. *In vivo* samples: Tissues were homogenized in lysis buffer (150 mM NaCl, 1% Triton X-100, 100 mM Tris-HCl, pH7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml pepstatin A), ultrasonicated at 4^0C, microfuged, and supernatants collected. Equal amounts of lysates (20 μg/lane) were separated on SDS-PAGE, transferred to filter paper, and immunostained using mouse anti-VEGF at 1:500 (ab3109; abcam) and rabbit anti-actin at 1:100 (A2066; Sigma-Aldrich). Cell line samples: Cells were cultured in six-well plates until 80-90% confluent, trypsinized, and equal amounts of total protein resolved by SDS-PAGE and blotted onto nitrocellulose membranes (162-0115; Bio-Rad) using NuPAGE Transfer Buffer (NP0006-1; Life Technologies). Membranes were blocked for 1-h in 5% BSA at room

temperature (RT) and probed overnight (O/N) with rabbit anti-eNOS at 1:500 (NB300-500; Novus Biologicals), mouse anti-caveolin-1 at 1:500 (sc-53564; Santa Cruz Biotechnology), goat anti-HMG CoA reductase at 1:100 (GTX88456; Genetex), rabbit anti-PLD2 at 1:100 (sc-25513; Santa Cruz Biotechnology), and rabbit anti-Actin at 1:100 (A2066; Sigma-Aldrich) or rabbit anti-GAPDH at 1:500 (sc-25778; Santa Cruz Biotechnology). Membranes were probed with anti-rabbit or anti-mouse secondary Ab at 1:3,000 and developed and quantified using a LI-COR Odyssey Infrared Imager.

Immunofluorescent Staining and Microscopy. Cryosections (8 μm) of fixed aortas from age-matched male mice on a normal chow diet were stained with rabbit anti-eNOS at 1:200 O/N and for 1 hr with DAPI and Alexa 647 anti-rabbit secondary antibody. Images were captured using a Leica TCS5 Confocal Microscope. Cells were cultured in 12-well plates on glass coverslips until 80-90% confluent, washed with PBS, fixed with 4% paraformaldehyde, permeabilized with Triton X-100, blocked with 5% goat serum for 1 hr, incubated for 1 hr at RT with primary antibodies followed by secondary antibodies and DAPI for another hour, and imaged as above.

Free-cholesterol measurements. In vivo samples: 4-6-week old male mice were placed on a high fat diet chow (HFD) for a total of 7 months. At time points 0, 3 and 7 months, blood was collected retro-orbitally. Serum lipoprotein levels were assessed using the HDL & LDL/VLDL Cholesterol Quantification Kit (K613-100; BioVision) following the manufacturer's protocol. For cell lines, Scramble and shPLD2 cells were plated in 100-mm tissue culture treated plates until 80-90% confluent, rinsed with PBS, trypsinized and stored at -80^{o}C until ready for cholesterol extraction. Cholesterol was extracted by lysing the cell pellets in 200 μL 7:11:0.1 chloroform:isopropanol:Nonidet P-40 and incubating at 25°C for 10 min. The cell debris was removed by centrifugation for 10 min at 15,000 x g and the supernatant collected, dried with a

rotary evaporator followed by 30 min in vacuum, and resuspended in 50 µL isopropanol. Cholesterol concentrations were measured using the enzyme cholesterol oxidase [24], which converts cholesterol to cholest-4-en-3-one, the formation of which can be monitored at 240 nm. Cholesterol samples were added to assay buffer (50 mM sodium phosphate pH 7.0, 0.025% Triton X-100, 0.020% bovine serum albumin) pre-incubated for 10 min with 125 nM cholesterol oxidase at 37 °C. The total reaction was incubated for 45 min at 37 °C. The samples were loaded into a 96-well UV transparent quartz microplate and the absorbance at 240 nm measured using a BioTek Synergy 2 plate reader. A standard curve of known cholesterol concentrations was prepared and measured in parallel to convert absorbance values of each sample to cholesterol concentrations.

NO Release Analysis into Cell Media. EA.hy926 cells were cultured in 24-well plates in normal growth medium until 80-90% confluent. NO level was assessed in 20 µl of reduced serum medium using a Nitrate/Nitrite Fluorometric Assay Kit (Cayman Chemical) following the manufacturer's protocol.

***Statistical analysis*.** Numerical data are presented as mean ± SEM. Student's t-test was used to compare the differences between two groups and one-way ANOVA with Bonferroni's Multiple Comparison Test to compare the differences between three or more groups. Significance was based on a value of $p<0.05$. Experiments were performed in duplicate unless otherwise noted in the Figure Legends. Statistical analysis was performed on the cumulative average values for each independent experiment (n = repeats [separate experiments]).

RESULTS

PLD2$^{-/-}$ mice have increased systemic blood pressure accompanied by decreased cardiac function. Tail-cuff measurements were used to determine the blood pressure of wild-type (WT) and *PLD2*$^{-/-}$ mice (Fig. 3-1A). Unexpectedly, the *PLD2*$^{-/-}$ mice were found to have increased systolic (Δ27 mmHg; $p<0.05$) and diastolic (Δ21 mmHg; $p<0.05$) BP. This increase is significant in the context of human hypertension, since increases of 20 mmHg systolic BP / 10 mmHg diastolic BP above the normal range are associated with two- or greater-fold differences in death rates from stroke, ischemic heart disease, and other vascular causes [25].

One of the major pathophysiology of prolonged hypertension is development of cardiac hypertrophy, since additional ejection force is needed to overcome the increased aortic blood pressure. The heart-to-body weight ratio in *PLD2*$^{-/-}$ mice was not significantly increased compared to WT mice (Fig. 3-1B). However, cardiac function in *PLD2*$^{-/-}$ mice as assessed by echocardiogram revealed a significant decrease in the % fractional shortening and ejection fraction (Fig. 3-1C, D), albeit still within the normal range. Similarly, the left ventricular diastolic volume was decreased by 15% in the *PLD2*$^{-/-}$ mice ($n=3$, $p=0.025$). These decreases are likely due to a compensatory response to increased blood pressure in the *PLD2*$^{-/-}$ mice.

The increased blood pressure in PLD2$^{-/-}$ mice is not associated with obesity or hyperlipidemia. Hypertension can evolve as a co-morbid disease linked to obesity, HDL cholesterol, and diabetes (the metabolic syndrome) [26]. However, the *PLD2*$^{-/-}$ mice were found to weigh significantly less than age-matched WT mice (Fig. 3-2A), and the levels of serum LDL and HDL levels were the same or lower than in WT mice, when placed on a high fat diet for 7 months (Fig. 3-2B, C). The ratio of total serum cholesterol to HDL is traditionally used as a

gauge to monitor the development of heart disease [27]; employing this assessment method, *PLD2*$^{-/-}$ mice would be predicted to be at lower risk of cardiovascular disease than WT mice (Fig. 3-2D). Thus, the increased BP would appear to arise from a mechanism distinct from the ones associated with the metabolic syndrome.

PLD2$^{-/-}$ aortas have decreased levels of endothelial nitric oxide synthase (eNOS), an effector of vasodilation. In surveying potential causes for the increased BP, we discovered, using immunofluorescent staining, that eNOS protein expression was dramatically reduced in the aorta of *PLD2*$^{-/-}$ mice (Fig. 3-3A). Quantitatively, the eNOS fluorescent signal was reduced by 62% ($\pm$ 2.9%, n=4, $p<0.001$). Moreover, virtually all the remaining fluorescence was localized diffusely in the cell rather than in punctate form at the plasma membrane. This was a very interesting finding, since the activity of eNOS is tightly regulated via several mechanisms including control of its subcellular localization [28, 29]. eNOS is thought to be active primarily when present in specialized plasma membrane domains rich in sphingomyelin, cholesterol, and caveolin, called caveolae [30-32]; cytoplasmic and Golgi-localized eNOS generate relatively little NO and are not activated by signaling events. This finding suggested that aortic endothelial cells in *PLD2*$^{-/-}$ mice might be failing to generate NO, a potent vasodilator. To test this hypothesis, we treated WT and *PLD2*$^{-/-}$ mice with the nitric oxide synthase inhibitor N-ω-nitro-L-arginine methyl ester (L-NAME) for a week and reassessed BP. Systolic and diastolic BP in the WT mice rose by 18% (Δ20 mmHg) and 17% (Δ14 mmHg), respectively, whereas no increase was seen for the *PLD2*$^{-/-}$ mice (Fig. 3-3B vs Fig. 3-1A). After both one week (Fig. 3-3B) and 2 weeks (not shown) of L-NAME treatment, BP was nearly identical in both the WT and *PLD2*$^{-/-}$ mice, suggesting that the PLD2 deficiency-induced loss of eNOS and endothelial cell NO production underlies the increased BP in the *PLD2*$^{-/-}$ mice. Similarly, L-NAME treatment decreased the % fractional

shortening, the % ejection fraction, and the left ventricular diastolic volume in the WT mice but not in the *PLD2*$^{-/-}$ mice, eliminating the difference between the mouse strains (data not shown, n=3). We next examined levels of VEGF in the lung and heart, since VEGF levels have been reported to decrease in some models of hypertension [33, 34]. Consistent with these reports, VEGF levels fell in WT mice after 15 days of L-NAME treatment and the resulting sustained hypertension (Fig. 3-3C-F; the decrease was highly significant in the lung and almost achieved significance ($p=0.06$) in the heart). In contrast, VEGF levels were initially at reduced levels in the *PLD2*$^{-/-}$ mice and were not affected by L-NAME treatment, suggesting that the PLD2-deficiency induced reduction in VEGF maybe the result of an eNOS-dependent mechanism.

PLD2 knock-down in the human umbilical vein cell line EA.hy926 decreases eNOS expression and NO production. To delineate the mechanism through which PLD2 regulates eNOS expression and to examine eNOS activity directly, we used a commercial lentiviral shRNA approach that employs three different shRNA sequences to establish a human umbilical vein cell line with greatly diminished PLD2 expression (shPLD2). A control lentivirus expressing scrambled shRNA sequences was used to generate the control "Scramble" cell line. The resulting stable shPLD2 pooled cell line exhibited a >90% knockdown of PLD2 mRNA as assessed by semi-quantitative qRT-PCR (Fig. 3-4A), and PLD2 protein was undetectable (Fig. 3-4B). Immunofluorescent confocal microscopy then revealed that PLD2 knockdown substantially decreased eNOS expression, especially on the plasma membrane (Fig. 3-4C), and quantitative western blotting indicated that the eNOS expression was 62% reduced in the shPLD2 cell pool ($p<0.01$, Fig. 3-4D, E). Finally, we examined NO production by the Scramble and shPLD2 endothelial cell lines. The shPLD2 cell line generated only 38% as much NO as the Scramble cell line ($p<0.05$, Fig. 3-4F). Treatment of the Scramble cells with the small molecule PLD2

inhibitor, NFOT [35], similarly reduced NO production (39% of the control value, as assessed by release of nitrite (NO_2^-) into the cell culture media), but NFOT had no further effect on the shPLD2 cells, confirming that NFOT mediates its effect through inhibition of PLD2 activity. This finding also demonstrates that PLD2 mediates its regulation of eNOS activity through PLD2 enzymatic activity rather than via a scaffolding mechanism, since ablation of the PLD2 protein and simple inhibition of its activity yielded the same outcome on NO production.

PLD2 knock-down increases expression of HMG-CoA reductase, a negative regulator of eNOS expression levels. HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, has been well studied as a negative regulator of eNOS function, through its production of mevalonate, which decreases eNOS mRNA stability [36]. Statins, which inhibit HMG-CoA reductase activity, have been shown widely to increase eNOS mRNA and protein levels and NO production [37]. Here, using western blotting, we found that HMG-CoA reductase levels were increased by 25% in the shPLD2 endothelial cells and in Scramble cells treated with the PLD2 inhibitor NFOT in comparison to Scramble cells (Fig. 3-5A, B). N-SIM (structured illumination) microscopy similarly revealed an increase in shPLD2 endothelial cell HMG-CoA reductase expression (Fig. 3-5C), and qRT-PCR demonstrated increased levels of HMG-CoA reductase mRNA in the NFOT-treated Scramble endothelial cells and shPLD2 endothelial cells ($p<0.01$, Fig. 3-5D).

Next, we tested whether an HMG CoA reductase inhibitor could overcome the PLD2-mediated decrease in eNOS protein expression levels and activity. Scramble endothelial cells treated with the PLD2 inhibitor NFOT had decreased expression levels of eNOS protein (Fig. 3-5E, F, lanes 1 and 2), as described above, whereas treatment with the HMG-CoA reductase inhibitor, Simvastatin, strongly increased eNOS protein expression (lane 3). The combined

treatment was intermediate in outcome (lane 4); with expression being elevated above the Scramble control, but not as high as Simvastatin alone. Similarly, eNOS protein expression was decreased in shPLD2 cells as described above (lane 5), and increased, but not fully, with exposure to Simvastatin (lane 6). Nonetheless, these findings suggest that the PLD2-deficiency caused an increase in HMG-CoA reductase expression level and activity underlies the decrease in eNOS protein expression. Finally, we observed a similar outcome through examining NO production: as previously mentioned, shPLD2 cells generated less NO than Scramble endothelial cells (Fig. 3-5G, lanes 1 vs 3), and Simvastatin increased the amounts of NO production in both the control and PLD2-knockdown cells (lanes 2 and 4), identifying a role for HMG-CoA reductase in the PLD2-deficiency-mediated inhibition.

Intracellular free cholesterol levels are decreased in PLD2-deficient endothelial cells, and when restored, bypass PLD2 deficiency to elevate eNOS expression. HMG-CoA reductase is regulated both transcriptionally and post-translationally in response to increased intracellular free cholesterol [38]. We found that free cholesterol levels are 9% reduced in shPLD2 cells ($p<0.001$, Fig. 3-6A). To establish whether this reduction is biologically significant, we supplemented the shPLD2 cells with 25 μM cholesterol for 24 hours, which increased the intracellular levels by 7%, bringing it almost back to normal levels (Fig. 3-6A). We then examined eNOS protein expression levels, and found that the cholesterol supplementation resulted in a 42% increase (Fig. 3-6B, C). Placing these observations together, our findings reveal a novel pathway in which PLD2 deficiency decreases intracellular cholesterol, leading to increased HMG-CoA reductase gene transcription and protein, which results in decreased eNOS expression, deceased production of the vasodilator NO, and, finally, hypertension (Fig. 3-6D).

R172C confers selective loss of function on PLD2. Returning to the human polymorphism linked to reduced blood pressure [12], we sought to address whether it functions differently than the wild-type PLD2 allele, since it clearly does not phenocopy PLD2 loss-of-function in mice. Human PLD2 overexpression in cells stimulates F-actin reorganization [39, 40], as illustrated in Fig. 3-7A, where both filopodia (arrowhead) and peripheral (chevron) and dorsal (*) membrane ruffles can be observed. In contrast, overexpression of R172C-hPLD2 did not provoke dramatic F-actin reorganization (Fig. 3-7B). Much of the R172C-hPLD2 protein appeared to localize to subcortical actin (arrow), in contrast to hPLD2 which localized primarily to the cell cortex. Overexpression of hPLD2 and R172C-hPLD2 increased eNOS protein expression levels in shPLD2 cells (Fig. 3-7C, D), and more importantly, restored NO production (Fig. 3-7E). Finally, we examined eNOS and caveolin-1 expression using N-SIM super-resolution microscopy. As before, eNOS protein levels were greatly diminished at the plasma membrane in the shPLD2 cells (Fig. 3-7F, G, J), and decreased expression was also observed for caveolin-1, a protein that interacts with PLD2 in lipid rafts in a lipid-dependent manner [41-43]. Overexpression of hPLD2 restored WT levels of expression for both eNOS and caveolin-1 (Fig. 3-7H, J). However, overexpression of R172C-hPLD2 rescued only the eNOS expression; no increase in caveolin-1 was observed (Fig. 3-7I, J). Together, these findings suggest that the R172C polymorphism does not alter eNOS function; hence, it would not lead to increased BP via this pathway. However, its contrasting effect on caveolin-1 is intriguing, as discussed below.

DISCUSSION

The regulation of BP is complex, involving numerous physiological and cellular pathways. Supporting roles for PLD2 in this process have been proposed, based on its contribution to the secretion of aldosterone [20, 21], via facilitation of AT1R signaling [19], and through promotion of VLDL formation [15, 16]. Based on these studies, the report that a polymorphism in human PLD2 negatively correlates with the development of hypertension appeared to support the hypothesis that PLD2 promotes increased blood pressure and that loss of PLD2 function should result in hypotension. Unexpectedly, we report here that mice lacking PLD2 exhibit hypertension (Fig. 3-1) due to reduced levels of eNOS (Fig. 3-3), and decreased production of NO (Fig. 3-4), an important physiological vasodilatory effector.

The proximal cause of PLD2-deficiency-induced eNOS reduction was identified to be a decrease in intracellular free cholesterol (Fig. 3-6), which upregulates HMG CoA-reductase transcription and enzyme half-life, a well-known negative regulator of eNOS (Fig. 3-5). The relationship between PLD2 function and intracellular cholesterol levels is poorly understood, but an intriguing possibility arises from a report that PLD2 may be involved in a pathway that destabilizes ABCA1 [44], which mediates the export of cholesterol and phospholipids from cells to HDL apolipoproteins. PLD2 loss, in this model, would lead to increased ABCA1 protein levels and activity, and consequently, reduced intracellular free cholesterol. This possibility represents an area for future exploration.

Reductions in intracellular cholesterol stimulate both transcriptional upregulation and decreased proteolysis of HMG-CoA reductase, which negatively regulates eNOS mRNA stability and hence protein expression. Simvastatin, an HMG-CoA reductase inhibitor, partially,

but not fully, rescued the decreased eNOS phenotype induced by PLD2 deficiency (Fig. 3-5), suggesting that HMG-CoA reductase upregulation is only one of the mechanisms affecting eNOS levels in this setting. It is well established that reductions in intracellular cholesterol also suppress eNOS activity [29, 32, 45, 46] through decreasing the association of eNOS with the plasma membrane in caveolae, where it can be activated by signaling events. Thus, while inhibition of HMG-CoA reductase blocks destabilization of eNOS mRNA, it does not address the underlying cholesterol deficiency, which also impacts eNOS activity.

The marked decrease in caveolin-1 in shPLD2 endothelial cells transfected with R172C-hPLD2 is intriguing in that caveolin-1 is a well-known negative regulator of eNOS activity [47]. Reduced caveolin-1 could lead to increased eNOS activity, increased NO production, and vasodilation, consistent with the resistance to hypertension reported to be associated with the human allele [12].

In vivo roles and potential opportunities for therapeutic interventions for PLD2 have been explored in recent years using PLD2$^{-/-}$ mice. PLD2 ablation has been shown to improve outcomes in a model of Alzheimer's disease [9], to suppress cancer growth and metastasis in a heterotopic implant model [10], and to improve outcomes in thrombotic disease models in combination with PLD1 ablation [4]. Finally, PLD2 inhibition has been reported to suppress influenza virus infection of a lung cell line *in vitro* and mouse lungs in an *in vivo* model [11]. While concerns regarding increased blood pressure are of lesser concern in the context of transient inhibition, such as would be performed for management of viral disease and acute thrombotic events, this issue may affect enthusiasm for pursuing PLD2 inhibition in the setting of long-term cancer or neurodegenerative treatment. In particular for cancer, some therapeutics, for example, VEGFR tyrosine kinase inhibitors, are independently associated with a substantially

elevated risk development of hypertension [48], and thus use of a PLD2 inhibitor in parallel with them could result in synergistic adverse effects. Nonetheless, murine studies do not always predict the outcomes of complex signaling pathways in humans. Given the potential utility of PLD2 inhibitors for cancer and acute thrombotic disease, in particular in combination with PLD1 inhibitors [2], an effect on blood pressure represents a topic that should be assessed carefully if and when PLD2 inhibitors enter the clinic.

FIGURES

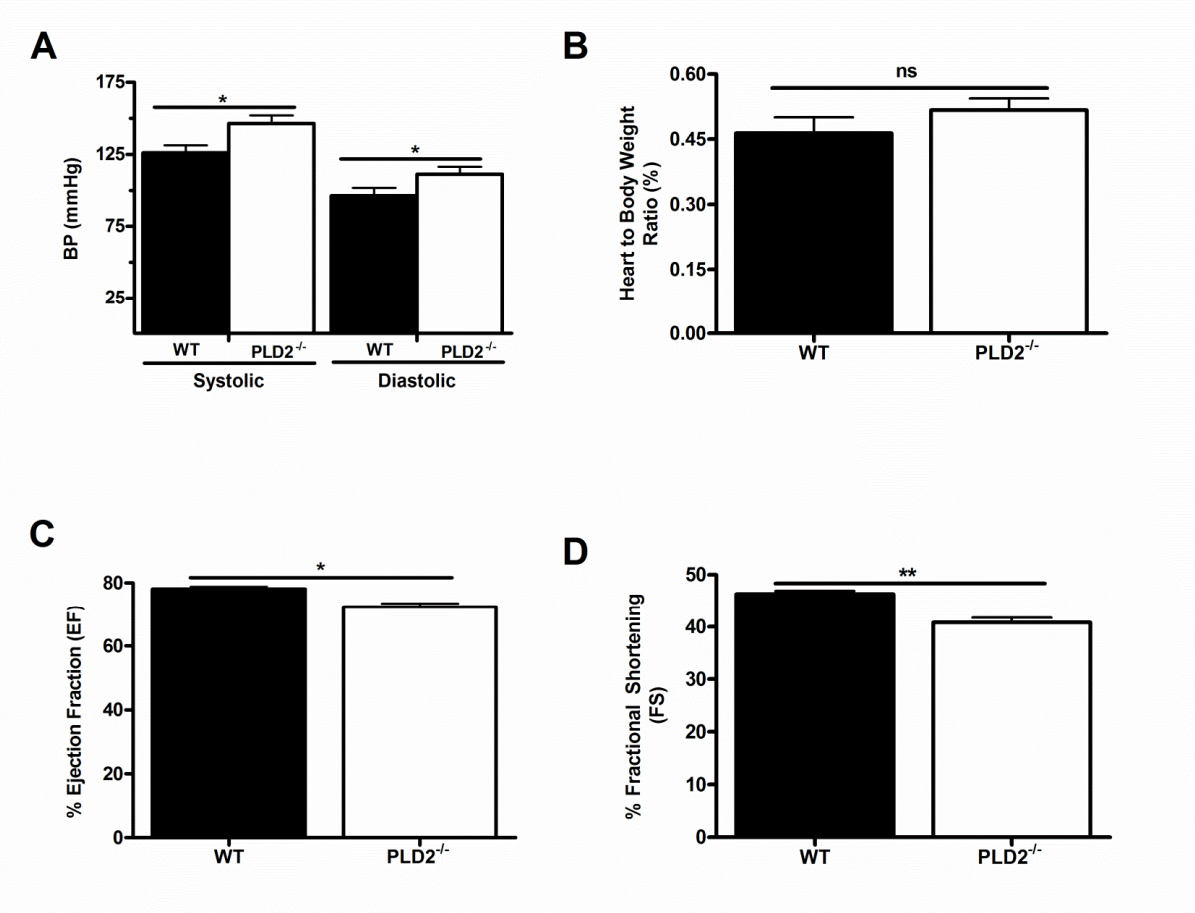

Figure 3-1. *PLD2*$^{-/-}$ mice have increased BP and altered cardiac function. (A) A non-invasive tail-cuff instrument was used to measure systolic and diastolic blood pressure as described in Methods on 5-month old male mice (n=7 mice for WT; 5 for *PLD2*$^{-/-}$; each data point represents the average of 15 measurements). (B) Heart-to-body weight ratio for *PLD2*$^{-/-}$ and WT mice (n=3 mice of each genotype). (C-D) Echocardiogram sonogram fractional shortening (FS) and percent ejection fraction (EF), performed as described in Methods (n=3). Mean ± SEM; *, $p<0.05$; **, $p<0.01$. Tail cuff blood pressure readings (A) and echocardiogram of Wildtype and PLD2$^{-/-}$ mice.

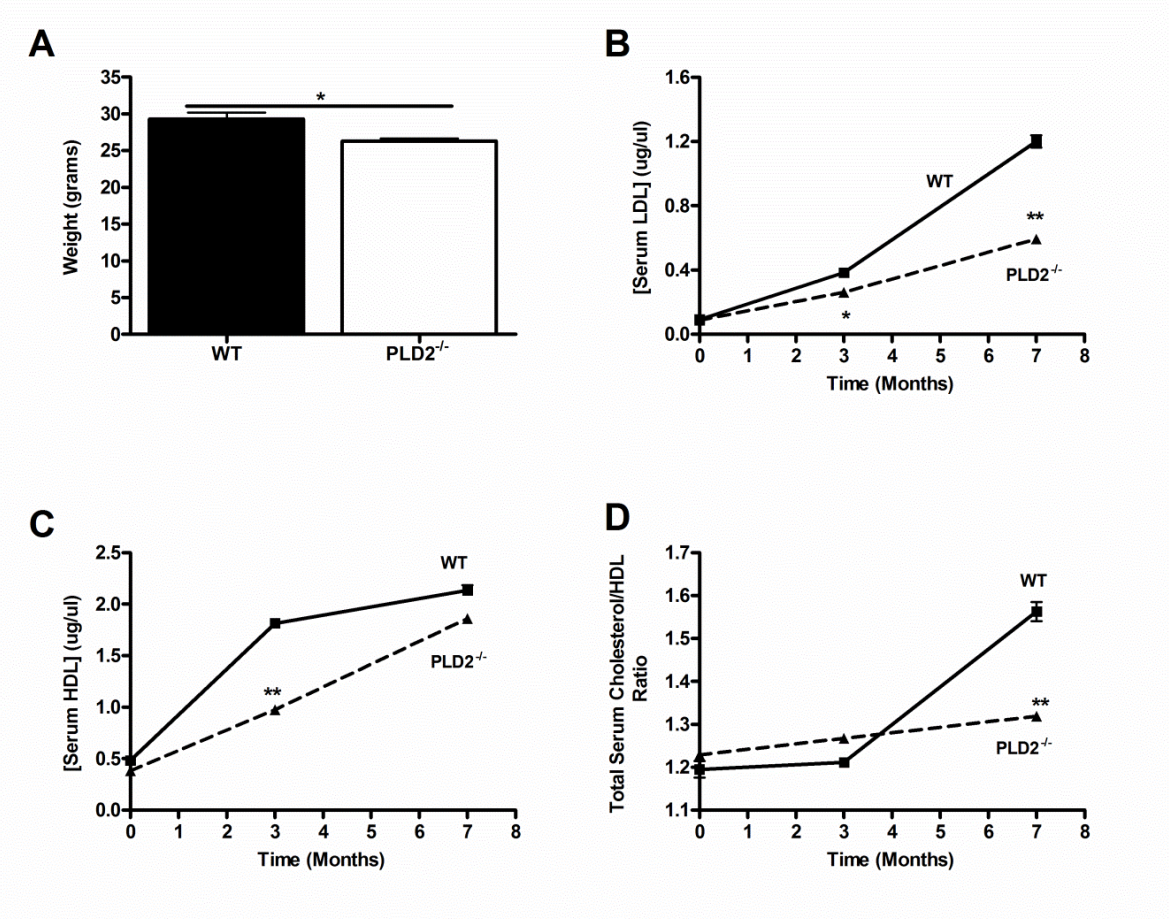

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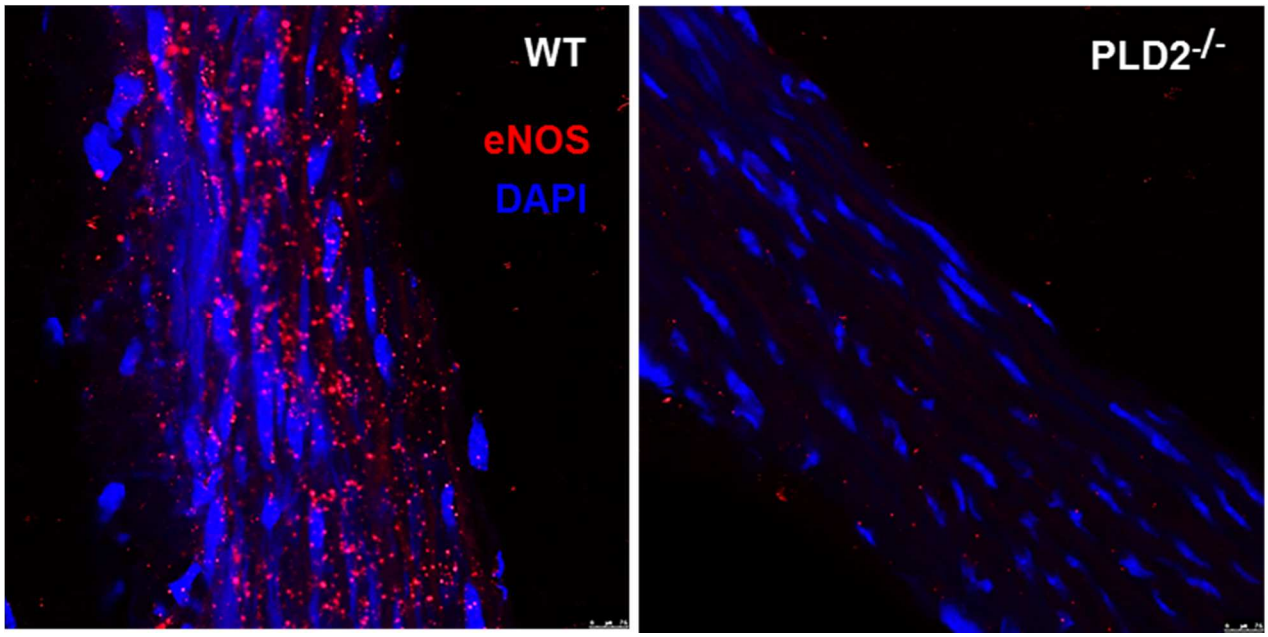
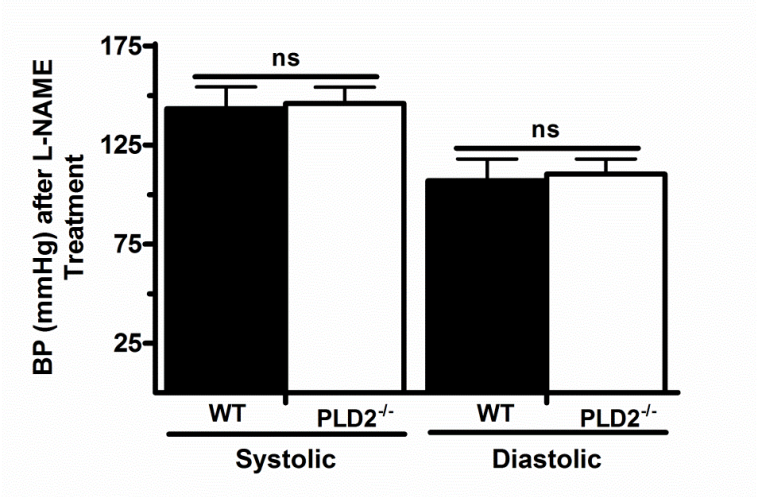
Figure 3-2. *PLD2*$^{-/-}$ mice are neither obese nor hyperlipidemic. (A) weight at 5 months, n=7 mice of each genotype. LDL (B), HDL (C), and total serum cholesterol to HDL ratio (D) at 0, 3, and 7 months on a high fat diet (n=3).

A


WT
eNOS
DAPI
PLD2-/-

B


BP (mmHg) after L-NAME Treatment
175
125
75
25
ns
ns
WT
PLD2-/-
WT
PLD2-/-
Systolic
Diastolic

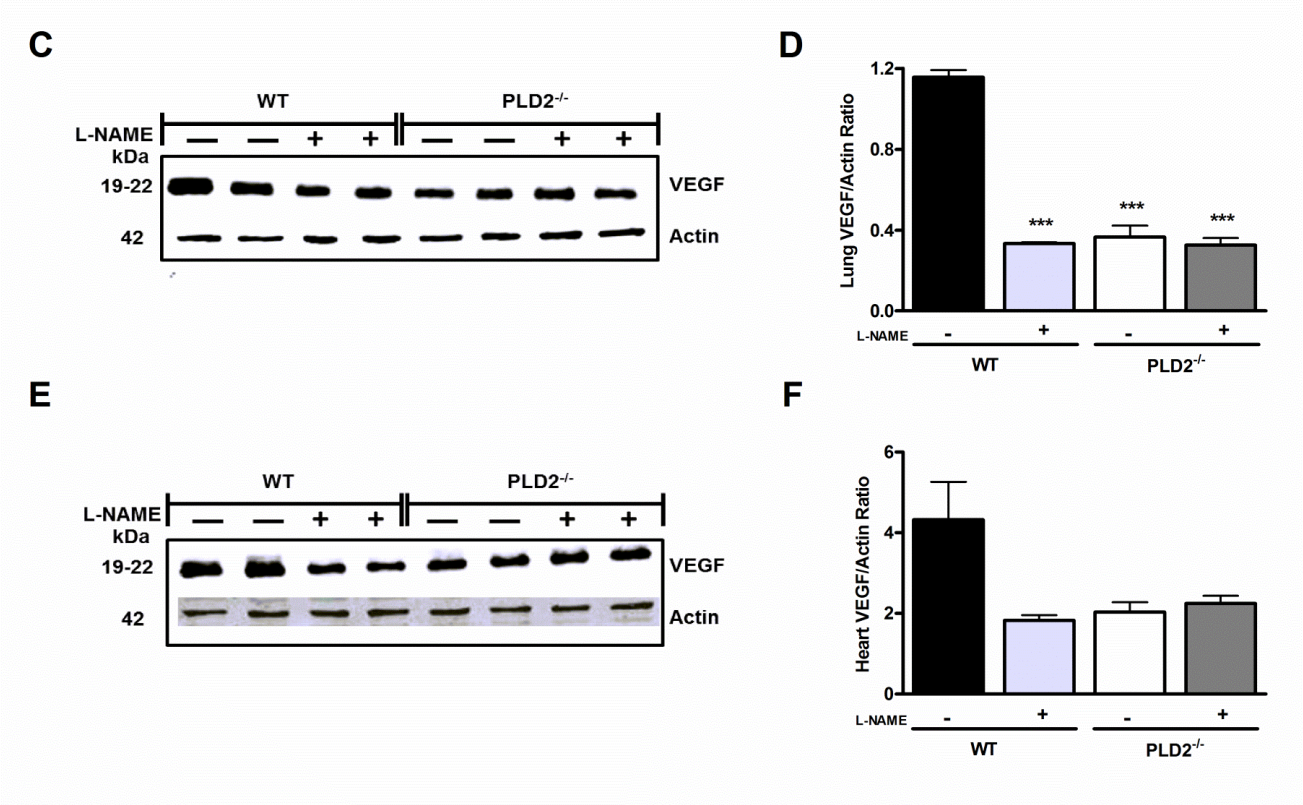
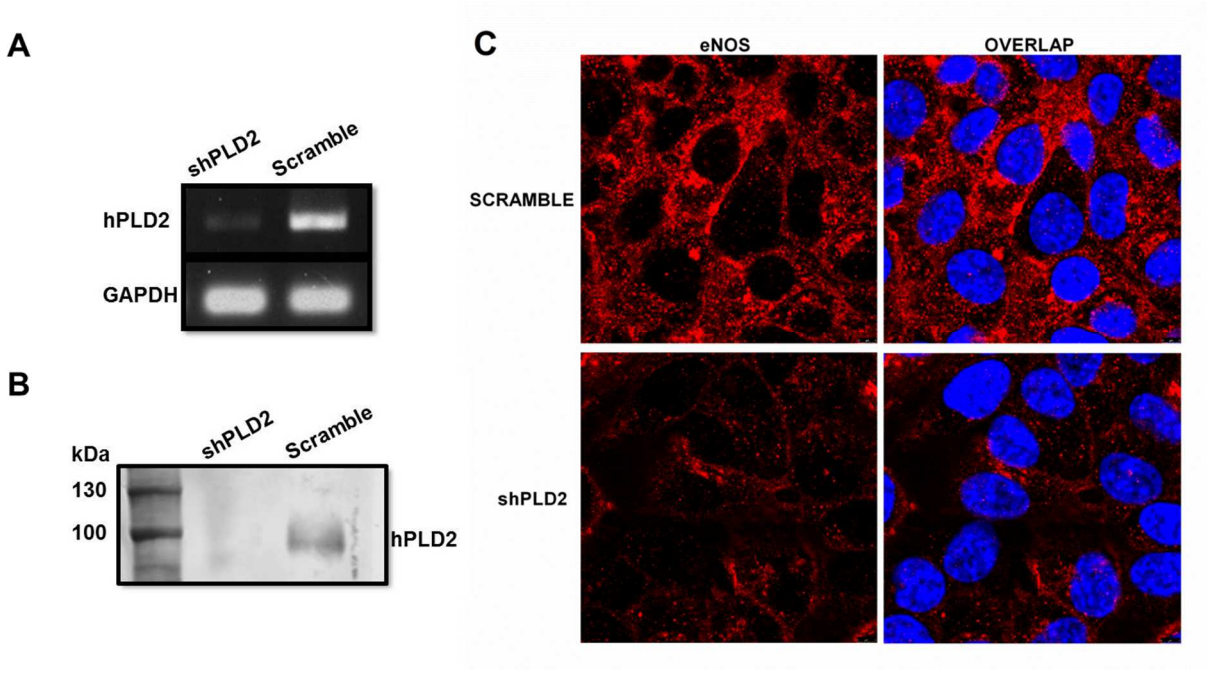


Figure 3-3. The decreased BP in *PLD2*$^{-/-}$ mice results from decreased levels of eNOS protein. (A) Immunofluorescent staining of eNOS in sections of aortas from WT and *PLD2*$^{-/-}$ mice. (B) Systolic and diastolic blood pressure following treatment with L-NAME for one week (n=4). (C-F) Measurements of VEGF protein levels by western blotting in *PLD2*$^{-/-}$ and WT mice lungs (C,D) and heart (E,F) were normalized to actin expression levels (n=4 mice of each genotype); ***, $p<0.001$.


A
shPLD2
Scramble
hPLD2
GAPDH
B
kDa
shPLD2
Scramble
130
100
hPLD2
C
eNOS
OVERLAP
SCRAMBLE
shPLD2

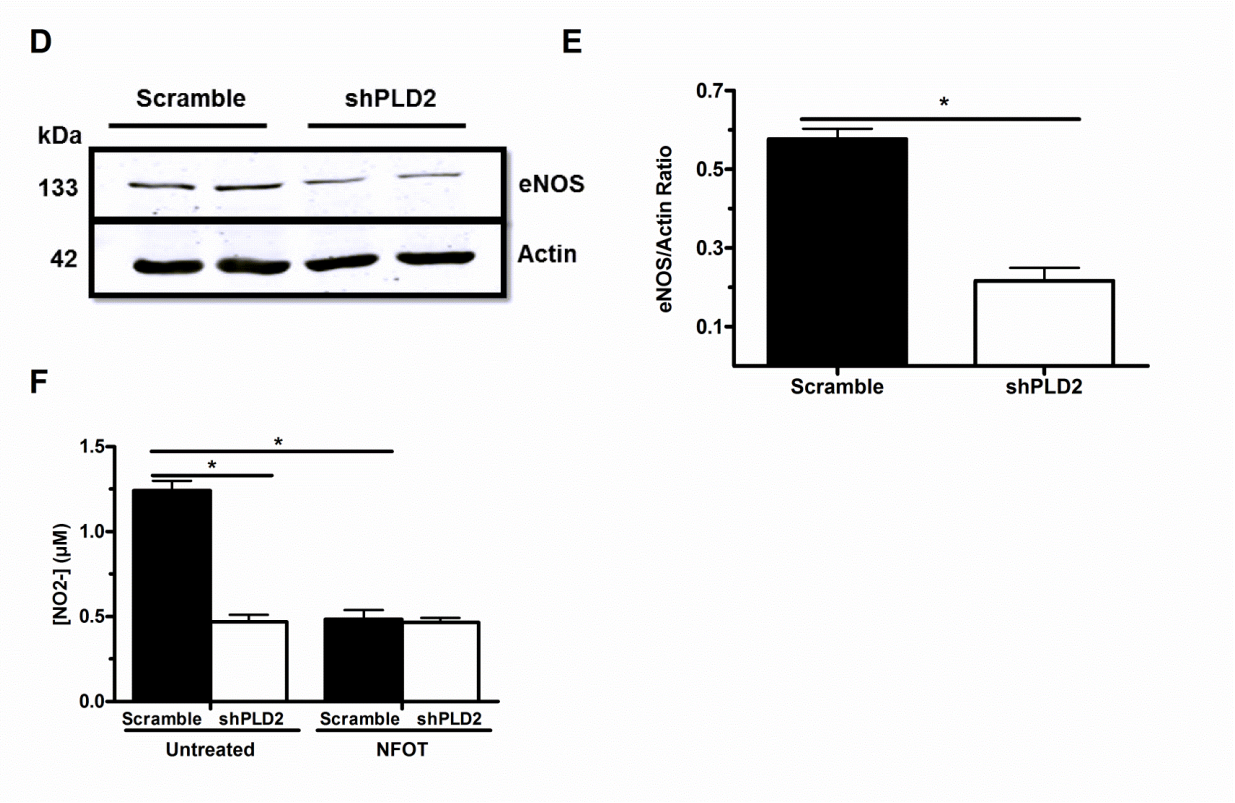
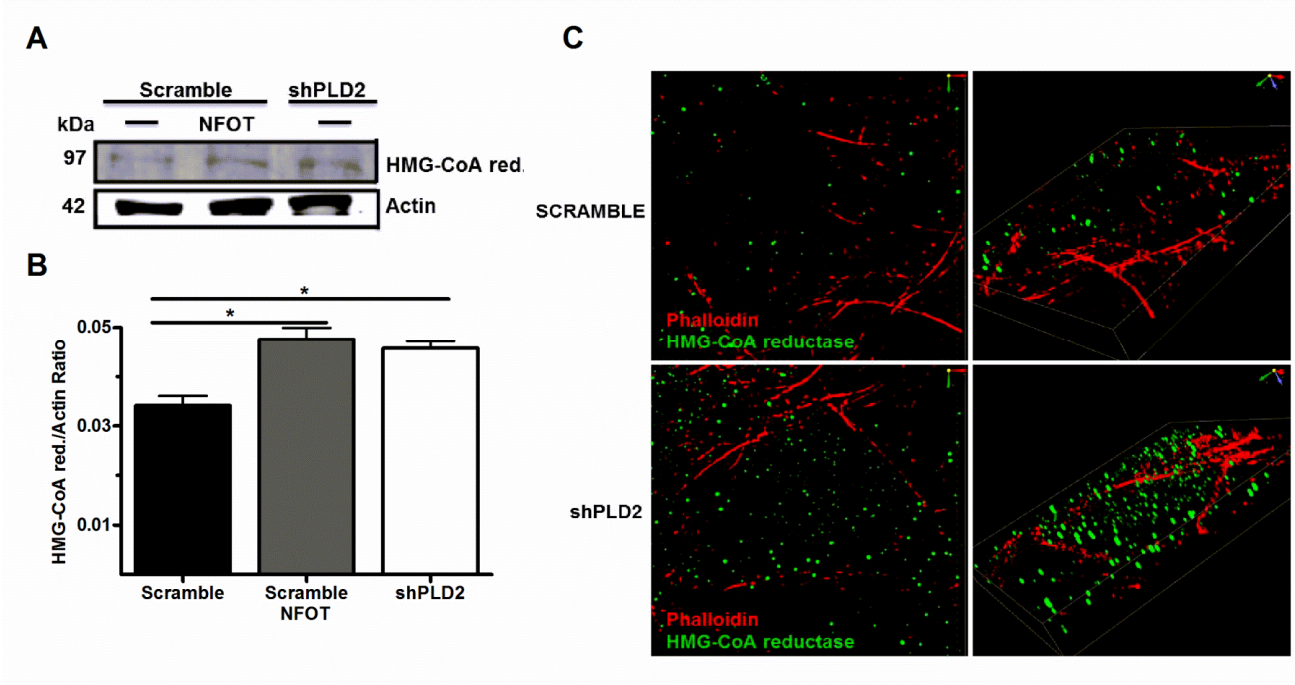


Figure 3-4. Stable shRNA knockdown of PLD2 in EA.hy926 endothelial cells decreases eNOS protein expression levels and NO production. (A, B) Stable *PLD2* knockdown cells (shPLD2 cells) were generated using shRNA lentiviral particles. In parallel, control shRNA sequences were used to generate a control cell line (Scramble cells). PLD2 knockdown was confirmed by qRT-PCR (A) and western blotting (B). (C) Confocal microscopy of eNOS protein expression as detected by immunofluorescent staining in Scramble and shPLD2 cells. (D) Western blotting of eNOS in Scramble and shPLD2 cells. (E) Quantification of eNOS western blot (n=3). (F) Measurement of nitrate production using a Griess Reaction Kit to quantify eNOS activity with and without treatment with a small molecule PLD2 inhibitor (NFOT) at 10 μM for 24 hours (n=7 experiments performed in duplicate).


A
Scramble
shPLD2
kDa
NFOT
97
HMG-CoA red.
42
Actin
B
HMG-CoA red./Actin Ratio
0.05
0.03
0.01
*
*
Scramble
Scramble NFOT
shPLD2
C
SCRAMBLE
shPLD2
Phalloidin
HMG-CoA reductase
Phalloidin
HMG-CoA reductase

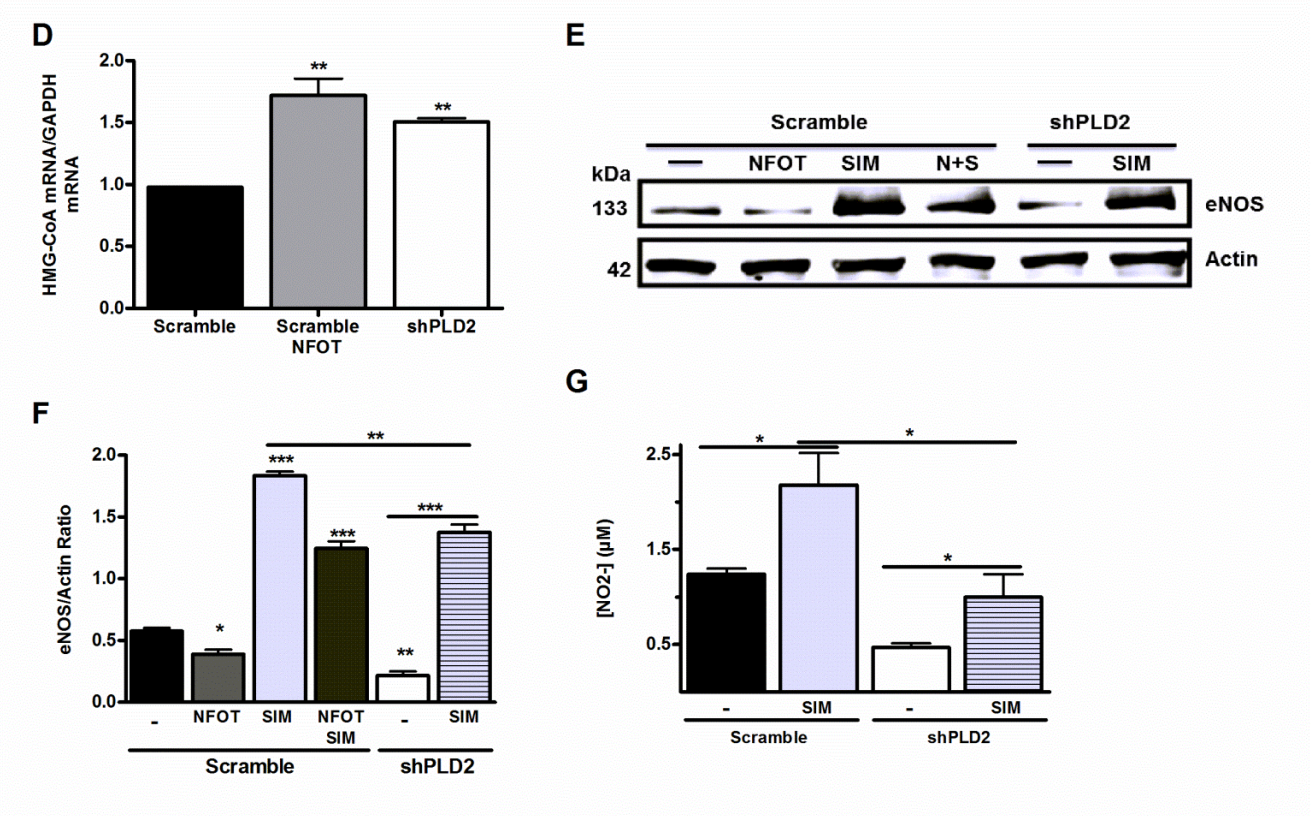
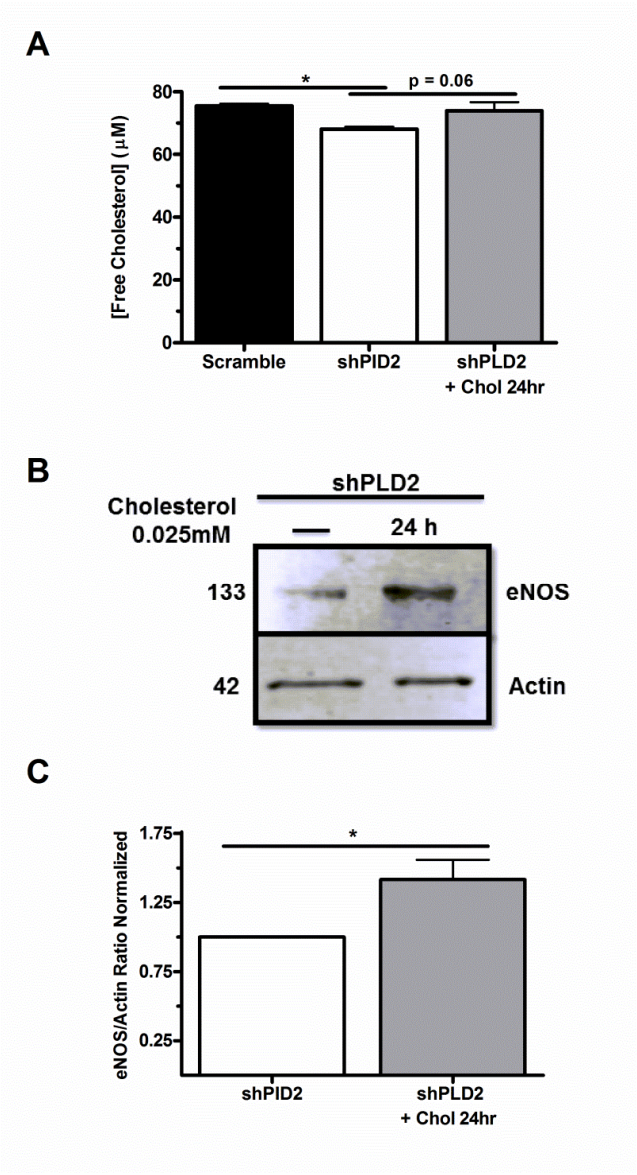
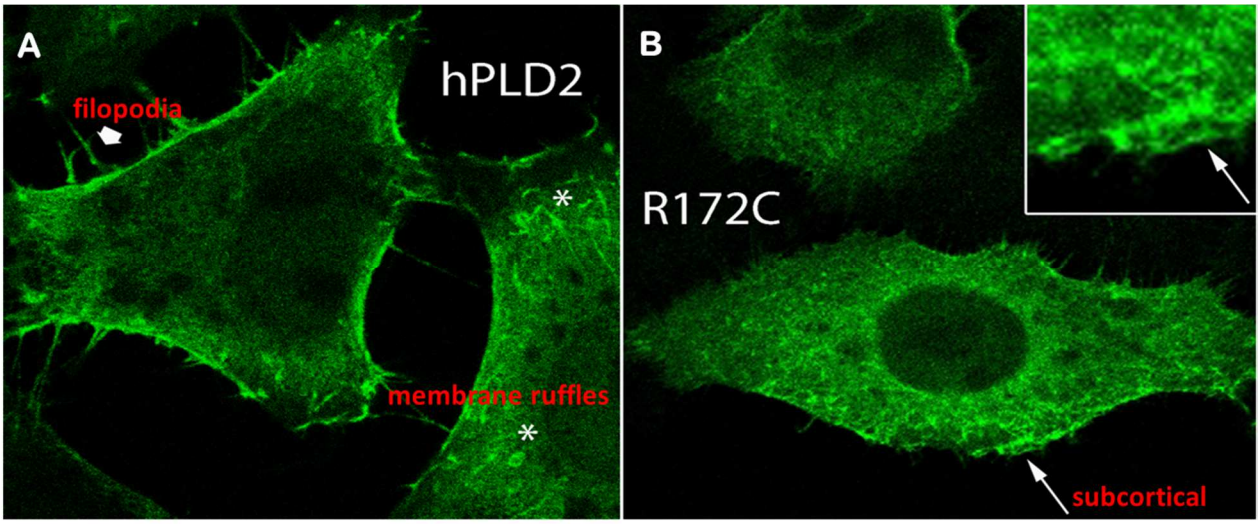


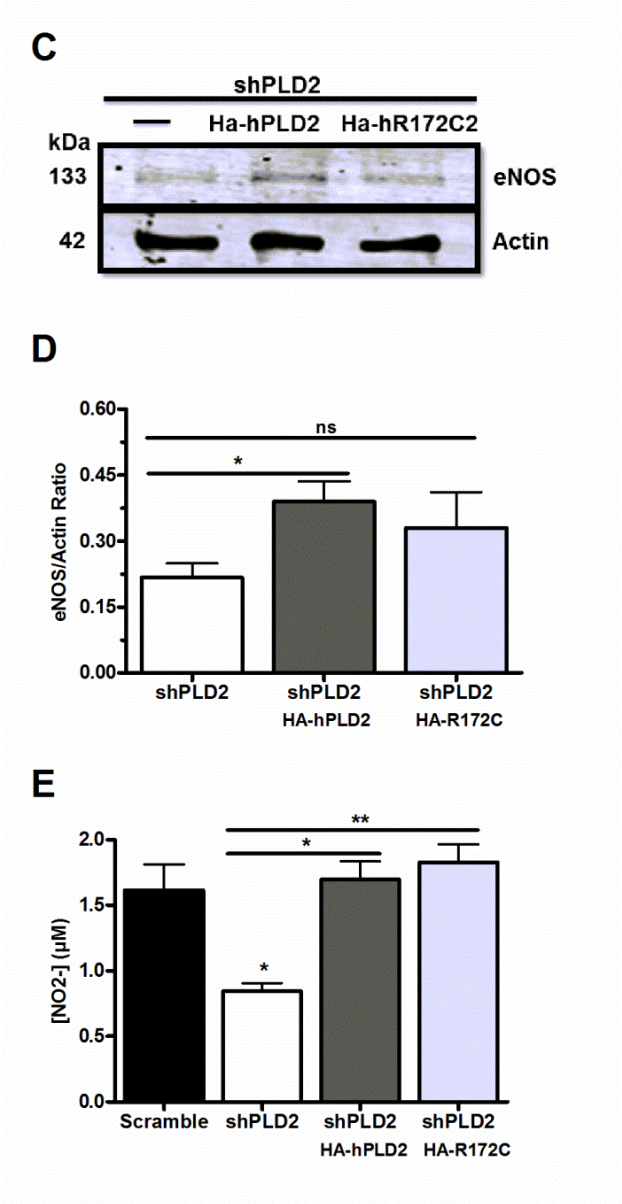
Figure 3-5. Upregulation of HMG-CoA reductase in shPLD2 cells decreases eNOS expression. (A) Western blot of HMG-CoA reductase in Scramble and shPLD2 cells. (B) Quantification of HMG-CoA reductase western blot (n=4 experiments performed in duplicate). (C) N-SIM microscopy of HMG-CoA reductase. (D) RT-PCR of HMG-CoA reductase in Scramble and shPLD2 cells (n=3). (E) Western blot of eNOS after treatment with NFOT and Simvastatin. (F) Quantification of eNOS western blot (n=3). (G) eNOS activity after treatment with Simvastatin (n=7).


A
*
p = 0.06
80
60
40
20
0
[Free Cholesterol] (μM)
Scramble
shPID2
shPLD2
+ Chol 24hr
B
shPLD2
Cholesterol
0.025mM
24 h
133
eNOS
42
Actin
C
1.75
1.25
0.75
0.25
eNOS/Actin Ratio Normalized
*
shPID2
shPLD2
+ Chol 24hr

D

Figure 3-6. Recovery of eNOS levels in shPLD2 cells following exogenous cholesterol supplementation. (A) Free cholesterol measurement assay monitored at 240nm after culture of cells with 0.025M exogenous cholesterol (in media) for 24 hours (n=4). (B) Western blotting of eNOS protein in shPLD2 cells with and without cholesterol supplementation. (C) Quantification of eNOS western blot (n=5). (D) Cartoon summary of model.


A
hPLD2
filopodia
membrane ruffles
B
R172C
subcortical


C
shPLD2
—
Ha-hPLD2
Ha-hR172C2
kDa
133
42
eNOS
Actin
D
eNOS/Actin Ratio
0.60
0.45
0.30
0.15
0.00
ns
*
shPLD2
shPLD2
HA-hPLD2
shPLD2
HA-R172C
E
[NO2-] (μM)
2.0
1.5
1.0
0.5
0.0
**
*
*
Scramble
shPLD2
shPLD2
HA-hPLD2
shPLD2
HA-R172C

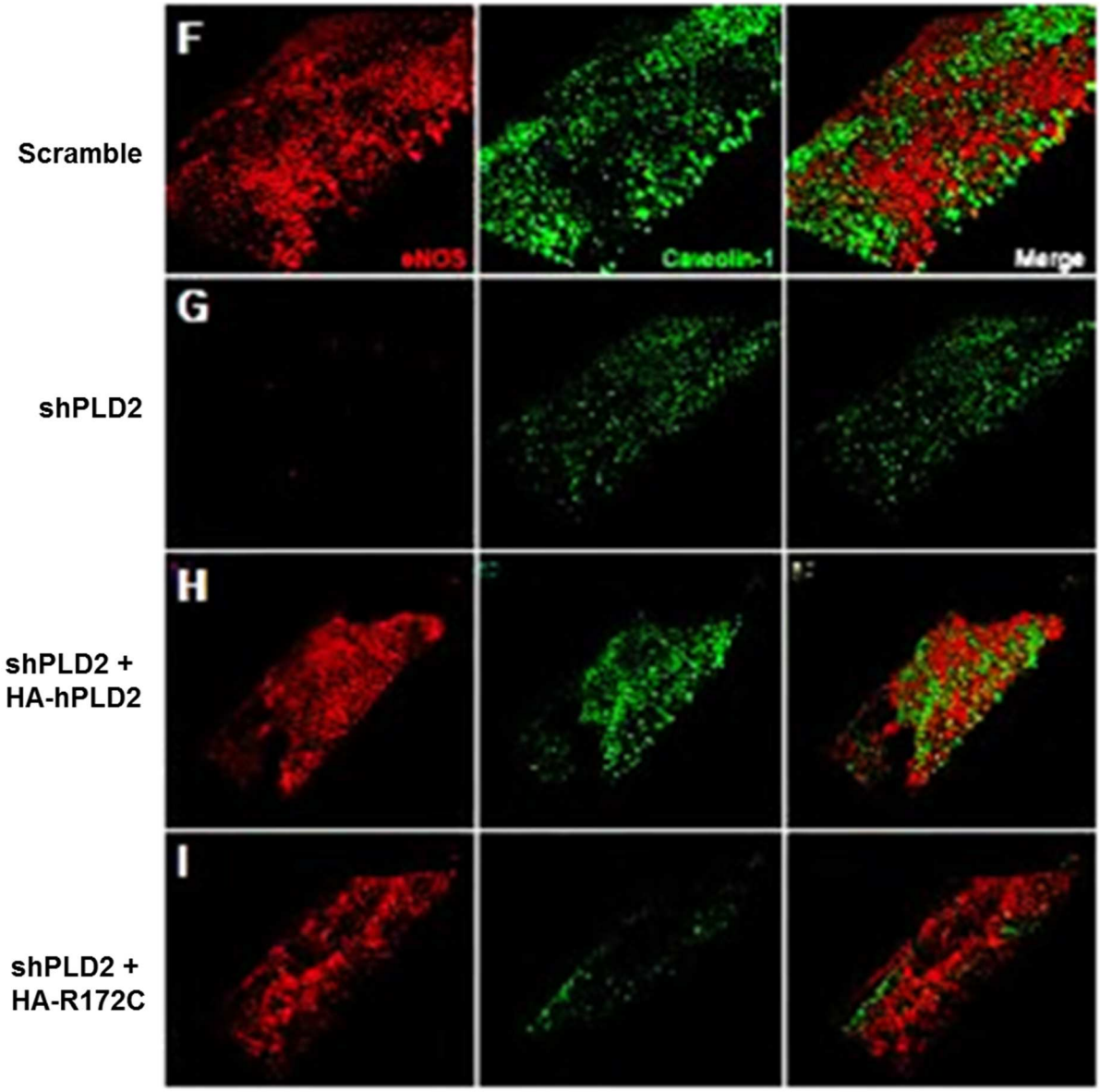


Figure 3-7. The human PLD2 polymorphism R172C does not alter eNOS signaling but does decrease caveolin-1 protein levels. Overexpression of HA-tagged hPLD2 (A) and HA-R172C-PLD2 (B) in HeLa cells, visualized using anti-HA immunofluorescent staining (green). Arrowhead, PLD2 localization in filopodia; chevron, in peripheral actin ruffles; *, in dorsal actin ruffles; arrow, in subcortical actin network. (C) Western blot of eNOS after transfection of HA-hPLD2 or HA-R172C-PLD2 into shPLD2 cells. (D) Quantification of eNOS by western blotting (n=3). (E) eNOS activity as measured by nitrate formation (n=7). (F-I) N-SIM microscopy of plasma membrane eNOS and Caveolin and (J) quantification of fluorescent staining density at the plasma membrane was determined using the histogram function of Photoshop (red, eNOS; green, caveolin-1).

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Chapter 4

Conclusions and Future Directions

CONCLUSION

In this dissertation, the role of Phospholipase D2 (PLD2) in blood pressure regulation via endothelial nitric oxide synthase (eNOS)/NO pathway has been described for the first time. This work introduced the concept that upon the loss of PLD2, either through genetic modification or using a specific PLD2 inhibitor (NFOT), the resulting quantity of eNOS, as well as the production of NO is significantly decreased. This decrease proved to be substantial enough to cause a significant increase in systemic blood pressure in $PLD2^{-/-}$ mice. Some insight has previously been given to the field of cardiovascular diseases on PLD2's role in the regulation of blood pressure through its effects on the renin-angiotensin-aldosterone system [1-4]. The current work, however, shows that despite its ability to decrease the release of aldosterone which would lead to vasoconstriction, the overall effect of PLD2 loss is a dramatic decrease in NO production and vasodilation in the chosen mouse model. Previous studies which initially introduced PLD2's role in blood pressure regulation solely used *in vitro* cell models whereas this study used a combination of *in vitro* and *in vivo* models. The use of both animal and cell models adds confidence to this study.

In our lab, $PLD2^{-/-}$ mice have been characterized as weighing significantly less than WT mice when on a normal chow diet and having a significant decrease in serum HDL and LDL levels. On the surface, such characteristics would suggest that $PLD2^{-/-}$ mice are protected from cardiovascular disease such as atherosclerosis and hypertension. However, $PLD2^{-/-}$ mice have increased systolic and diastolic blood pressure when compared to WT mice. This increase in

PLD2$^{-/-}$ mice blood pressure was then shown to be due to a significant decrease in eNOS and NO production.

The regulation of eNOS has been widely studied, and is shared by multiple proteins and fluctuations in calcium and cholesterol content. Upon further investigation, we found that PLD2 knockdown endothelial cells have a significant decrease in their unesterified (free) cholesterol content, when compared to wildtype endothelial cells. PLD2 knockdown endothelial cells are seen to respond to this decrease in free cholesterol content by significantly increasing their translation of the rate limiting enzyme, HMG-CoA reductase which is rate-limiting in the bio-synthesis of cholesterol. Interestingly, HMG-CoA reductase is one of the many proteins responsible for the negative regulation of eNOS [5, 6]. HMG-CoA reductase targets, and destabilizes, eNOS mRNA resulting in an overall decrease in eNOS protein levels [7]. Finally, we showed that eNOS protein levels, and activity in PLD2 knockdown endothelial cells can be recovered with a 24-hour incubation with 0.025mM free cholesterol, and with the transfection of wildtype PLD2

Taken together, this data suggests that the loss of PLD2 initiates a domino effect which ultimately leads to a decrease in eNOS protein levels and activity resulting in an overall increase in blood pressure, and shed new light on the role of PLD2 in the development of hypertension.

FUTURE DIRECTIONS

1. PLD2 and blood pressure regulation:

This dissertation elaborates on PLD2's role in blood pressure regulation via a cascade of effects which include the activities of Cav-1, HMG-CoA reductase and eNOS. Future studies, which measure the rate at which new cholesterol is synthesized by PLD2 knockdown endothelial cells, are needed to further understand the role of cholesterol in this model. Such a study will provide further knowledge of whether PLD2's role in the change in cholesterol content of these cells is due to an inhibition or slowing down of the de novo cholesterol bio-synthesis. Similarly, the localization of cholesterol in the plasma membrane of endothelial cells is also a major regulator of eNOS activity. Future studies should also directly monitor the movement or lack of movement of de novo cholesterol from the endoplasmic reticulum to the plasma membrane of endothelial cells by Cav-1 in PLD2 knockdown cells.

The mechanism proposed in this dissertation focused on changes in cholesterol content, followed by an increase in HMG-CoA reductase mRNA and protein abundance, which led to a decrease in eNOS abundance, following PLD2 silencing (Fig. 3-6D). However, research has shown that eNOS activity is widely regulated by many factors. The presence and activity of PLD2 in endothelial cells may also be linked to the phosphorylation state of eNOS. eNOS has several phosphorylation sites which lead to both the enzyme's activity and inhibition. Therefore, the loss of PLD2 may lead to changes in one or more of these regulation sites, and hence, eNOS phosphorylation should also be monitored via western blot analysis following PLD2 inactivity. In a study performed by Dimmeler et al., it was shown that eNOS is susceptible to an activating

phosphorylation at its serine 1177 amino acid site by the serine/threonine protein kinase Akt [8]. Dimmeler et al. also showed that HMG-CoA reductase's enzymatic activity ultimately leads to the inhibition of Akt, which prevents this activating phosphorylation signal [9]. Here I propose that the increased HMG-CoA reductase protein levels may also lead to an increased inhibition of Akt, which may decrease the serine 1177 phosphorylation, and activation of eNOS. This alternative mechanism of PLD2's effect on eNOS activity is proposed in Figure 4-1, and requires further investigation.

The transfection of PLD2 knockdown endothelial cells with the point mutated HA-PLD2-R172C, lead to an increase in eNOS protein levels. The activity of eNOS in HA-PLD2-R172C transfected cells is also significantly higher than that of the non-transfected PLD2 knockdown shPLD2 endothelial cells. Cav-1 protein levels also increases following shPLD2 endothelial cells transfection with HA-PLD2-R172C. Future experiments, which quantitatively measure the concentration of Cav-1 protein in HA-PLD2-R172C transfected cells are still needed. Similarly, further studies which monitor the co-localization of eNOS/HA-hPLD2 or HA-hR172C/Cav-1 are also required. Our preliminary data suggest that PLD2 and eNOS co-localize creating a protein complex with Cav-1 primarily at the plasma membrane; however, some co-localization can also be seen in the cytosol (Fig. 4-2). Interestingly, this pattern of co-localization changes following the transfection of shPLD2 endothelial cells with HA-PLD2-R172C. The eNOS/HA-hR172C/Cav-1 complex was no longer found at the plasma membrane but was more strongly found in the cytosol. Additionally, there was a major increase in the number of Cav-1/HA-hR172C complexes formed in the HA-PLD2-R172C transfected shPLD2 endothelial cells compared to those transfected with HA-PLD2.

Finally, the presence of PLD2/eNOS co-localization suggests that PLD2's effect on blood pressure via eNOS may not strictly be through an indirect pathway (affecting cholesterol content and HMG-CoA reductase protein levels) but may also be through a direct pathway involving the creation of an eNOS/PLD2/Cav-1 complex. Obtaining a better understanding of whether, and how these three proteins directly interact, will elucidate the role of PLD2 in the regulation of blood pressure via the eNOS/No pathway and possibly how point mutation R172C affects the activity of PLD2.

2. PLD2 and cholesterol internalization:

Along with the production of PA, PLD2 can also transphosphatidylate lysophosphatidyl choline to produce the LysoPA analog, cyclic PA (cPA) [10]. cPA has been reported to function as an antagonist of peroxisome proliferator-activated receptor-γ (PPARγ) [10], which is relevant to the development of atherosclerosis. In our lab, PLD 1 and $2^{-/-}$ mice have also been shown to be very useful mice models in the study of atherosclerosis development, treatment and prevention.

Development of atherosclerosis

Blood vessels are composed of three major layers: the inner layer (tunica media; primarily composed of a single layer of endothelial cells), the middle layer (tunica media; primarily composed of vascular smooth muscle cells (VSMC)), and an outer adventia; primarily composed of mast cells and nerve endings. One major cause of atherosclerosis is an increase in serum low-density lipoprotein (LDL) which delivers cholesterol from the liver and intestines to all cells and to leukocytes (primarily monocytes) that transmigrate across the endothelium into the intimate of the blood vessel [11]. These leukocytes can migrate through the vessel due to damage to the endothelium which causes an increase in endothelial cell "stickiness." These endothelial cells are termed as being activated and allow an increase in leukocyte adhesion to their cell surface. Once in the intima, the transmigrated monocytes mature and differentiate into macrophages that produce reactive oxygen and nitrogen species leading to oxidation of LDL (ox-LDL) [11]. ox-LDL is then taken up by the macrophages via CD36, a class B scavenger receptor [12]. The ox-LDL accumulation in macrophages creates a positive feedback loop - stimulating PPARγ, which increases CD36 expression, further increasing the uptake of ox-LDL [13]. With continued influx

of ox-LDL, macrophages develop into foam cells, the initial visible characteristic of plaque formation.

Once initiated, atherosclerotic lesions will continue to progress in size and complexity ultimately leading to narrowed blood vessels, thrombosis, stroke and cardiac infarction. The vascular smooth muscle cells found in the media begin to migrate into the intima where they begin to also internalize ox-LDL and differentiate into foam cells. In the growing plaque there is also an increase in the synthesis of extracellular macromolecules, including collagen, proteoglycans and elastin [11]. Subsequently, apoptosis of the foam cells begins to occur which leads the release of the internalized lipid which then accumulates in the growing plaque. This central region which bears the accumulating lipids and macromolecules is termed the necrotic core of atherosclerotic lesions [11]. The growing plaque is also characterized by the presence of cholesterol crystals.

Normally, to help prevent excess accumulation of cholesterol, and the development of atherosclerosis, macrophages use the reverse cholesterol transport pathway to assist in the removal of unwanted cholesterol from the vessel wall. By doing so, macrophages can protect themselves from the development of cholesterol toxicity. The transmembrane protein ATP-binding cassette transporter A1 (ABCA1) is used to facilitate the actions of the reverse cholesterol transport pathway. In combination with apolipoprotein A-1 (apoA-1) and high-density lipoprotein (HDL), ABCA1 allows for the export and transport of excess cholesterol from the vessel wall to the liver for excretion from the body [14, 15]. Previous studies using $ABCA1^{-/-}$ mice have shown that the lack of ABCA1 in macrophages is substantial enough to

significantly hinder cholesterol efflux making these mice more susceptible to the development of atherosclerosis [16, 17].

The liver is a central organ in the production of many of the proteins and peptides involved in the development of atherosclerosis and other cardiovascular and liver diseases. The liver is also important for storing fuel needed by the body, making many essential proteins needed for homeostasis and metabolism, synthesizing and distributing cholesterol and lipoproteins as well as the final internalization of LDL and other lipoproteins by receptor-mediated endocytosis and clearing the body of toxins and drugs. Many forms of liver disease include the liver's inability to rid itself of the excess cholesterol harvested from other tissues and the blood via the reverse cholesterol pathway. The buildup of excess liver cholesterol is most often seen in alcoholic liver disease, fatty liver disease (hepatic steatosis), alcoholic hepatitis and cirrhosis; all of which can culminate into the formation of chronic liver failure. Excess cholesterol in the liver is sometimes attributed to increased alcohol and drug intake, lack of regular exercise, increased consumption of high fat content meals and irregularities in the function of reverse cholesterol transport. When functioning properly, reverse cholesterol transports unwanted cholesterol by the combined actions of ABCA1, ApoA-1 and HDL to the liver where the cholesterol is excreted or is converted to bile salts. The cholesterol and or bile salts are then transported from the liver into bile for excretion via feces. This ensures proper removal of excess cholesterol from the body.

Research has shown that the rate of reverse cholesterol transport also needs to be properly regulated for the liver to efficiently rid the peripheral tissues of excess cholesterol. The initial studies which established the importance of reverse cholesterol transport from vasculature by

macrophages was first performed by Ross and Glomset [11]. These studies showed that in normally functioning vasculature, the removal of excess arterial cholesterol is efficiently performed my macrophages. However, in vasculature exhibiting endothelial dysfunction there is an increase in the rate of arterial cholesterol deposition versus arterial cholesterol removal. Following this initial observation, further research has shown that macrophages and HDL are both important in the prevention of cholesterol toxicity in arteries [18, 19]. In rats, studies mimicking an increased rate of reverse cholesterol transport showed an increase in fatty accumulation in the liver partly due to the liver's inability to increase the speed at which it transports the excess cholesterol into bile for biliary cholesterol secretion [20]. Previous studies in our lab has shown that PLD2$^{-/-}$ mice aortas exhibit an increase rate of plaque deposition and progression when placed on a high fat diet. Similarly, when crossed on an ApoE background ApoE/PLD2$^{-/-}$ mice also had a significant increase in atherosclerosis but at a much faster rate of plaque deposition than the single PLD2$^{-/-}$. Here, we hypothesize that PLD2$^{-/-}$ leads to a decrease in liver lipid droplet size and quantity due to a decrease in reverse cholesterol transport via ABCA1.

The report of PLD2-generated cPA acting as a PPARγ antagonist adds another element to this complex field. The PPARs are transcription factors that play key roles in the regulation of triglyceride metabolism, lipoprotein accumulation, and fatty acid storage [21]; accordingly, PPAR activity connects to the development of atherosclerosis, during which LDL and monocyte transmigration across the endothelium increases [22]. The potential contribution of PLD2-generated cPA to CVD via PPARγ antagonism is still unclear.

MATERIALS AND METHODS

Animals. *PLD2*$^{-/-}$ mice (C57BL/6) [23], generously provided by Dr. Gil di Paolo (Columbia University), were bred in the Stony Brook University animal facilities. Mice were fed standard chow or high-fat diet chow and water *ad libitum* and kept on a 12:12-h dark-light cycle. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). Male mice were fed normal chow or chow containing 40% fat at 7 weeks of age (n=6 mice /group). FIPI was administered 2x daily via IP injections of 3mg/kg FIPI in 4% DMSO/96% saline [7].

Tissue Oil Red O Staining. Following HFD, mouse liver tissue was embedded in OCT compound then snap-frozen in liquid nitrogen. Tissues were sectioned at 6-8 μm followed by staining with Oil Red O (Sigma-Aldrich) for lipid deposition following the manufacturer's protocol.

Primary Macrophage Isolation. Femurs from 3-6-month-old mice were cleaned of excess muscle and fat and flushed with 4mL of extraction media (DMEM + 10% FBS) to collect the bone marrow cells. The cell suspension was allowed to settle for 60 sec to remove clots and the supernatant centrifuged at 1000 x g for 10min at RT to pellet the myeloid progenitor cells. The pellet was resuspended in 4mL extraction media and re-centrifuged 3 times to wash the cells. Pellets were resuspended in 10mL of BMM-high (L-sup (supernatant of L- 929 cells, 30% by volume), FBS (20%), pyruvate (1%), DMEM (50%), 2.5% Pen/Strep) and plated on 10mm plastic petri dishes to initiate differentiation of the progenitor cells into macrophages [24, 25].

Medium was replaced on day 3 of culture; on day 5, macrophages are recovered and seeded to either 6 or 24-well plates at least 12 hours prior to experimentation. The macrophages were seeded at 1.5×105 cells/well in 24-well plates for Ac-LDL internalization and confocal imaging or 2.5×106 cells/well in 6-well plates for Western blot analysis.

PCR Primers.

ABCA1 forward: AAAACCGCAGACATCCTTCAG and

reverse: CATACCGAAACTCGTTCACCC.

LCAT forward: CCCACCAGCAGGATGAATACTAC and

reverse: AGGCTATGCCCAATGAGGAA.

GAPDH forward: CATGTTCCAGTATGACTCCACTC and

reverse: GGCCTCACCCCATTTGATGT.

Western Blots. Cells were cultured in six-well plates until 80-90% confluent then trypsinized. Equal amounts of total protein was resolved by SDS-PAGE and blotted onto nitrocellulose membranes (162-0115; Bio-Rad) using NuPAGE Transfer Buffer (NP0006-1; Life Technologies). Membranes were blocked for 1-h in 5% BSA at room temperature (RT) and probed overnight (O/N) with rabbit anti-ABCA1 at 1:500 (ABCAM) and rabbit anti-Actin at 1:100 (A2066; Sigma-Aldrich). Membranes were probed with anti-rabbit or anti-mouse secondary Ab at 1:10,000 and developed and quantified using a LI-COR Odyssey Infrared Imager

Ac-LDL Cholesterol internalization. Primary macrophages were seeded and incubated in media containing 10 μg/mL Alexa Fluor 488-AC-LDL for 4 hours. Cells were rinsed for 5 minutes with PBS (repeated 3x) then fixed with 4% paraformaldehyde for 10 minutes. Cells were rinsed for 5 minutes with PBS (repeated 3x) then stored for at 4^0C until time for imaging using a Leica TCS5 Confocal Microscope.

Primary Macrophage Oil Red O Staining. Following Ac-LDL incubation, primary macrophages were rinsed with PBS then stained with Oil Red O (Sigma-Aldrich) for lipid deposition following the manufacturer's protocol.

Immunofluorescent Staining and Microscopy. Human umbilical vein cell line EA.hy926 (CRL-2922; ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with L-glutamine (2 mM), 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were transduced with either Control shRNA Lentiviral Particles-A or with PC-PLD2 shRNA (h) Lentiviral Particles (Santa Cruz Biotechnology), which contain a puromycin-resistance gene to enable selection of stable cell lines or pools. The culture medium was changed 24 hrs following transduction and the cells cultured another day. Puromycin (0.5 μg/ml) was then added for positive selection. The resulting stably-transduced EA.hy926 cell pools were denoted "scramble" and shPLD2, respectively.

Cells were cultured in 12-well plates on glass coverslips until 80-90% confluent, washed with PBS (repeated 3x) then fixed with 4% paraformaldehyde for 10 minutes then rinsed for 5 minutes with PBS (repeated 3x), permeabilized with Triton X-100 and blocked with 5% goat

serum for 1 hr. Cells were then stained with rabbit anti-ABCA1 at 1:200 (ABCAM) for 1 hr, rinsed for 5 minutes with PBS (repeated 3x) then incubated for 1 hr with DAPI and Alexa 647 anti-rabbit secondary antibody. Cells were rinsed for 5 minutes with PBS (repeated 3x) then stored at 4^0C until time for confocal microscopy. Images were captured using a Leica TCS5 Confocal Microscope.

Statistical analysis. Numerical data are presented as mean ± SEM. Student's t-test was used to compare the differences between two groups and one-way ANOVA to compare the differences between three or more groups. Significance was based on a value of $p<0.05$. Experiments were performed in duplicate unless otherwise noted in the Figure Legends. Statistical analysis was performed on the collection of average values for each independent experiment (n = repeats).

PRELIMINARY RESULTS

PLD2$^{-/-}$ mice weigh less than and have a significantly smaller liver-to-body weight ratio than WT mice. PLD2$^{-/-}$ mice were placed on a 40% fat HFD for 7 months. Compared to WT mice, PLD2$^{-/-}$ mice body weight trend lower than WT mice ($p < 0.06$, $n = 6$, Fig. 4-3A). Two weeks prior to the end of the study, WT mice on the HFD were treated for 2 weeks (2x per day) with small molecule inhibitor FIPI via IP injections at dose 3mg/kg FIPI in 4% DMSO/96% saline. FIPI treated WT mice also experienced a slight decrease in body weight following completion of the study.

Used as a marker for liver enlargement due to accumulation of fats in the liver cells, the liver-to-body weight ratio (LTBR) of mice on HFD was taken ($n = 6$, Fig. 4-3B). Prior to the HFD, there was no significant change in the liver-to-body weight ratios of WT vs. PLD2$^{-/-}$ mice (LTBR ~5%). However, following the diet, there was a greater enlargement of livers in WT mice (LTBR ~8%) compared to FIPI treated WT (LTBR ~4.5%) and PLD2$^{-/-}$ (LTBR ~5.5%).

PLD2$^{-/-}$ mice liver maintain a fairly normal morphology compared to WT mice following an HFD. Morphological evaluation of livers following 7 mos HFD showed fatty accumulation in the livers of all experimental groups ($n=6$, Fig. 4-4). However, WT mice experienced a more severe fatty accumulation (Fig. 4-4A) whereas PLD1, and 2$^{-/-}$ mice experienced mild-to-moderate fatty accumulation (Fig. 4-4C and D). Treatment with FIPI was sufficient to decrease the fatty accumulation experienced by WT mice (Fig. 4-4B).

The regulation of liver fat accumulation is the result of a balance between fat acquisition from peripheral tissues, and liver disposal via conversion to bile salt and excretion through the feces. A 40% HFD increases the delivery of fatty acids to the liver thereby leading to the development of a fatty liver. Magnification of WT+FIPI livers show remnants of fatty accumulation surrounding the liver cells' nucleus (Fig. 4-4F) suggesting a shift towards fat removal from these cells. Using the NIH's ImageJ software, WT livers were also shown to have a significantly higher number of lipid droplets in their liver (Fig. 4-4I). Similarly, when the size of the droplets was measured, WT liver droplets were significantly larger than all other experimental groups (Table 4-1). Interestingly, despite PLD1$^{-/-}$ mice livers having significantly less lipid droplets, the size of their droplets was 2x the size of PLD2$^{-/-}$ mice.

PLD2$^{-/-}$ mice have a decreased fibrotic fiber staining than WT mice following a HFD. Chronic increase in the accumulation of fats and triglycerides in the liver is generally accompanied by the development of nonalcoholic steatohepatitis (NASH), characterized by inflammation of the liver and liver damage which arises from a significant accumulation of fats in the liver. Livers suffering from NASH also experience a vast amount of liver remodeling characterized by the formation of scar tissue (fibrosis). WT and PLD$^{-/-}$ livers were stained with Masson's trichrome following the 7 mos 40% HFD (n=6, Fig. 4-5). PLD2$^{-/-}$ mice liver had a decrease in fibrotic fiber staining compared to WT, WT+FIPI and PLD1$^{-/-}$ mice. Moderate type 1 collagen staining (blue) can be seen around the central vein of WT, WT+FIPI and PLD1$^{-/-}$ mice, with very mild staining in the latter.

PLD2$^{-/-}$ mice have decreased liver Oil Red O staining than WT mice following a 4 week HFD. Oil Red O (ORO) is commonly used to stain fat globules in liver cells. WT and PLD2$^{-/-}$ mice livers were frozen and sectioned following a 4 week 40% HFD (n=6, Fig. 4-6). WT mice liver had a significantly stronger ORO staining than PLD2$^{-/-}$ mice, suggesting that PLD2$^{-/-}$ mice are less susceptible to fat accumulation.

PLD2$^{-/-}$ primary macrophages experience more severe accumulation of 488-Ac-LDL than WT mice following 4 hours incubation. Since the excess cholesterol is not circulating in PLD2$^{-/-}$ mice blood (based on serum cholesterol measurements) nor accumulating in their liver cells, the rate of cholesterol deposition in primary macrophages and blood vessels was measured. To do so, Ac-LDL internalization was measured. WT and PLD2$^{-/-}$ primary macrophages were incubated for 4 hours with Alexa 488-Ac-LDL. PLD2$^{-/-}$ primary macrophages had an increased Alexa 488-Ac-LDL accumulation (n=4 femurs from 4 different mice per group, Fig. 4-7). Cells were grouped based on the severity of Alexa 488-Ac-LDL accumulation, where stage 0 = no Alexa 488-Ac-LDL accumulation and 5 = most severe Alexa 488-Ac-LDL accumulation.

PLD2$^{-/-}$ primary macrophages have increased Oil Red O staining than WT mice macrophages following incubation with Ac-LDL. PLD2$^{-/-}$ primary macrophages incubated with Ac-LDL (which is taken up by CD36) and stained with ORO show an increase in cholesterol accumulation in the PLD2$^{-/-}$ macrophages compared to WT macrophages (n=7 femurs from 7 different mice per group, Fig. 4-8). Unlike the liver cells, only 47% of WT macrophages became ORO+ under the experimental conditions, whereas 67% of the PLD2$^{-/-}$ macrophages were positive.

PLD2$^{-/-}$ primary macrophages have a significantly slower response to LXR agonist T0901317 (T09). To determine whether the increased Ac-LDL accumulation seen in PLD2$^{-/-}$ primary macrophages is due to an increase in lipid influx or a decrease in lipid efflux, western blot of primary macrophage extracts, were probed for ABCA1 protein which mediates excess cellular cholesterol removal. Under basal conditions, PLD2$^{-/-}$ primary macrophages have a 30% increase in basal ABCA1 protein levels compared to WT primary macrophages (Fig. 4-9A, Lane "0"). However, when stimulated by the LXR agonist T0901317 (T09), PLD2$^{-/-}$ macrophages have a 75% decrease in ABCA1 levels. These data suggest potential alterations in PPARγ-regulated ABCA1 transporter.

PLD2$^{-/-}$ primary macrophages also exhibited a decrease in ABCA1 mRNA levels (Fig. 4-9B) when compared to WT primary macrophages. Similarly, PLD2$^{-/-}$ primary macrophages also have a decrease in Lecithin–cholesterol acyltransferase (LCAT) mRNA levels. During HDL synthesis, LCAT is the enzyme responsible for the conversion of free cholesterol into cholesteryl ester which is used to create HDL.

PPARγ regulates cholesterol efflux from macrophages via ABCA1 to HDL [26, 27] through increasing ABCA1 expression via the PPARγ-Liver X Receptor (LXR)-ABCA1 pathway [28]. Previous studies have shown that PLD2 and ABCA1 activities are very closely linked [29, 30]. Similarly, PLD2$^{-/-}$ mice primary macrophages have an altered ABCA1 protein level following stimulation with LXR agonist T09. This decrease in ABCA1 protein levels could impart an overall decrease in cholesterol efflux from macrophages and blood vessels to the liver for

excretion, resulting in an increase in modified LDL accumulation in the macrophages and blood vessels of PLD2$^{-/-}$ mice, and the associated decrease in fatty accumulation in their livers. Supporting this finding, PCR of ABCA1 in PLD2$^{-/-}$ mice also showed a decrease in ABCA1 mRNA levels. These data suggest the rate of LDL efflux by PLD2$^{-/-}$ mice may be slower than that of WT mice. Further experiments need to be performed to conclude the current work. These experiments would include a more in depth monitoring of the rate of cholesterol influx versus efflux of cholesterol in macrophages and liver cells. The present experiments provide some insight into the mechanisms responsible for PLD2's role in the development of atherosclerosis. These results suggest the excess cholesterol consumed by PLD2$^{-/-}$ mice is localizing to the aorta via macrophage internalization and is not being efficiently shuttled to the liver for cholesterol excretion.

FUTURE WORKS

Further studies need to be conducted on the rate of both influx and efflux of cholesterol in PLD2$^{-/-}$ primary macrophages. The preliminary data presented in this dissertation suggest that the rate of reverse cholesterol transport in PLD2$^{-/-}$ mice macrophages is slower than that of WT mice based on the levels of HDL and ABCA1 in these cells, as well as the quantity of lipid accumulation in the livers of mice. However, not much is known about the rate of influx of cholesterol into PLD2$^{-/-}$ mice macrophages. It is expected that the rate of LDL internalization by PLD2$^{-/-}$ mice macrophages may also be lowered compared to WT mice, however, since PLD2$^{-/-}$ mice macrophages' ABCA1 levels are also lowered the net movement of their cholesterol may be inward resulting in the increased lipid accumulation in macrophages and the decrease in the liver. Such data would lead to a better understanding of whether the increase in Ac-LDL staining in PLD2$^{-/-}$ primary macrophages is truly due to a decrease in efflux rate.

Similarly, hepatic lipids need to be extracted and liver triglyceride and cholesterol measurements need to be performed to further confirm our findings that the excess cholesterol in PLD2$^{-/-}$ mice following a HFD is more significantly directed to the blood vessels and not the liver of these mice. Because of the cholesterol distribution to the blood vessels, PLD2$^{-/-}$ mice are expected to be protected from the development of nonalcoholic fatty liver disease. Therefore, plasma acetoacetyl-CoA synthetase (AACS), dipeptidyl-peptidase 4 (DPP4), glutamine synthetase (GLUL), and glutathione *S*-transferase (GST) levels need to be measured. These proteins are well known to be associated with hepatic lipid deposition and thus are observed as biomarkers for nonalcoholic fatty liver disease. As a control, mice on a HFD should also be supplemented

with either 900 ppm Betaine or 1% DHA, both of which are commonly used to lower hepatic lipid deposition and are also shown to decrease the expression of the biomarkers associated with nonalcoholic fatty liver disease [31].

These preliminary results also lay the foundation for determining the cause of the difference in body weight of Wildtype, PLD1$^{-/-}$, and PLD2$^{-/-}$ mice. Body weight regulation is crucial in the development of many obesity-related comorbidities, including hypertension, atherosclerosis and diabetes. Therefore, understanding the mechanism by which PLD2$^{-/-}$ mice maintain a lower body weight than their wildtype and PLD1$^{-/-}$ counterparts will help in the development of medicines for the regulation of weight gain and subsequently the prevention of these comorbidities. One way to further the understanding of PLD2$^{-/-}$ mice mechanism for regulating their body weight is through the study of their white adipose fat pad tissue weight, size, and number. The physiological and metabolic profiles of rodent white adipose fat pads are commonly studied to help better understand the human white fat adipose depots. Finally, Wildtype, PLD1$^{-/-}$ and PLD2$^{-/-}$ mice should also be monitored for the development of hyperglycemia, hyperinsulinemia, and hypoadiponectinemia. All of which are characteristic in the development of NASH in humans.

FIGURES

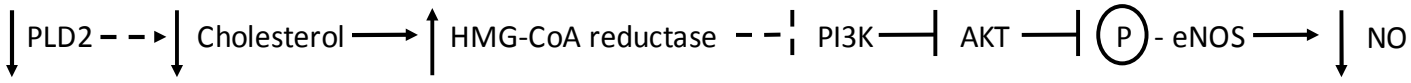


Figure 4-1. Proposal of alternate mechanism of PLD2's effects on eNOS phosphorylation and activation.

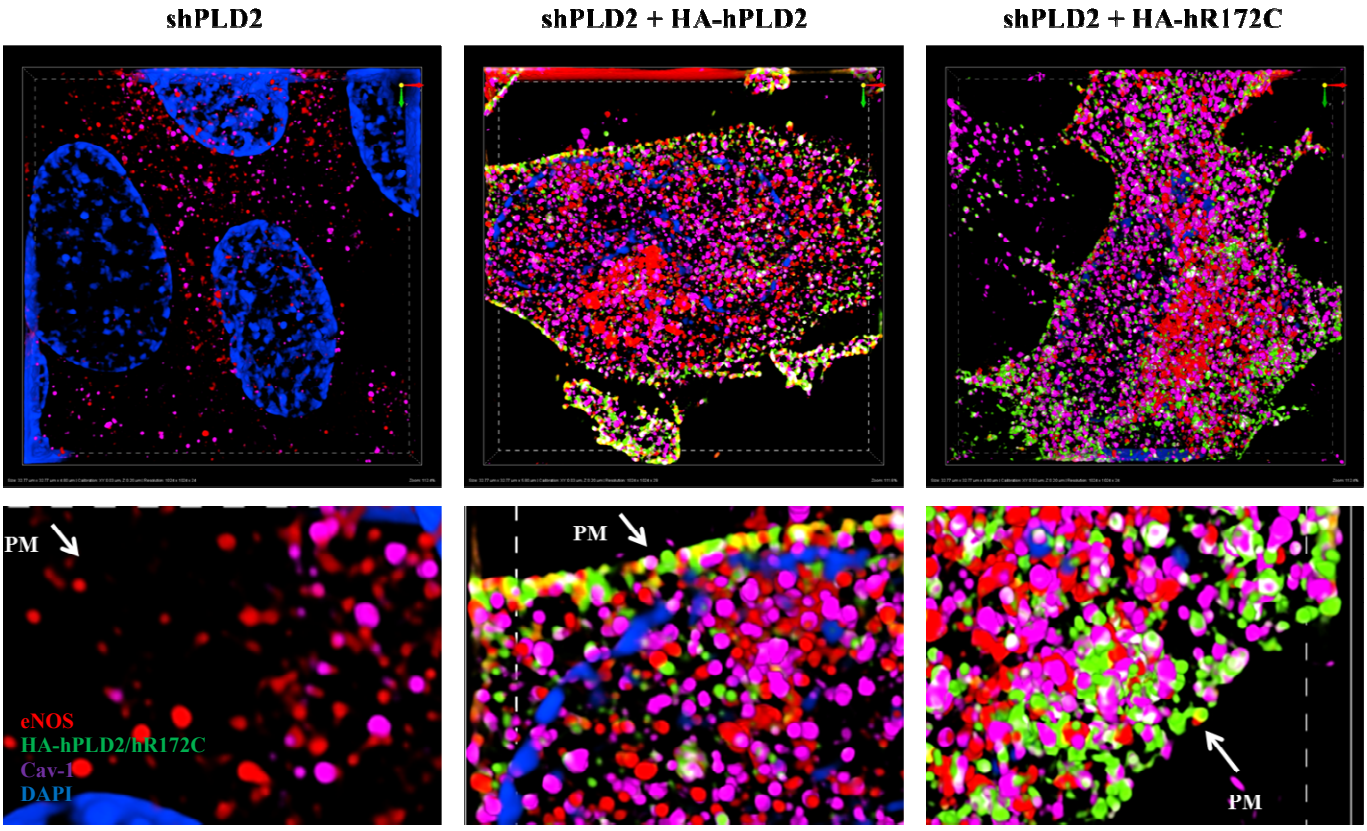


Figure 4-2. Change in the co-localization of eNOS/HA-hPLD2 or HA-hR172C/Cav-1 in transfected shPLD2 endothelial cells. HA-hPLD2 localizes primarily to the plasma membrane (arrow; PM) of shPLD2 cells. HA-hPLD2 also seems to co-localize with eNOS and Cav-1 at the PM. The presence of an HA-hR172C and Cav-1 complex is seen at the PM of shPLD2 cells with a stronger HA-hR172C/eNOS/Cav-1 complex seen in the cytosol of shPLD2 cells. SIM microscopy imaging of shPLD2 endothelial cells transfected with either HA-hPLD2 or HA-R172C.

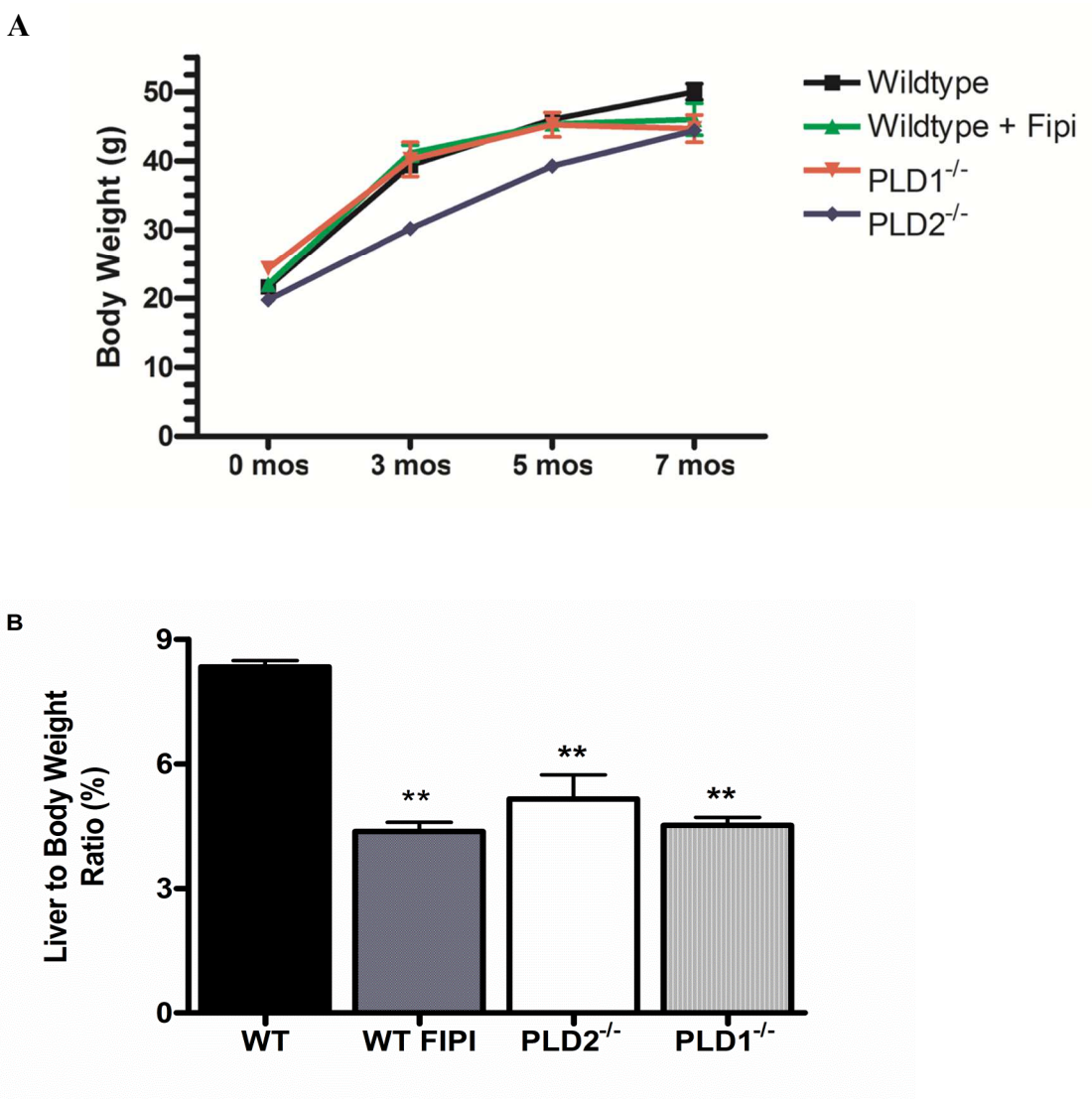
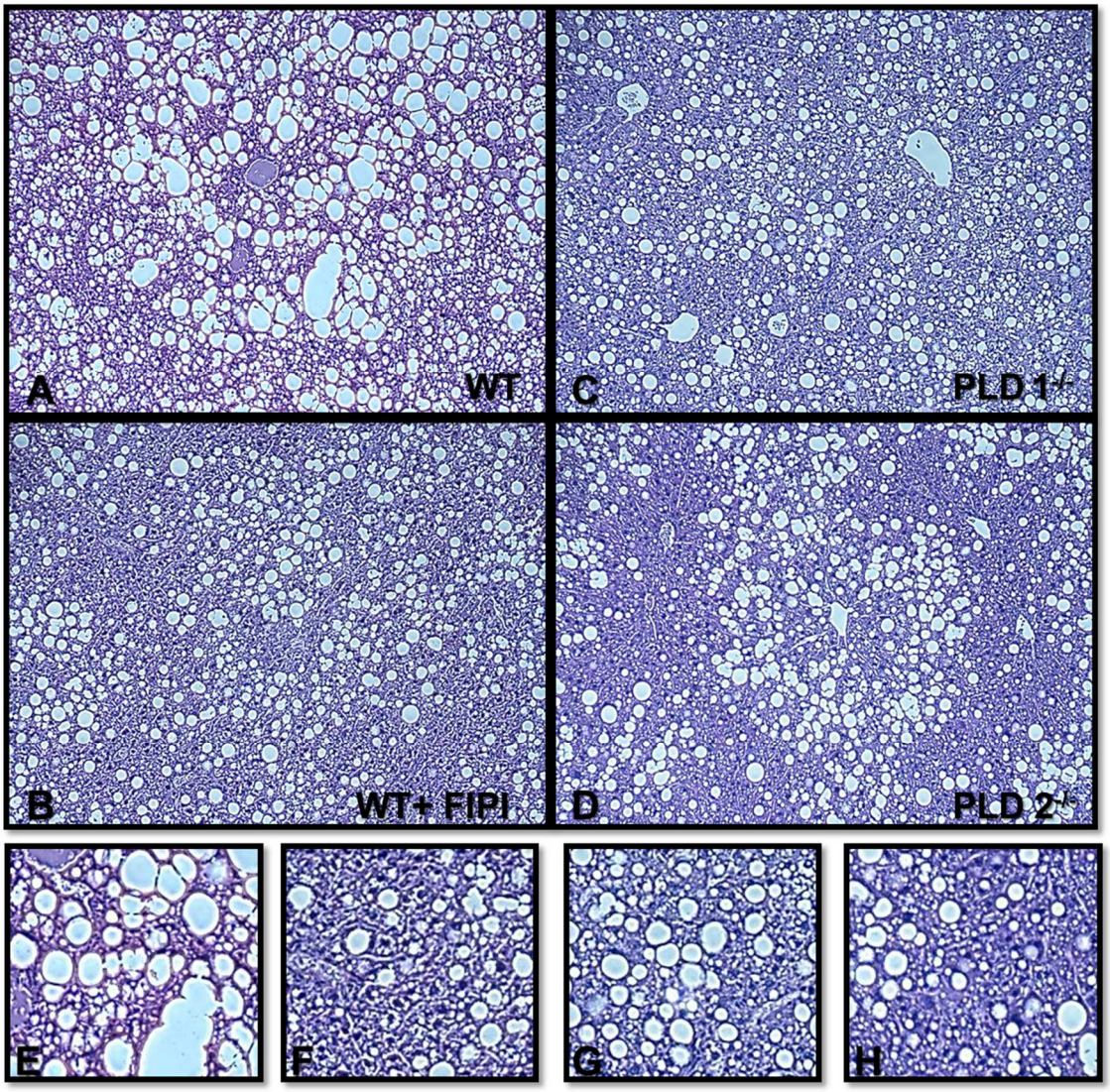


Figure 4-3. PLD2$^{-/-}$ mice weigh less than WT mice and have a significantly smaller liver-to-body weight ratio. Body weight in grams (A) and liver-to-body weight ratio (B) following a 7 months high fat diet on in WT, PLD2$^{-/-}$ and FIPI treated WT mice (n=6 per group).


A
WT
C
PLD 1-/-
B
WT+ FIPI
D
PLD 2-/-
E
F
G
H

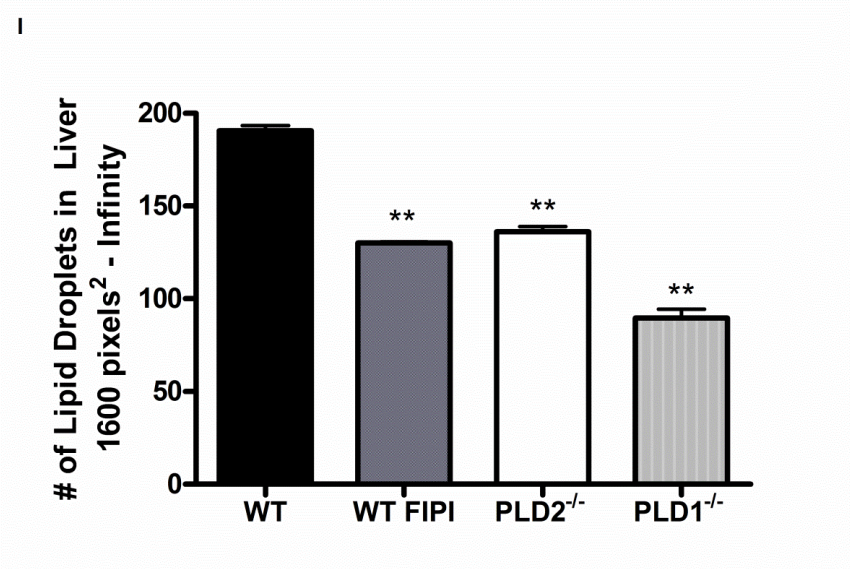


Figure 4-4. PLD2$^{-/-}$ mice liver maintain a fairly normal morphology than WT mice following an HFD. Representative Hematoxylin and Eosin staining of livers from (A) WT (B) PLD1$^{-/-}$ (C) FIPI treated WT and (D) PLD2$^{-/-}$ mice following a 7 months high fat diet (n=6 per group). Original magnification x100. (I) Image J quantification of number of lipid droplets in WT, PLD1$^{-/-}$, FIPI treated WT, and PLD2$^{-/-}$ mice livers.

Pixel^2 Value	**WT**	**WT+F**	**PLD1$^{-/-}$**	**PLD2$^{-/-}$**
Max	58,112	8,187	36,857	13,937
Min	2,526	2,506	2,507	2,504

Table 4-1: Image J quantification of size of lipid droplets in livers ranging from smallest lipid droplet (Min) to largest lipid droplet (Max).

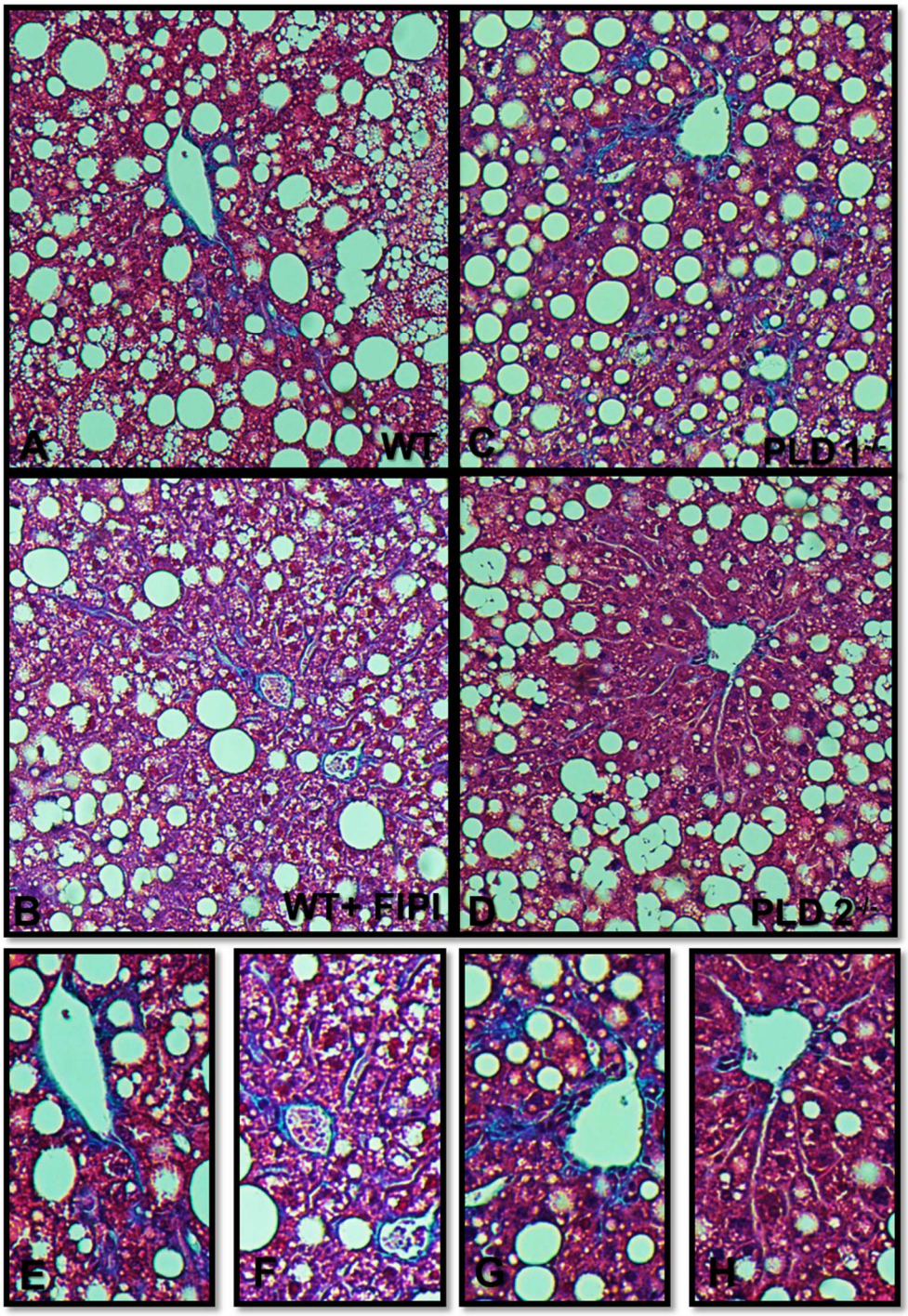


Figure 4-5. PLD2$^{-/-}$ mice have less fibrotic fiber staining than WT mice following a HFD. Representative Masson's Trichrome staining of livers from (A) WT (B) PLD1$^{-/-}$ (C) FIPI treated WT and (D) PLD2$^{-/-}$ mice following a 7 months high fat diet (n=6 per group). Original magnification x100, with higher magnification x200.

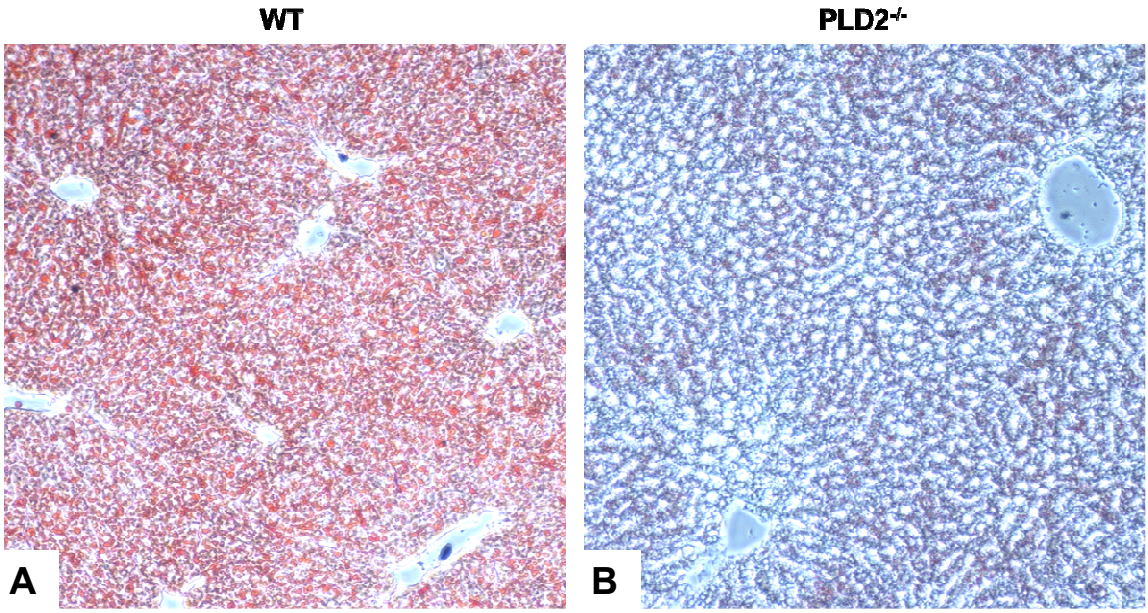


Figure 4-6. PLD2$^{-/-}$ mice livers have decreased Oil Red O staining than WT mice following an HFD. Representative Oil Red O staining of livers from (A) WT (B) PLD2$^{-/-}$ mice following a 7 months high fat diet (n=6 per group).

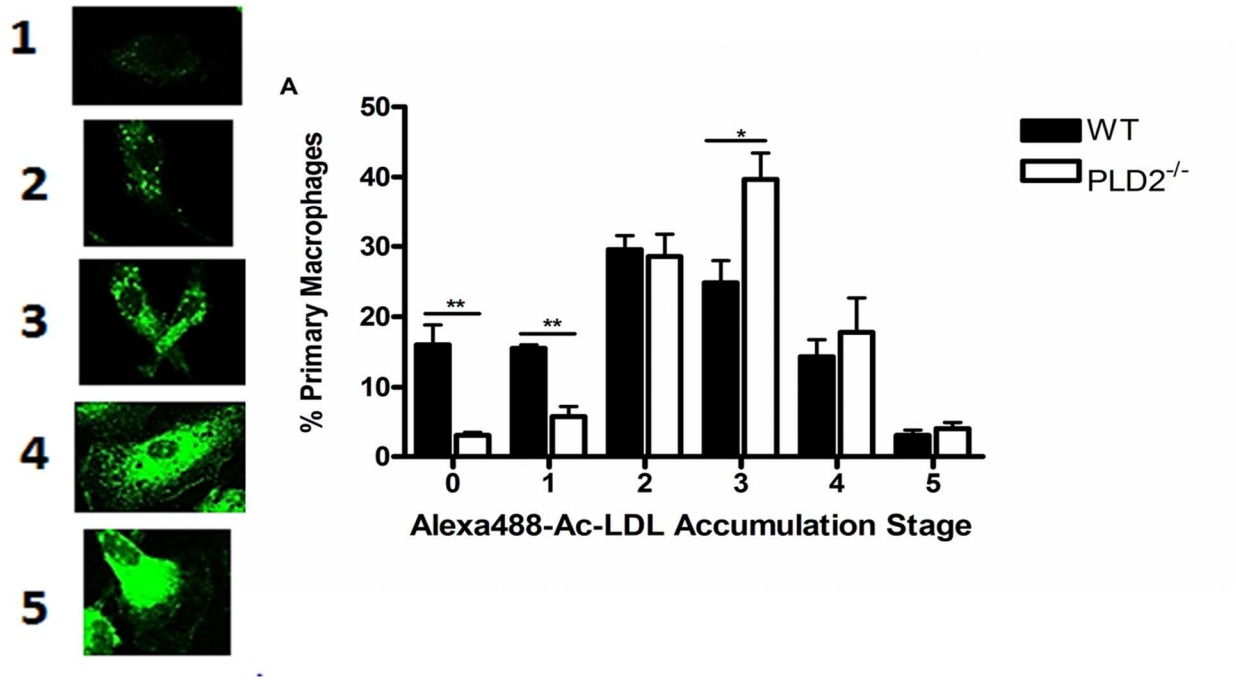


Figure 4-7. PLD2$^{-/-}$ primary macrophages experience more severe accumulation of 488-Ac-LDL than WT mice following 4 hours incubation. Representative quantification and staging of fluorescent staining in primary macrophages from WT and PLD2$^{-/-}$ mice, following 4-hours incubation with 488-Ac-LDL (n=7 femurs from 7 different mice per group).

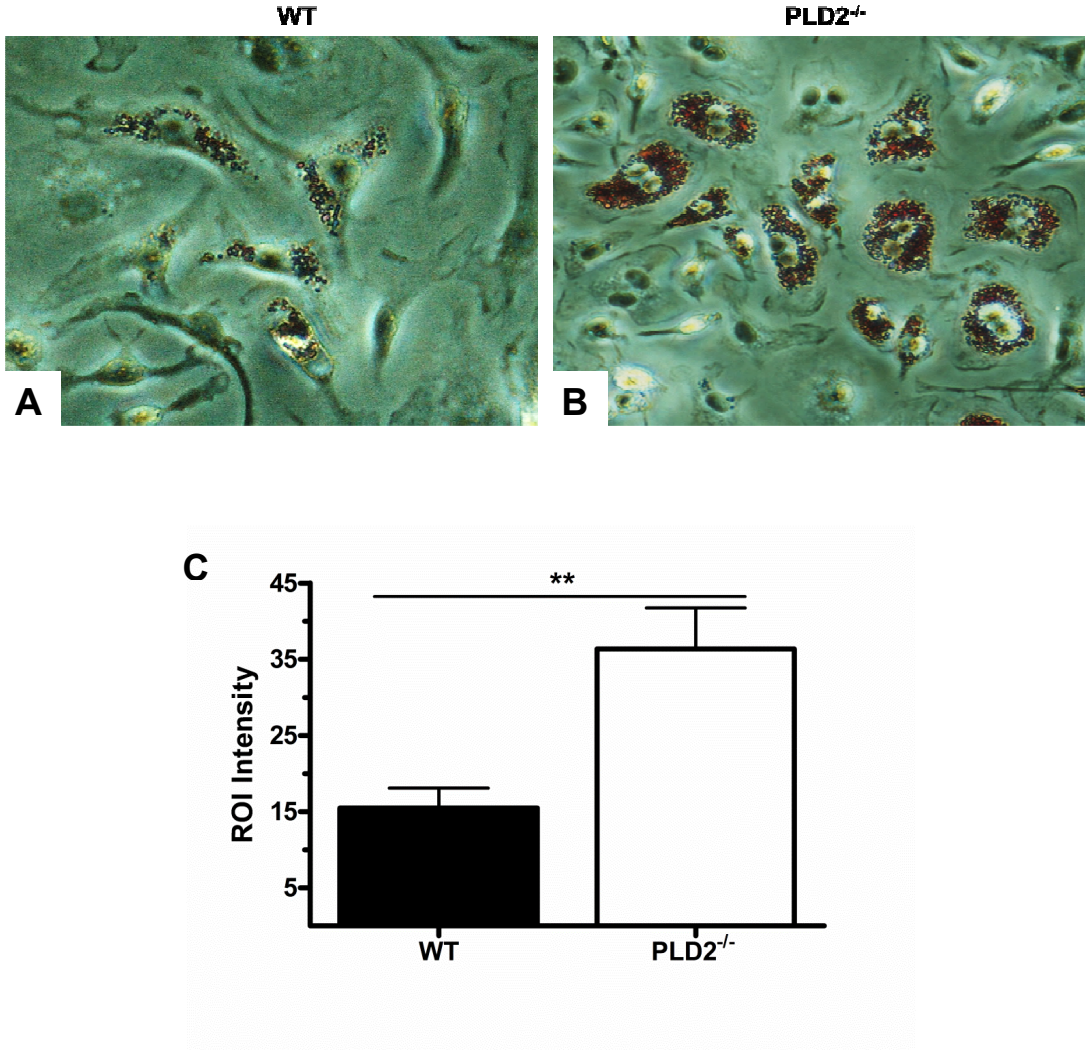


Figure 4-8. PLD2$^{-/-}$ primary macrophages have higher Oil Red O staining than WT mice macrophages following incubation with Ac-LDL. Representative Oil Red O staining of primary macrophages from (A) WT (B) PLD2$^{-/-}$ mice macrophages following 4-hours incubation with Ac-LDL (n=7 femurs from 7 different mice per group). (C) Image J quantification of Oil Red O staining.

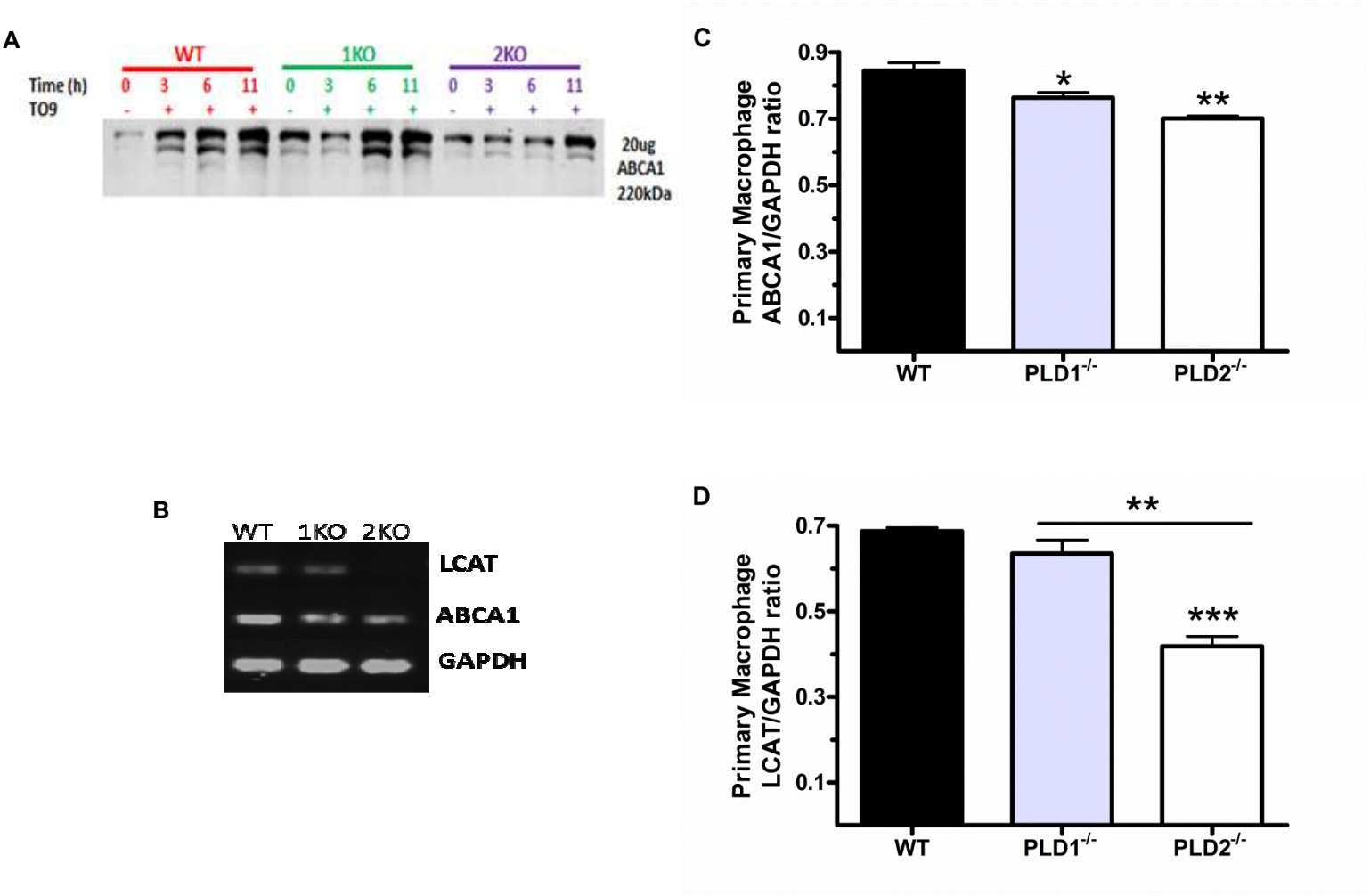


Figure 4-9. PLD2$^{-/-}$ primary macrophages had a significantly slower response to LXR agonist T0901317 (T09) than PLD1$^{-/-}$ and WT cells. (A) Western blot analyses of WT, PLD1$^{-/-}$ and PLD2$^{-/-}$ primary macrophages following 0, 3, 6, and 11 hour treatments of LXR agonist T09. (B) LCAT and ABCA1 mRNA levels of WT, PLD1$^{-/-}$ and PLD2$^{-/-}$ primary macrophages. Quantitative analyses of ABCA1 (C) and LCAT (D) mRNA of WT, PLD1$^{-/-}$ and PLD2$^{-/-}$ primary macrophages.

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Appendix A

List of eNOS Activators

List of eNOS Activators	
eNOS Activators	**Reference**
Bradykinin	[1]
ATP	[1]
ADP	[2]; [3]
Estradiol/Oestradiol	[4]; [5]
VEGF	[6]; [7]
Acetylcholine (Ach)	[8]; [9]
Endoglin (CD105)	[10]; [11]
Protein kinase B	[12]; [13]
Hsp90	[14]; [15]; [16]
PP1	[17]; [18]
PP2A	[19]; [20]; [21]
NOSTRIN	[22]; [23]; [24]
NOSIP	[25]
Caveolin	[26]; [27]
Calmodulin	[26]; [28]; [29]
SIP and LPA	[30]
Thrombin	[31]; [32]

Table A-1. List of eNOS Activators: Platelet-derived lipid mediators – sphingosine 1-phosphate (SIP) and lysophosphatidic acid (LPA). Phosphatases – serine-threonine protein phosphatase 1 (PP1), serine-threonine protein phosphatase 2A (PP2A) and calcineurin. eNOS TRafficking INducer protein (NOSTRIN). eNOS Interacting Protein (NOSIP).

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Appendix B

Synopsis of ABCA1

ATP-binding cassette A1 (ABCA1)

The ATP-binding cassette (ABC) protein ABCA1 plays a major role in the regulation of normal and healthy cell/tissue cholesterol content. The ABC protein family includes 49 transmembrane transporters which transport several substrates across membrane bilayers [1]. As hinted by its name, this family of transporters functions in an energy dependent manner requiring the hydrolysis of ATP. Cholesterol is a large component of the Golgi and plasma membranes, synthesized from acetyl-coenzyme A (acetyl-CoA), and is significant in maintaining the fluidity of these membranes [2]. Cholesterol is also the precursor molecule to steroid hormones as well as to bile acids, adding to the importance of this molecule in eukaryotes. The cholesterol found in specific areas of the plasma membrane known as lipid rafts, have been shown to play a vital role in allowing signal transduction in cells [3].

The maintenance of cholesterol levels is highly crucial to the quality of life in organisms. This is because cholesterol overload is toxic to cells and can lead to the development of many diseases such as atherosclerosis and hypertension. ABCA1 function is widely accepted as one which allows for the efflux of cholesterol and phospholipids out of cells leading to the maturation of high density lipoprotein (HDL). $ABCA1^{-/-}$ mouse models have shown that the prevention of translation of liver ABCA1 will lead to an 80% decrease of HDL while the loss of intestinal ABCA1 leads to a 25% decrease in HDL [4, 5]. Several studies have also shown that mutations in ABCA1 correlate to the development of atherosclerosis as well as some HDL deficiencies. Some of these deficiencies include familial hypoalphalipoproteinemia caused by

ABCA1$^{-/+}$ and Tangier disease caused by ABCA1$^{-/-}$ [6, 7]. In these diseases, the efflux of excess cholesterol to apolipoprotein-A1 (ApoA1) by ABCA1 is compromised preventing the formation of HDL as well as preventing the removal of excess cholesterol from the organism through reverse cholesterol transport. As a result, there is an increase of cholesterol ester accumulation in the tissues of these individuals. Diseases which arise from an inability to properly efflux cholesterol are generally always correlated with at least one mutation of one of the 49 human ABC transporters. Current research have unmasked 73 different mutations in ABCA1 alone which result to some degree of change in cholesterol efflux [8].

Two major models have been created to explain the efflux of cholesterol from cells by ABCA1. These include the flippase model [9] which states that the substrate lipid will diffuse into the binding site of the ABC transporter when the transporter is allosterically opened. Once the substrate and ATP has bind to the transporter, the transporter dimerizes and changes to a closed conformation which flops the lipid substrate to the exoplasmic leaflet of the lipid bilayer. ABC transporters employing this mechanism can also flip substrate lipids to the cytosolic leaflet of the lipid bilayer. In a variation of the flippase model a “flopless activation” (termed “flopless” because during this movement, the lipid’s polar headgroup does not need to be reoriented) [10, 11] of ABC transporters is also proposed. In this model, following the binding of the substrate lipid and ATP, the ABC transporter changes to its closed conformation. However, once closed, the lipid does not flop out of the transporter and instead awaits the arrival of an acceptor molecule (like HDL or albumin) to bind to the ABC transporter with the assistance of ApoA1. Once the acceptor molecule has bind to the transporter, the substrate lipid is then able to diffuse onto the acceptor.

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