THE THERAPEUTIC POTENTIAL OF ALTERING LIPID FLUX FOR

DIABETIC RETINOPATHY

A Thesis in

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by

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Diabetic retinopathy is a debilitating complication of diabetes and a leading cause of vision loss. Diabetic retinopathy is a multifactorial disease that affects multiple cell types within the retina resulting in changes in glial function, the vasculature and neuronal apoptosis. However, the fundamental mechanisms contributing to vision loss remain undefined.

Protein Kinase C (PKC) has been identified as a putative contributor to diabetic retinopathy. Therefore, we sought to explore PKC isotype function within the retina further. In R28 cells, an in vitro model of retinal neurons, activation of PKCs through exogenous application of diglycerides or phorbol esters impairs insulin-induced phosphorylation of Akt, identifying a possible role of PKCs in causing insulin resistance in retinal neurons. By immunohistochemistry, PKC isoforms demonstrate discrete immunolocalization within multiple layers of the retina, suggesting that PKCs may be involved in pathological features other than that proposed for the retinal vasculature. However, in examining the translocation and lipid microdomain localization of PKC isotypes from control and streptozotocin-induced diabetic rats, no significant alterations were observed. Furthermore, analysis of phosphorylation sites on PKCs that can be regulated by upstream kinases or a major downstream target of PKCs revealed no significant alterations. Therefore, we observed no global evidence of PKC activation and if PKCs are involved in diabetic complications it may be in discrete subpopulations of retinal cells. Alternatively, the pathological role of increased diglyceride concentration in diabetes may be independent of PKC.
Despite these negative data, diglyceride metabolism, a regulator of PKCs, may still be involved in diabetic complications. In fact, inhibition of diglyceride metabolism and resultant decrease in phosphatidic acid (PA) formation has been demonstrated to recapitulate some diabetic complications within the retina. We showed previously that mTOR-p70 S6K prosurvival signaling is diminished in the diabetic rat retina and that mTOR signaling is involved in insulin-mediated retinal neuronal survival. Therefore, further understanding of the mechanisms by which mTOR is regulated could have therapeutic implications for retinal neuronal cell death in diabetes. We show that insulin is able to activate p70 S6K in R28 retinal neurons. Furthermore, insulin and PKC activation synergize to further augment p70 S6K. To begin to understand the mechanisms responsible for this synergy, we investigated the role of lipid and non-lipid mediators to negatively regulate the tuberin/hamartin complex, an inhibitor of mTOR. We demonstrate that insulin and PKC differentially phosphorylate and inactivate tuberin. In addition, our data suggests that PKC, but not insulin, activation of mTOR is regulated by MAPK signaling cascade as well as phosphatidic acid, a metabolite of diacylglyceride. This dichotomy between insulin and PKC/phosphatidic acid activation of mTOR signaling is also a function of intact cholesterol-enriched microdomains. As the retina in diabetes is in an inflamed state, we determined that phosphatidic acid and insulin synergistically inhibited interleukin-1β-induced apoptosis in a rapamycin-sensitive manner. These results demonstrate that phosphatidic acid and insulin differentially and synergistically activate mTOR signaling to promote retinal neuronal survival. In addition, these data suggest that therapeutic modalities that elevate endogenous phosphatidic acid
concentrations may be effective in circumventing insulin resistance in diabetic retinopathy.

To further explore implications of altered lipid metabolism, sphingolipids were also analyzed. Dysregulated sphingolipid metabolism has been associated with insulin resistance and cellular death in many model systems and diseases. It is thus hypothesized that diabetes alters sphingolipid metabolism contributing to neuronal pathologies in diabetic retinopathy. Ceramide content as well as ceramide metabolites were measured after 2, 4 and 8 weeks of streptozotocin (stz)-diabetes by ESI/MS/MS. After 4 and 8 weeks of diabetes a ~30% decrease in total ceramide content was observed, concomitant with a significant ~30% increase in monohexosylceramides (cerebrosides) levels in fed diabetic rats compared to their age-matched controls. Acute insulin therapy as well as a short-term lowering of glucose via fasting did not affect the augmented flux through the glycosphingolipid biosynthetic pathway. This altered sphingolipid metabolism in diabetic retinas occurred without any significant changes of fatty acid composition from total lipid extracts, as assessed by gas chromatography. Interestingly, the retina contains a unique fatty acid profile that is enriched in palmitic and stearic acid compared to other tissues. As a positive control, we observed decreases in 16:1 and 18:1 fatty acids in lipid extracts from diabetic livers.

To assess the putative biological consequences of altered glycosphingolipid flux, we treated R28 retinal neurons with glucosylceramide synthase inhibitors. This led to increased insulin activation of p70 S6K. Inhibition of glycosphingolipid synthesis also suppressed glucosamine- and IL-1β-induced death. Consistent with these inhibitor studies, glycosphingolipid metabolism may regulate stress responses associated with cell
death. It is speculated that an increase in cerebrosides, and possibly higher order glycosphingolipids could contribute to the pathogenesis of diabetic retinopathy by contributing to insulin resistance resulting in neuronal cell death.

Taken together, this body of work supports the contention that altered lipid flux (sphingolipid, phospholipid) may contribute to the pathogenesis of diabetic retinopathy. Thus, dysfunctional lipid flux may be a contributor to metabolic stress in diabetes and therapeutic strategies to restore homeostatic lipid flux may be a viable approach for treatment of diabetic retinopathy.
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Chapter 1
Introduction

Diabetic retinopathy arises from a combination of alterations that lead to vision loss. These changes not only include increased cellular death, impaired insulin receptor signaling, alterations in protein kinase C, diglycerides and triglycerides, more recent data reveals changes in sphingolipid and glycosphingolipid flux.

1.1 Diabetes and Diabetic Retinopathy

Diabetes is a major health problem in the US, that affects approximately 20 million people and costs upwards of $98 billion in medical and indirect costs [1]. Those persons afflicted by diabetes have higher prevalence of many health complications including that of the cardiovascular, renal, peripheral nervous and visual systems. These complications arise from defective insulin production and/or insulin signaling. As a consequence, people affected by diabetes have a metabolic defect in the ability to produce energy from sugars and to respond to insulin-mediated signaling support.

As just mentioned, people with both type 1 and type 2 diabetes are more at risk for several visual problems. There is an approximate two-fold increase in the likelihood of cataracts and glaucoma compared to normal individuals, and a dramatic increase in the incidence of retinopathy [2]. Among people who are affected by diabetes, about 90% will exhibit retinopathy. Among these people, 25% will have some degree of vision loss with
progression to legal blindness 5% of the time [3]. This amounts to 20,000 new cases of legal blindness a year, making diabetic retinopathy one of the leading causes of blindness.

Diabetic retinopathy is a multifactorial disease of the retina, which affects multiple cell types within the retina. Many contributing factors to the pathogenesis of diabetic retinopathy have been identified, but how hyperglycemic conditions and dysfunctional insulin signaling cause them remains largely undefined. These different pathological changes include neuronal [3-10] and endothelial apoptosis and subsequent formation of acellular capillaries [11, 12], blood-retinal barrier breakdown [13], neovascularization and glial reactivity [5, 14], which all consequently hinder the normal vision process. By definition, retinopathy is defined as, “any of various noninflammatory disorders of the retina, including some that cause blindness” [15]. There is increasing evidence implicates an immune component in this disease; such as leukocyte adhesion/infiltration at the vasculature [16, 17] and microglial activation [5, 14] in the retina. This immune response likely contributes to the pathologies just mentioned, thus the term retinopathy is a misnomer.

1.2 Neuronal Apoptosis in Diabetic Retinopathy

The neurons of the retina receive and transmit visual cues from the external environment to the brain. Therefore, neuronal cell death can and does diminish one’s vision. Neuronal cell death in the retinas of humans with diabetes was demonstrated in 1961, specifically atrophy of ganglion cells and degeneration of the inner nuclear layer of
the retina was observed [18]. Degeneration of the inner plexiform and ganglion cell layers [19] and axonal degeneration due to ganglion cell body dysfunction [20] have also been described in human histological studies. More recent studies have demonstrated increased TUNEL positive neurons, a marker of nicked-DNA, which occurs in cells undergoing apoptosis, in human diabetic retinas [3]. In addition, ganglion cells in diabetic retinas express several proapoptotic molecules, suggesting that these cells may be more vulnerable to apoptosis [9]. Indeed, focal areas of retinal thinning have also been described in patients with diabetic retinopathy [21], and nerve fiber layer defects occur in patients with minimal or no vascular lesions [22]. Furthermore, decreased contrast sensitivity [23, 24] and color vision [25] have been described in patients with diabetes, further indicating dysfunction within the neural retina. These findings of neurodegeneration and diminished vision are also supported by electrophysiological tests. A recent review of the literature summarized these electroretinograms, which revealed diminished oscillatory potentials of the inner retinal layers within 6 months, within the macula after a year and the outer layers of the retina after 10 years of diabetes [26]. Hence, there is a neuronal pathology to diabetic retinopathy.

In order to investigate the mechanisms that cause retinal neuronal dysfunction, animal models of diabetes were utilized to determine if a similar pathophysiology is observed. In streptozotocin(stz)-induced diabetic rats, apoptosis was observed in ganglion cells. This apoptosis was prevented by nerve growth factor (NGF) treatment [4]. A later study further explored this and reported about a 10-fold increase in apoptotic cells in rats with stz-induced diabetes, with a reduction of the inner nuclear and the inner plexiform layer thickness. This increased rate of apoptosis was inhibited by insulin treatment [3].
Other laboratories have also demonstrated increases in apoptotic retinal neurons [10] and necrosis in the inner retinal layers and ganglion cell layers as well as apoptosis in the photoreceptors [6]. Similar to humans with diabetes, these rats demonstrate a reduction of the a- and b-waves of electroretinograms suggesting alterations in the outer and inner retina respectively [27-29]. This retinal neuronal dysfunction is not specific to the stz-induced diabetic rat model as increased neuronal cell death were observed in the retinas of an stz-mouse model [7] and the Ins2Akita mouse model of diabetes [30].

The classification of cellular death as necrosis or apoptosis is often imprecise. In several studies mentioned in the preceding paragraph, apoptosis is referred to as the cause of neuronal cell death by utilizing TUNEL staining to detect fragmented DNA. However, DNA degradation can also occur in necrosis and autophagic cell death [31, 32]. Many putative causes of diabetic retinopathy, such as ischemia [33], oxidative stress [34] and proinflammatory cytokines [35-37] can cause necrosis [32, 38]. Therefore, the use of the term apoptosis in this document may be an inaccurate description of the type of cell death that is actually occurring.

1.3 Insulin and the Retina

1.3.1 The retina is an insulin responsive tissue:

As the primary cause of diabetes is a failure to produce and/or effectively respond to insulin, several investigations into the role of insulin in the retinal physiology have been undertaken. As already mentioned, systemic insulin treatment diminishes apoptosis
in the diabetic retina [3], therefore it is imperative to understand the role of insulin in the retina. Insulin signals through various downstream molecules to regulate cellular responses. The pathways emphasized and discussed herein are shown in an overview in Fig. 1.1 are not meant to be all encompassing.

Immunohistochemistry studies have demonstrated that the insulin receptor (IR) is localized throughout all layers of the retina [39]. Studies that have investigated retinal IR kinase activity revealed that like brain IR kinase activity, it remains constant under both fed and fasted conditions [40, 41]. This is in contrast to liver IR kinase activity, which diminished in the fasted state [40]. Interestingly, the retina IR exhibits higher basal tyrosine phosphorylation in comparison to other tissues, such as muscle [41]. These results could be a reflection of decreased phosphatase activity on the IR or the presence of other IR activating factors, such as IGF-II [42, 43], light-induced activation of the IR [44] and/or estrogen (17β-estradiol and 17α-estradiol) [45, 46]. Another possibility is a local production of insulin in the retina. The mRNA for preproinsulin has been detected in the retina [47]. Immunoreactivity for insulin has been observed in the fetal and neonatal rat retina [48] as well as in cultured retinal glial cells [48]. Also, locally produced insulin has been demonstrated to regulate chick retinal neurogenesis during development [49]. However, it is currently unknown if this mRNA is translated and properly processed to produce a functional insulin protein in the mature retina. Regardless of the mechanism of this higher basal activity, systemic administration of insulin via the portal vein injection further increased the IRβ subunit tyrosine phosphorylation, a marker of activation, hence systemic insulin is a likely contributor to retinal IR activation [41].
Fig. 1.1: Insulin signaling pathway
In both the stz-induced diabetic rat and Ins2^Akita mouse models of diabetes the activity of the insulin receptors are reduced by approximately 25-30% [30, 50], which was further diminished after 12 weeks of stz-induced diabetes to nearly 50% [50]. Surprisingly, in an stz-mouse model, increased content and phosphorylation of the IR was observed, whereas no changes in the insulin resistant obese ob/ob mouse were seen compared to controls [51]. This disparity may be due to differences in the duration of diabetes, as the ob/ob and stz-induced diabetic mice were examined after 7-days of diabetes versus the longer time periods examined in the stz-induced diabetic rat and Ins2^Akita models. The contribution of diminished IR activity in the stz-induced diabetic rat and Ins2^Akita diabetic mice to neuronal cell death in diabetic retinopathy remains unknown. Investigation of retina-specific knockouts of the IR will provide information regarding the significance of the IR to retinal homeostasis in these animals.

1.3.2 Proximal insulin signaling and diabetes

1.3.2.1 Insulin receptor substrate (IRS)

While activation of the IR is an essential first step in mediating the effects of insulin, this means very little without the coordinate regulation of downstream effectors. The insulin receptor substrate (IRS) binds to the IR and serves as an adaptor protein to regulate PI3K activity. In the rat retina, IRS-2, but not IRS-1 demonstrates increased tyrosine phosphorylation upon insulin stimulation, both in \textit{ex vivo} and \textit{in vivo} retinas [41, 52]. Other IRS isoforms, IRS-3 and IRS-4, do not appear to be expressed in the retina.
In the STZ-induced diabetic rat model, within 12 weeks there is a significant decrease in IRS-2 content and tyrosine phosphorylation [50]. Again, surprisingly, in the STZ-mouse and ob/ob mouse models of diabetes an increase in both IRS-2 content and phosphorylation is seen in response to diabetes when compared to controls [51].

The importance of IRS-2 has recently been demonstrated in the retinas of IRS-2 knockout mice [53]. These authors demonstrate that IRS-2 immunolocalized predominantly within the outer plexiform layer (OPL) and photoreceptor inner segments with less intense immunoreactivity observed in the ganglion cell layer (GCL) and inner plexiform layer. IRS-2 was not detected in photoreceptor outer segments or in the retinal pigment epithelium [53]. Localization to the OPL and photoreceptor inner segments is relevant, as IRS-2 knockout mice exhibit increased photoreceptor apoptosis, suggesting IRS-2 is necessary for proximal signaling and survival. The ERG responses in these animals were diminished in proportion to photoreceptor cell death [53]. Accordingly, diminished IRS-2 signaling may potentially contribute to the decreased ERG response in the outer retina after 10 years of diabetes in humans [26].

The significant histopathological and electrophysiological abnormalities in the IRS-2 knockout mice are in stark contrast to IRS-1 knockout mice. Despite the fact that IRS-1 is widely distributed throughout the retina, including the GCL, INL, and photoreceptor outer segments, IRS-1 knockout mice exhibited normal retinal morphology [53]. It was recently postulated that IRS-1 may be inhibited by Grb14 acting as a dominant negative inhibitor of IRS-1 function in the retina [54]. This hypothesis is consistent with the recent observation that Grb14-knockout mice exhibit enhanced IRS-1 phosphorylation and activation of Akt [55], though this has not yet been examined in the
retina. Uncoupling Grb14 and IRS-1 could serve as possible mechanism to increase insulin sensitivity within the retina. It appears that different IRS isoforms can exhibit discrete roles; in fact IRS-2 knockout mice develop diabetes [56, 57], whereas IRS-1 knockout mice do not, though they demonstrated glucose intolerance [58]. Thus, the mechanism of IRS-1 signal suppression is unknown, but interfering with this negative regulation could prove to be detrimental to the retina.

**1.3.2.2 Phosphatidylinositol-3-Kinase (PI₃K) and Akt**

The p85 subunit of phosphatidylinositol-3-kinase (PI₃K) is downstream of IRS and/or the IR itself and becomes activated by directly interacting with these proteins. The PI₃K complex of p85/p110, in turn, phosphorylates the inositol ring of phosphatidylinositol at the 3’ position, creating PI₃ lipids at the membrane. The phosphorylation of these lipids is important as they in turn facilitate activation of other kinases by binding to the pleckstrin homology domain of Akt isotypes as well as PDK1 (Phosphoinositide-dependent protein kinase 1) [59, 60]. Like IRS, PI₃K is also activated in response to insulin in the retina [41]. Also of importance is the fact that stz-induced diabetes diminished PI₃K activity [50]. Subsequently, in ob/ob and stz-mouse models, the expression and phosphorylation of PDK1 and Akt are greatly diminished [51]. In stz-induced diabetic rats, Akt content remains constant with diminished activity of Akt-1 and Akt-3 isoforms [50]. In all three models mentioned, Akt is, as expected, stimulated by insulin treatment. Interestingly though, only Akt-1, but not Akt-2 or -3 have increased activity in response to insulin in the rat model [41].
In mice, disruption of the gene that encodes p85α, but allowing expression of the splice variants p50α and p55α, results in a marked decrease in IRβ associated PI3K activity in the retina. Interestingly, the phosphorylation of Ser\(^{473}\) of Akt was unaffected in these mice’s retinas and there was no significant change in retinal morphology, though the photoreceptor outer segments appeared to be shorter. The lack of an overt effect may be due to expression of the p50α and p55α splice variants, which are needed to prevent lethality [61]. A p50α and p55α knockout, that retains p85α expression, has also been studied. These mice demonstrate increased insulin sensitivity and increased glucose transport in isolated muscle tissues and adipocytes [62] though the effects of this knockout on the retina remains unexamined. It would therefore be reasonable to investigate alterations in these three splice variants in the retina as they exhibit different functions to positively and negatively regulate insulin signaling [63].

As described above, Akt-1 and -3 isoforms demonstrate impaired activation in the diabetic retina, but the importance of specific Akt isotypes in the retina remains unknown. Specific Akt knockouts have revealed potentially relevant information for diabetes and the retina. Such studies have revealed that for mouse development and survival the order of Akt isoform importance is Akt-1 > Akt-3 > Akt-2 [64]. This is not meant to diminish the importance of the Akt-2 isoform. In fact, knockout studies have revealed Akt-2 [65, 66], but not Akt-1 [67] or Akt-3 [68] to be an essential mediator in the maintenance of normal glucose homeostasis. Furthermore, in Akt-2 knockout males, insulin resistance progresses to a severe form of diabetes with pancreatic beta cell failure [66]. Additionally, an autosomal dominant mutation of Akt-2 has been implicated in
severe insulin resistance and diabetes mellitus in humans [69]. In contrast, Akt-1 knockout mice have revealed that it is a necessary component of angiogenesis and vascular permeability, though this has yet to be examined in the retina [70, 71]. Further understanding of how selective activation of specific Akt isoforms occurs and isoform specificity towards downstream substrates could provide means of selectively activating/inhibiting isotypes therapeutically.

1.3.2.3 mTOR and p70 S6K

Other downstream effectors of insulin signaling include the mammalian target of rapamycin (mTOR) and the downstream target p70 S6 Kinase (p70 S6K) (Fig. 1.2). These molecules control nutrient- and growth factor-stimulated protein synthesis and regulate additional kinases to facilitate cellular growth and survival. Though information as to the roles of these kinases in the retina is still minimal, there is evidence that these two kinases in the retina are stimulated by insulin and have reduced activity and/or phosphorylation in stz-induced diabetes [50]. The consequences of this reduction in vivo are unknown, but in vitro studies have demonstrated that mTOR signaling suppresses autphagic-induced cellular death [72-74]. Further in vitro studies demonstrate that p70 S6K activation decreases serum deprivation-induced apoptosis in retinal neurons in response to insulin. It was demonstrated that a dominant negative p70 S6K inhibited insulin’s ability to prevent serum deprivation-induced apoptosis and that overexpression of a wild-type p70 S6K increased the ability of insulin to prevent apoptosis [75]. This may be due, in part, to the ability of the mTOR inhibitor rapamycin, to partially block
insulin-induced inactivation of the forkhead transcription factor (FKHR). Furthermore, co-transfection of wild-type p70 S6K and FKHR resulted in increased FKHR accumulation in the cytosol in response to insulin [75]. The p70 S6K may also regulate cell survival by inactivating the pro-apoptotic protein BAD via phosphorylation at Ser\(^{136}\) [76].

It has been determined in culture, that postmitotic retinal neurons require protein synthesis for survival, though it was not examined if this was mTOR-dependent mRNA translation [77]. There is also evidence that that an mTOR-riCTOR (Fig. 1.2) signaling complex can directly enhance Akt activation through phosphorylation to mediate cellular survival [78, 79]. Also of importance is that knockout of ribosomal protein S6 in mice, a downstream substrate of mTOR and p70 S6K signaling, leads to glucose intolerance [80].

Conversely, suppression of mTOR signaling may be therapeutic for diabetic complications. In models of retinopathy of prematurity and choroidal neovascularization, the mTOR inhibitor rapamycin inhibited neovascularization [81]. Activated mTOR signaling has also been implicated in diabetic nephropathy [82]. Furthermore, p70 S6K has been demonstrated to directly phosphorylate IRS-1 to inhibit downstream insulin signaling [83, 84]. In fact, in p70 S6K knockout animals, there is increased insulin sensitivity [85].
1.4 Putative mechanisms of retinal insulin resistance and apoptosis

Though decreased ligand (insulin) is the primary defect in type I diabetes, insulin resistance has been implicated in both type 1 and type 2 diabetes [87]. Therefore, understanding the underlying mechanisms by which impaired/altered insulin signaling and hyperglycemia contribute to retinal pathology in diabetes may provide insights for therapeutic intervention.
1.4.1 Protein Kinase C

One of the most widely implicated contributors to the pathogenesis of diabetic retinopathy has been the lipid regulated Protein Kinase C (PKC). PKCs belong to the AGC family of kinases and are involved in a wide variety of cellular responses. There are at least 11 different PKC isoforms that are further subdivided into three subfamilies depending on their sensitivity to the lipid diacylglycerol (DAG) and/or calcium. The classical PKC isoforms, $\alpha$, $\beta$I, $\beta$II, and $\gamma$ are sensitive to DAG and calcium, whereas the novel PKC isoforms, $\varepsilon$, $\delta$, $\eta$, $\mu$ and $\theta$ are sensitive to DAG, but not calcium. Lastly, the atypical PKC isoforms, PKC$\zeta$ and $\lambda$/$\iota$ are insensitive to both calcium and DAG [88].

Despite this discrimination based upon calcium/DAG sensitivity, many other lipids have been shown to directly regulate PKC isoforms. These lipids include phosphatidic acid [89, 90], lysophosphatidic acid [91], ceramide [92-98], free fatty acids [98-100], sphingosine [101], and PI$_3$ lipids [102-104]. Additionally, phorbol esters also activate the classical and novel PKC isoforms. The roles of PKCs are to mediate transmembrane signals at the plasma membrane, though they have also been shown to mediate signaling within subcellular organelles, including the nucleus and Golgi apparatus. Specific PKCs have been implicated in diverse functions, including opposing functions such as apoptosis and survival. This diversity of function may be due to varied subcellular localization, a multitude of activating cofactors and target substrates, and prolonged versus transient activation [105, 106].

The activation of PKC, like most kinases, also depends upon activating phosphorylation by upstream kinases as well as autophosphorylation. This regulation is
quite complex, and unlike Akt and p70 S6K where specific phosphorylation events tend to correlate to activity, this is largely untrue for PKCs. For PKCs, phosphorylation events are typically priming events for maturation and future activation. For example, classical PKCs can be activated by any changes in basal phosphorylation state [88, 107]. Also of note, recombinant PKCδ from bacteria can have activity without being basally phosphorylated by the major priming enzyme PDK1 [108]. Therefore, a lack of an effect, or an effect putatively caused by a PKC isotype based upon changes in activity, phosphorylation and/or translocation is not conclusive due to this complexity.

1.4.1.1 PKC and diabetes

Studies of the roles of PKC isoforms in diabetes are mired with conflicting data. Many investigators argue that increased activity of PKC isotypes, specifically PKCβII are responsible for many diabetic complications. PKCβII in the rat retina demonstrated the greatest increase in membrane translocation in the diabetic state [109] and in cultured retinal endothelial cells, PKCβII and δ had the greatest increase in translocation in response to hyperglycemic conditions [110]. Due to these findings, a PKCβ inhibitor, ruboxistaurin/ LY333531, has been investigated as a potential therapeutic for diabetic complications, including retinopathy.

The use of this orally available PKCβ inhibitor has shown promise in in vivo models. George King’s group has demonstrated that the inhibitor ameliorated diabetes-induced decreased glomerular filtration rate, albumin excretion rate, and retinal circulation in rats [111]. This group has also demonstrated that increasing DAG content
in the retina, through the reduction of DAG flux to phosphatidic acid by the inhibition of DAG kinase, mimicked the effect that diabetes has on retinal circulation [112]. Others have shown that in an ischemic neovascularization porcine model, ruboxistaurin inhibited the formation of new vessel formation, suggesting a therapeutic role in preventing neovascularization in diabetic retinopathy [113]. Moreover, ruboxistaurin also inhibited retinal leukostasis [114, 115]. Also of importance is the discovery of polymorphisms in the 5’-upstream region of PKCβ altering the affinity of the Sp1 transcription factor, which may contribute to susceptibility to diabetic complications [116].

Unfortunately, human clinical trials utilizing ruboxistaurin for diabetic complications have been less successful. Two large, long-term multicenter clinical trials, the PKC-DMES (PKC-Diabetic Macular Edema Study) and the PKC-DRS (PKC-Diabetic Retinopathy Study) are ongoing. Preliminary results from the PKC-DMES reported that treatment with ruboxistaurin did not significantly alter the progression of diabetic macular edema or delay the use of laser photocoagulation treatment [117]. However, another report suggested some success with this drug for patients that demonstrate a higher baseline of vascular leakage [118]. The PKC-DRS demonstrated that ruboxistaurin was not efficacious in reducing the progression of diabetic retinopathy. They did however report a significant reduction in the occurrence of moderate visual loss [119]. A more recent publication revealed that this PKC inhibitor decreased diabetes-induced changes in retinal hemodynamic properties [120].

Other clinical trials with ruboxistaurin revealed some benefit with diabetic nephropathy, with reduced albuminuria and maintenance of glomerular filtration rate [121]. Ruboxistaurin also appeared beneficial in a subgroup of patients with less severe
peripheral neuropathy by improving nerve fiber function [122]. In the future, PKC β inhibition may prove to be a useful adjunct to current therapy for diabetic complications. However, these modest effects must be weighed against the uncertain risk of long-term adverse effects from chronic doses of, and subsequent long-term inhibition of PKCs, on normal physiological processes.

The modest effect of ruboxistaurin may be due to the selectivity of this inhibitor for PKCβ. Though it was initially reported that PKCβII demonstrated the most translocation in comparison to other PKC isotypes in the diabetic retina, increased translocation of other PKC isotypes were also observed [109]. The resultant leap to anoint PKCβ as a therapeutic target is accordingly questionable, as other PKC isotypes have been demonstrated to be involved in diabetic complications. Some relevant examples include evidence that PKCα may regulate altered neurotransmitter levels in the diabetic brain [123]. Hyperglycemia increased endothelial cell permeability via the activation of PKCα [124]. Also, PKCα, but not β isoforms was activated in response to high glucose in Schwann cells [125]. In vitro, glycated albumin induced insulin resistance through a PKCα−dependent inhibition of IRS in L6 skeletal muscle cells [126]. Additional in vivo and in vitro studies have also implicated PKCθ in causing insulin resistance in skeletal muscle [127, 128]. In fact, PKCθ, but not PKCα demonstrates upregulated expression in skeletal muscle from patients with type 2 diabetes [129, 130]. Additionally, in dorsal root ganglia isolated from diabetic rats, increased PKCα, but not β, as well as decreased PKCδ was observed [131]. However, PKCδ, but not classical PKC isotypes contributed to the production of reactive oxygen species in adipocytes from
insulin resistant mice [132]. PKCδ also impaired nerve regeneration in diabetic rats [133].

Both PKCβII and PKCε were implicated in diabetes-induced cardiomyopathy [134, 135]. *Schaffer et al.* has reported that PKCβ is downregulated in the diabetic heart [136] and *Giles et al.* reported that PKCα and δ were increased, but not β and ε [137] and yet *Liu et al.* reported increases in all PKC isotypes examined [138]. A significant increase in membrane-associated PKCα, δ, ε and a significant decrease in membrane PKCβII content were also observed in diabetic rat glomeruli compared with normal animals [139].

Furthermore, *Bosch et al.* demonstrated that they could not detect changes in PKC isotype expression or the hexosamine pathway in muscle and adipose tissue from insulin-resistant Zucker rats at various time points despite their hyperglycemic state [140]. Investigation of sciatic nerves from rats with diabetes to determine the cause of diabetic neuropathy has demonstrated impaired PKC activation in diabetes, rather than activation. Sciatic nerves of stz-induced diabetic rats show decreased PKC activity [141], impaired phosphorylation of the Na,K-ATPase α subunit due to defective PKC activation [142, 143], decreased inositol phospholipid pools and decreased arachidonic acid containing diacylglycerols [144-146]. Additionally, mononuclear cells isolated from human diabetic patients demonstrate an impaired PKC translocation [147]. Furthermore, inhibition of PKC augmented the proapoptotic effects of high glucose on retinal pericytes [148], thus *in vivo* inhibition of PKC may possibly abrogate diabetic-induced changes on these pericytes. Also, studies in PKCβ knockout mice have revealed that PKCβ inhibition may
only be marginally effective in combating diminished retinal oxygenation in diabetes [149].

The disparity and lack of cohesiveness of all the PKC studies point to two major underlying factors; (1) there are differences in terms of PKC isotype responsiveness between different models of diabetes and the animal species studied and (2) there are differences in PKC isotype responsiveness between different tissue/cell types within the same animal. Therefore, it is not surprising for a lack of a robust effect of ruboxistaurin in the retina for human patients with diabetes. Thus, PKCβ as a unifying factor for diabetic complications is unlikely to solve the enigma of diabetes complications and investigations into PKCs requires more rigorous examination and interpretation.

This statement is not meant to disparage the importance of PKCs. There is clear evidence that PKC isoforms can directly phosphorylate and regulate the IRβ subunit [150], IRS [151, 152] and Akt [153] to diminish insulin signaling. Conversely, activation of PKC is needed for insulin-induced glucose uptake in adipocytes in vivo [154], though PKCβ knockout animals demonstrate a slight increase in glucose transport [155]. On the other hand, PKCs, specifically PKCα [156] and PKCβII [157], can have a positive effect on insulin signaling and Akt activation. It is hence imperative to determine definitive mechanisms for how PKC are regulated and dysregulation in diabetes as well as their immediate substrates.

To illustrate this point, there is growing acceptance that increased DAG synthesis is not responsible for PKC activation. This is due to evidence that high glucose-induced increases in DAG are formed through the phospholipase D-phosphatidic acid
phosphohydrolase pathway, which typically produces saturated/monounsaturated DAGs and not from PLC-mediated PIP$_2$ hydrolysis, which predominantly produces polyunsaturated DAGs (Fig. 1.3) [158-161]. Additionally, inositol phosphate products are not elevated in diabetes, which would be caused by PIP$_2$ hydrolysis [162, 163]. This point is important as saturated DAGs are poor activators of PKCs compared to polyunsaturated DAGs [164-167]. Also of note is that inflammatory cytokines tend to produce ether-linked diglycerides, but not ester-linked diglycerides. These ether-linked diglycerides can inhibit, rather than activate PKCs [168, 169]. More recent data suggests a role for oxidative stress as an activator of PKCs. Glycated albumin has been demonstrated to activate PKCs through oxidative stress [170-174]. This oxidative stress mediated-PKC activation may occur through redox modification of the regulatory domain [175, 176] or increased tyrosine phosphorylation [174, 177-179].
Fig. 1.3: Mechanisms for DAG synthesis
It should also be noted that increased membrane localization does not always correspond to increased PKC activity. Pools of PKCs already localized at the membrane can be activated and increased activity has been observed without translocation [180-183]. Furthermore, membrane-localized PKCs can be regulated through interactions with scaffolding proteins such as caveolin [184] and 14-3-3 protein [185-187]. As mentioned previously, other molecules, like fatty acids, which are altered and increased in diabetes, such as oleic and arachidonic acids can also stimulate PKC activation [98-100]. Alterations in DAG and fatty acids are also important, because they serve as first and second messengers, and precursors and intermediates for the production of other lipid-derived messengers, such as triglycerides. In fact, acyl CoA:diacylglycerol acyltransferase-1 knockout mice, which have impaired triglyceride synthesis, demonstrate decreased adiposity and increased insulin sensitivity when fed a high fat diet [188]. Likewise, altered lipid flux through monoacylglycerol acyltransferase, a much less studied enzyme in physiological diacylglycerol formation, may also be involved in increased diacylglycerol in some diabetic tissues [189]. The diabetes field to this point has focused largely on diglycerides and triglycerides, with the roles of other lipid classes, such as the sphingolipids relatively understudied.

1.4.2 Sphingolipids

The sphingolipid ‘rheostat’ [190] has been described where pro-apoptotic sphingolipids exist in a balance with pro-survival sphingolipids (Fig. 1.4). Disturbance of this balance can lead to cellular death or growth arrest in the case of ceramide
accumulation, or alternatively to proliferative disorders (i.e., cancer, angiogenesis) in the case of formation of sphingosine-1-phosphate (S1P). In fact, the coordinate biochemical regulation of ceramidases and sphingosine kinases (SphK) may serve as a critical control point regulating the dynamic flux between these metabolites. In addition, endogenous ceramide levels can be reduced by activation of ceramide kinase, glycoceramide synthases, or sphingomyelin synthase and enhanced via ceramide synthase (de novo) or sphingomyelinases. In a similar scenario, activation of S1P lyase or S1P phosphatase could reduce endogenous S1P content (Fig. 1.5). Pharmacological or molecular manipulations of any of these enzymes have the potential to reset the critical homeostatic balance between sphingolipid metabolites. Thus, understanding shifts in this balance may identify new therapeutic targets for various diseases.

Fig. 1.4: The Tao of sphingolipid metabolism
Fig. 1.5: Sphingolipid metabolism
1.4.2.1 Altered sphingolipid metabolism and diabetes

Several studies have demonstrated increased ceramide content in insulin-resistant skeletal muscle from rats [191, 192] and humans with type 2 diabetes [193, 194]. Furthermore, patients with type 2 diabetes have increased sphingolipid metabolism as evidenced by increased sphingosine and sphinganine in their plasma [195]. A recent review by Summers and Nelson puts forth a hypothesis that suggests that the similarities of type 2 diabetes, Metabolic Syndrome X, and Cushing’s Syndrome may be mediated through altered sphingolipid metabolism [196]. Also of importance is a recent paper demonstrating hyperglycemic activation of SphK1 in the aorta and heart of streptozotocin-induced diabetes in rats [197]. Additionally, increased sphingosine and S1P has been correlated with glomerular mesangial cell proliferation in stz-induced diabetic rats [198, 199], suggesting that pharmacological manipulation of sphingolipid flux might be efficacious in complications of diabetes.

In fact, sphingoid mimetics are beneficial in animal models of type 1 diabetes. A sphingosine-1-phosphate mimic, FTY720, serves as an endothelial differentiation gene (EDG) receptor agonist and has been demonstrated to be an effective immunosuppressant without many of the side effects associated with other drugs such as cyclosporin. FTY720 can prevent islet allograft rejection in rodents and nonhuman primate models and cure autoimmune diabetes in diabetic mice [200-202]. The mechanisms by which FTY720 can regulate immune responses are only recently being described, but may include sequestering of lymphocytes from the circulation to lymph nodes and Peyer’s patches, which involves the SphK2 isoform [203]. This redistribution effectively reduces T cell
numbers at the sites of inflamed tissue or graft sites. Recent evidence suggested that FTY720 may also inhibit S1P lyase, which will decrease S1P degradation, thus providing another mechanism of action in addition to the phosphorylated form activating EDG receptors [204].

Indication of the importance of sphingolipid metabolism in the retina comes from several diseases, such as Farber’s (defective acid ceramidase) and Niemann-Pick (defective sphingomyelinase), where this defective sphingolipid metabolism can cause vision loss by killing retinal neurons. Recently a mutated ceramide kinase-like protein has been described to be responsible for a form of retinitis pigmentosa (RP) [205]. Though the activity and regulation of this ceramide kinase-like protein remains elusive [206], modulation of ceramide levels has been demonstrated to be important in other forms of RP not caused by a genetically altered sphingolipid enzyme. By overexpressing neutral ceramidase, which forms sphingosine at the expense of ceramide, photoreceptor degeneration was suppressed in Drosophila models of RP [207].

1.4.2.2 Sphingolipids: putative mechanisms for diabetic complications.

While alterations in sphingolipids have been demonstrated in diabetes and modulating sphingolipid flux has proven beneficial in animal models of diabetes, the underlying mechanism by which sphingolipids actually cause or contribute to these complications are under active investigation.

Ceramide is viewed as a pro-apoptotic sphingolipid, which exerts its effects through second messenger [208, 209] and biophysical activities [210-212]. Ceramides
accumulate in response to stress and proapoptotic stimuli, such as the inflammatory cytokines IL-1β and TNFα, which are upregulated in diabetes systemically and in the retina [35-37]. Ceramides may contribute to apoptosis through the inhibition of PI3K/Akt [213] and ERK [93] signaling as well as activating the stress-activated MAPK p38 and SAPK [92] and by regulating mitochondrial permeability and golgi fragmentation [214]. Ceramide levels are increased in insulin-resistant skeletal muscle [191-194], so it was not surprising that ceramide can interfere with insulin action and proximal signaling elements such as Glut4-mediated glucose transport, IRS and Akt signaling [215-221].

Multiple biochemical mechanisms have been described by which ceramide can inhibit insulin signaling. These include the inhibition of IRS tyrosine phosphorylation [220], the activation of PP2A, a putative target of ceramide, which in turn dephosphorylates and subsequently turn off Akt [222-225]. Ceramide can also directly activate PKCζ [92, 94, 153, 213, 226], which in turn, phosphorylates Akt at Ser/Thr34, an inhibitory post-translational modification that prevents Akt from binding to PI3-lipids [153]. Yet another target may be the PI3-lipid phosphatase PTEN. Ceramide has recently been demonstrated to recruit and activate PTEN, which led to diminished Akt activation as a consequence of PI3-lipid reduction [227]. In fact, increasing evidence also supports a role of PTEN in insulin resistance. Adipose-specific disruption of PTEN resulted in increased insulin sensitivity and resistance to stz-induced diabetes [228]. A muscle-specific disruption also caused enhanced insulin sensitivity and is protective for developmental diabetes in response to a high-fat diet [229]. Additionally, a liver-specific PTEN knockout also enhanced insulin sensitivity, but interestingly also led to increased fatty acid synthesis and a fatty liver phenotype [230].
Additional evidence for the role of ceramide in diabetes includes ceramide-induced vascular permeability, which is antagonized by S1P [231] as well as ceramide-induced reduction of protein synthesis through the suppression of mTOR signaling and system A amino acid transport [232].

Several studies by the Summers laboratory have demonstrated that the fatty acid palmitate induced the formation of both diglycerides and ceramides and caused diminished insulin-stimulated Akt activation [233-235]. However, inhibition of ceramide metabolism with de novo synthesis inhibitors [234] or increasing the catabolism of ceramide through the overexpression of the acid ceramidase isotype [235] prevented this saturated fatty acid-induced insulin resistance. These findings support the role of ceramides, but not DAG, in this model of insulin resistance. Furthermore, palmitate-induced inhibition of insulin expression in pancreatic islets [236] beta cell apoptosis [237-239] and apoptosis of cultured bovine retinal pericytes [240] are also mediated through increased de novo ceramide production.

Another example that may support the notion of altered sphingolipid flux is that hyperglycemia can induce the formation of arachidonic acid release and eicosanoid production [241]. This is significant, as recent publications have demonstrated that ceramide-1-phosphate acts as a direct substrate to activate phospholipase A₂ and produce arachidonic acid [242]. Furthermore, CerK and SphK coordinately work together to produce the prostenoid PGE₂ via arachidonic acid and COX-2 induction, respectively [243]. This fact coupled with evidence of hyperglycemia-induced activation of SphK [197], further supports the potential of sphingolipid-based therapeutics for diabetic complications, including retinopathy. Fascinatingly, PKCs have been demonstrated to be
positive regulators of both CerK [244] and SphK [245, 246]. Another possible connection between ceramides and DAGs may be in triglyceride synthesis. It has been reported that ceramide can increase glucose incorporation into triglycerides [247], a metabolite of DAGs. For these reasons the interplay between PKC regulation and sphingolipid regulation in diabetic complications merits investigation.

1.4.2.3 Altered glycosphingolipid metabolism and diabetes

The glycotransferases, glucosylceramide synthase and galactosylceramide transferase, catalyze the first committed step for glycosphingolipid metabolism by transferring glucose or galactose to ceramide to form cerebrosides which are metabolized into the more complex sulfatides, lacto(neo)ceramides, globosides and gangliosides (Fig. 1.6). Changes in glycosphingolipid metabolism have been observed in diabetes. A study from 1975 revealed that diabetic patients with hyperlipoproteinemia type IV demonstrated increased cerebroside levels [248]. Insulin resistance has also been shown to be negatively correlated with sulfatide concentration and positively correlated with sulfated lactosylceramide concentration in humans with type 2 diabetes [249]. Other groups have demonstrated that glycosphingolipids may be involved in the pathogenesis of diabetes by contributing to insulin resistance in adipocytes through the upregulation of the ganglioside forming enzyme GM3 synthase [250, 251] and/or involved in renal complications [252, 253].
Increases in cerebroside-sulfatide in the cerebellum of diabetic guinea pigs [255] and altered sulphatide fatty acid profile in type 2 diabetic pancreas have also been reported [256]. Furthermore, increases of specific gangliosides have been observed in livers from stz-induced diabetic rats [257, 258]. Autoantibodies to sulphatides [259], GT3 [260], GD3 [261] and GM2-1 [262, 263] have been identified in patients with type 1 diabetes. This may contribute to the inflammatory pathology by facilitating the immune
destruction of pancreatic beta cells. In fact, neonatal treatment with the diabetic autoantigen sulphatide delayed the onset of diabetes BB rats [264].

Therapeutically, the glycosphingoid mimetic, OCH, which is an analog of α-galactosylceramide, inhibited the development of insulitis and diabetes in non-obese diabetic mice [265]. α-Galactosylceramide treatment also prevented diabetes in this mouse model [266-268]. This effect is possibly mediated through the stimulation of natural killer T cells inducing T helper 2 cytokine production [269, 270]. A glucosylceramide synthase inhibitor was also demonstrated to diminish renal hypertrophy [252]. It may therefore be therapeutically desirable to manipulate glycosphingolipid metabolism for diabetes.

1.4.2.4 Glycosphingolipids: putative mechanisms for diabetic complications.

Glycosphingolipids have been demonstrated regulate many cellular processes that are relevant to diabetes. Lactosylceramide can mediate VEGF-induced angiogenesis [271] and glycosphingolipids themselves can stimulate VEGF production [272], which in turn could possibly modulate vascular permeability and/or neovascularization in diabetes. It has also been reported that glucosamine-induced inhibition of retinal pericyte proliferation is mediated, in part, through increased glycosphingolipid metabolism [273].

Altered glycosphingolipid metabolism may also mediate the proinflammatory processes in diabetes. Sulphatides and galactosylceramide can modulate cytokine production in human mononuclear cells [274] and whole blood cultures [275]. Glycosphingolipids can activate microglial cell lines to produce the inflammatory
mediators iNOS, TNFα, and IL-1β through PKC and NADPH oxidase [276] and PKA [277].

While ceramide has been implicated in TNFα-induced insulin resistance [278], breakdown of sphingomyelin by sphingomyelinases also provides a source of ceramides for further metabolism. In fact, Fernández-Checa’s group has demonstrated that TNFα activates acid sphingomyelinase, which initiates a chain of events to produce the ganglioside GD3, leading to an apoptotic response in the liver [279]. Also of importance is that GM3 has been implicated in insulin resistance in response to TNFα [250] and that mice deficient in GM3 synthase, and thus GM3 and its metabolites, exhibit increased insulin sensitivity [280]. This may be mediated by altering the localization of the insulin receptor within lipid microdomains [251]. Also of consequence is GD3, which has been implicated in Fas (CD95)-induced apoptosis [281, 282] and regulation of mitochondrial permeability and apoptosis [283, 284], and GT1b which has been demonstrated to inhibit Akt activation [285].

The effects of altered glycosphingolipid metabolism within the retina are clearly manifested in several lysosomal storage diseases, such as Tay-Sachs, Sandhoff, GM1 gangliosidosis, Gaucher’s and Krabbe’s, where glycosphingolipid accumulation can lead to cellular death and a proinflammatory environment in the retina, as well as varying degrees of vision loss, including blindness. Of interest, it has been demonstrated that in GM1 gangliosidosis, GM1 accumulation induces the endoplasmic reticulum (ER) stress response [286].
The ER stress response arises from alterations in the ER’s homeostatic environment, such as nutrient deprivation and the accumulation of untranslated proteins, which can contribute to insulin resistance and cell death. Mice deficient in X-box-binding protein-1 (XBP-1), a transcription factor that modulates the ER stress response, develop insulin resistance, demonstrating that ER stress is a central feature of peripheral insulin resistance [287]. ER stress-induced apoptosis can also be inhibited by treatment with IGF-1 in mouse insulinoma cells. Furthermore, reduction of insulin receptor expression enhanced susceptibility to ER stress-induced apoptosis. This increased apoptosis was associated with a reduction in phosphorylated Akt and the downstream target GSK3β. In fact, depletion of GSK3β by use of small interfering RNA made cells resistant to ER stress-induced apoptosis [288]. It has also been demonstrated that systemic expression of ORP150 (oxygen-regulated protein), an ER chaperone protein, in Ins2Akita mice improves insulin resistance through enhanced glucose uptake, accompanied by suppression of oxidized protein [289]. Moreover, ORP150 enhanced the insulin sensitivity of myoblast cells treated with hydrogen peroxide (reactive oxygen species) [289] leading to diminished gluconeogenesis and amelioration of glucose tolerance in the liver of diabetic mice [290]. It was also demonstrated that IL-1β and IFN-γ-induced depletion of ER calcium and subsequent activation of the ER stress pathway, potentially contributed to beta-cell death [291].

Other evidence supporting a role for ER stress in complications of diabetes may be related to the neurotoxic excitatory amino acid glutamate [292, 293]. Glutamate toxicity has been implicated in diabetic retinopathy [292, 293]. This putatively would occur through excessive stimulation of NMDA (N-methyl-D-aspartate) receptors, that are
activated by the co-agonists NMDA (or glutamate) and glycine. When NMDA is administered to the retina, it induces apoptosis that can be blocked by a SAPK/JNK inhibitor. While SAPK/JNK activation also occurs independently of ER stress, many additional lines of evidence suggest a role of glutamate in ER stress. Glutamate results in the release of calcium from intracellular stores, another hallmark of ER stress. Also of note, glycosphingolipids can stimulate JNK [286], increase intracellular calcium release and increase the sensitivity of neurons to glutamate-induced apoptosis [294, 295]. In addition, increased caspase-3 cleavage is observed in the diabetic retina. During ER stress, caspase-12 cleavage occurs and can lead to caspase-3 cleavage/activation. Lastly, VEGF expression can be stimulated by ER stress conditions [296-299]. Though no direct evidence of an activated ER stress response in the retina in diabetes has been reported, these many lines of evidence suggest that it may be a contributor and requires investigation.

Finally, the possibility of increased polyunsaturated and lyso- forms of glycosphingolipids (i.e. glycosylated sphingosine) may also have implications for diabetic pathology. It has been postulated that the accumulation of lysosphingolipids, but not parental sphingolipids, triggers the apoptotic cascade in neuronal cells of patients with sphingolipidosis [300]. For example, lyso-GM1a induced apoptosis in mouse neuroblastoma cells [300] as does a polyunsaturated GM1a [301]. The physiological relevance of these lyso and polyunsaturated derivatives of glycosphingolipids remain poorly defined, but have been found to be a minor constituent of several tissues [302-304]. It should also be noted that polyunsaturated ceramides have been observed [305], though this physiological function of these are undefined.
Taken together alterations in lipids metabolism have clear physiological and pathological outcomes. Understanding and characterizing these alterations and the effects they cause have important relevance for diabetic complications, including retinopathy. We will document in this thesis that augmenting DAG flux and/or increasing phosphatidic acid concentration synergistically enhances insulin receptor-induced neuronal cell survival. We will also document that diminishing sphingolipid flux and resultant generation of glycosphingolipids has the potential to prevent retinal neuronal cell death in diabetes. We conclude that dysfunctional lipid flux may be a contributor to metabolic stress in diabetes and therapeutic strategies to restore homeostatic lipid flux may be a viable approach for treatment of diabetic retinopathy.
Chapter 2

Protein Kinase C: A mediator of diabetic retinopathy?

2.1 Introduction

Protein Kinase C (PKC) is an important regulator of cellular homeostasis including cellular growth, proliferation and apoptosis. There are at least 11 different PKC isoforms that are further subdivided into three subfamilies depending on their sensitivity to the lipid diacylglycerol (DAG) and/or the metal cofactor calcium. The classical PKC isoforms of α, βI, βII, and γ are sensitive to DAG and calcium, whereas the novel PKC isoforms of ε, δ, η, μ and θ are sensitive to DAG, but not calcium. The atypical PKC isoforms of PKCζ and λ/ι are insensitive to both calcium and DAG [88].

PKCs, particularly the βII isoform, have received much attention for diabetic complications. PKCβII exhibits the greatest increase in membrane translocation in the diabetic rat retina [109] and in cultured retinal endothelial cells PKCβII and δ demonstrate the greatest increase in translocation in response to hyperglycemic conditions [110]. The use of an orally available PKCβ inhibitor, ruboxistaurin, has demonstrated promise in in vivo models. George King’s group has demonstrated that this compound ameliorated diabetes-induced alterations in retinal circulation in diabetic rats [111]. This group has also demonstrated that increasing DAG content in the retina, through the reduction of DAG flux to phosphatidic acid via the inhibition of DAG kinase, mimics the effect that diabetes has on retinal circulation [112]. Consistent with a potential
therapeutic effect for diabetic retinopathy, others have shown that in an ischemic neovascularization porcine model, this compound inhibited the formation of new vessel formation [113]. Moreover, this compound also inhibited retinal leukostasis [114, 115]. Human clinical trials have suggested some utility of this drug in patients that demonstrate a higher baseline of vascular leakage [118] and it has demonstrated the ability to inhibit diabetes-induced changes of retinal hemodynamic properties [120].

The majority of studies on PKCs and diabetic retinopathy focus on the vascular pathology, while the retina is mostly neuronal and to date studies on retinal neurons with this PKC inhibitor have not been validated. Therefore the objective is to gain insights into the putative roles of PKCs in the neuronal retina and determine putative consequences for activated PKCs within diabetic neurons.

2.2 Materials and Methods

Materials. Bovine insulin was purchased from Sigma (St. Louis, MO). Laminin and cell permeable cAMP were from BD Biosciences (Franklin Lakes, NJ) and MP Biomedicals Biomedicals (Irvine, CA) respectively. Phorbol 12-myristate 13-acetate (PMA) and 4α-phorbol 12-myristate 13-acetate (4α-PMA) were purchased from Promega (Madison, WI). The cell permeable 1,2-Dioctanoyl-sn-Glycerol (DAG) was obtained from Avanti Polar Lipids (Alabaster, AL). Polyclonal rabbit anti-phospho-p42/44 MAPK (Thr202/Tyr204), rabbit anti-p42/44 MAPK, phospho-MARCKS, and phospho-PKC antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-rabbit and anti-mouse IgG-horseradish peroxidase conjugated and anti-PKC antibodies were
obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The PKC inhibitor bisindolylmaleimide-I (BimI) and the mTOR inhibitor rapamycin were from EMD Biosciences (San Diego, CA). The MEK inhibitors, PD98059 and U0126, and the SAPK (SP600125) and p38 inhibitors (SB202190) were purchased from Biomol (Plymouth Meeting, PA).

Animals. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were fasted overnight and given a single intraperitoneal injection of streptozotocin (65 mg/kg; Sigma, St. Louis, MO), freshly dissolved in 10 mM sodium citrate buffer (pH 4.5). Diabetes was confirmed 6 days later by blood glucose greater than 250 mg/dL (Lifescan, Milpitas, CA). Age-matched control and diabetic rats were monitored regularly by weight and blood glucose tests. Rats were housed in accordance with the Institutional Animal Care and Use Committee guidelines, and the study protocol adhered to the ARVO Statement for the Use Animals in Ophthalmic and Vision Research. Rats were maintained by the Juvenile Diabetes Research Foundation Animal Core Facility at Penn State University and group housed in solid plastic bottom cages with bedding as well as ad libitum food (Teklad Global 18% Protein Rodent Diet) and water under a normal 12-hour light-dark schedule. In some cases a group of animals received subcutaneous insulin injections twice daily for 3 days prior to their sacrifice with 5U Humulin® Regular/5U Humulin® Ultralente as described previously [306]. Retinas were dissected [307] at the indicated times and snap frozen in liquid N2.

C57BL/6J Ins2^Akita^ heterozygote mice (Jackson Laboratory, Bar Harbor, ME) were bred in the Juvenile Diabetes Research Foundation Animal Core Facility at Penn State University, in accordance with the Penn State College of Medicine Institutional
Animal Care and Use Committee guidelines. All methods involving animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were housed in pairs in plastic cages in a pathogen-free environment, with continuous access to food and water on a 12-hour light–dark schedule. Diabetic phenotype and genotype was confirmed 4.5 weeks after birth by blood glucose >250 mg/dL. Retinas were removed after 24 weeks of diabetes and frozen in liquid N₂. At the time of sacrifice the average blood glucose for the diabetic mice was 305mg/dL compared to 122.8mg/dL for the sibling controls.

Cell Culture. R28 cells were a generous gift from Dr. Gail M. Seigel, State University of New York, Buffalo [308]. They were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 5.5 mM glucose supplemented with 10% newborn calf serum (Hyclone). The cells were differentiated to neurons on laminin-coated plates or coverslips with addition of 25μM cell-permeable cAMP as previously described [309].

Western Blot Analysis. Western blot analyses were performed essentially as described previously with some modifications [92]. Briefly, treated R28 cells were washed in ice-cold Dulbecco's phosphate-buffered saline solution and lysis buffer [50 mM HEPES, 137 mM NaCl, 5 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM NaVO₄, 1% NP-40, Protease Inhibitor Cocktail (Roche)] was added. Cell lysates were cleared by centrifugation, and the Bio-Rad DC protein assay was utilized to determine protein concentration. Typically 30μg of protein lysate per sample was separated on a 4-12% NuPAGE gels (Invitrogen) and transferred to nitrocellulose membranes. The membranes were subsequently blocked in 5% BSA (1 hour) and incubated with the primary overnight (1:1000 dilution). Membranes were subsequently washed 3 x 10 min in TBST and
incubated with an HRP-conjugated secondary for 2 hours (1:5000 dilution), washed in TBST (3 x 10 min) and visualized by ECL (GE Healthcare, Piscataway, NJ). The bands were visualized by enhanced chemiluminescence (ECL) and quantified using ImageQuant (Molecular Dynamics, Sunnyvale, CA), or GeneTools SynGene (K&R Technology, Frederick, MD) software.

**Cellular fractionation.** R28 cells were treated as described in the text and particulate/microsomal fractions were isolated as described by others [109]. Additionally, a detergent-free method of isolating lipid rafts/low-buoyant domains was performed also as described by others [310]. Briefly, cells were harvested in sodium carbonate buffer (pH 11.0) and homogenized. Homogenates were adjusted to 4 ml with 90% sucrose in MES-buffered saline (MBS) (25 mM Mes, pH 4.5, 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM NaF, protease inhibitor tablet) to a final concentration of 45% sucrose. Samples were layered at the bottom of a 14 × 89 mm tube containing 4 ml each of 35% and 5% sucrose in sodium carbonate/MBS and centrifuged in a Beckman SW41ti rotor at 200,000g for 16-20 h at 4°C. Serial fractions of 1ml were removed and analyzed by western blotting.

**Akt isoform-specific kinase assays.** Akt isoform-specific kinase assays were performed essentially as previously described [311] with some modifications [312]. The supernatants (200 µg protein) of R28 cell homogenates were subjected to immunoprecipitation (overnight at 4°C) with 2 µg of anti-Akt-1, -2 (Santa Cruz), -3 (Upstate Biotechnology) primary antibody. The antibody:antigen complex was then incubated with Gammabind G Sepharose (Amersham) for 1 hour at 4°C. The immunoprecipitates were washed and incubated in assay buffer [20 mM HEPES (pH 7.2),
25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10µM cold ATP, 5mM MgCl₂ and 1 mM dithiothreitol] at 35°C for 10 min, in the presence of a protein kinase A inhibitor peptide (1 µM, Upstate), GST-GSK3 (3µg assay, Cell Signaling), and [γ-32P]ATP (10 µCi/assay). The amount of 32P incorporated into GSK3 was determined by SDS-PAGE and transferred to nitrocellulose and exposed to film. The radioactive bands corresponding to GSK3 were cut out and measured by scintillation counting. The observed cpm values were corrected for nonspecific binding by subtracting the background values (no primary antibody immunoprecipitation) and normalized to the total amount of Akt immunoprecipitated by reprobing the blots for Akt.

**Immunohistochemistry and Confocal Microscopy.** Retina sections (10µm) on glass slides were fixed in 2% paraformaldehyde for 10 min at room temperature and subsequently rinsed in PBS (2 x 10 min). Slides were blocked in 10% donkey serum (Jackson ImmunoResearch, West Grove, PA) for 1 hour prior to incubation with the primary antibody overnight at 4°C. Slides were subsequently washed with PBS containing 0.1% triton-x (3 x 20 min). Primary antibodies were PKCα (1:100), PKCβI (1:100), PKCβII (1:50), PKCe (1:200), PKCζ (1:50), Akt-1 (1:100), Akt-2 (1:50), Akt-3 (1:500). The slides were then incubated with the secondary antibody (CY2-conjugated donkey anti-rabbit or anti-mouse (1:1000; Jackson ImmunoResearch) and nuclear stain Hoechst (0.5µg/ml) for 1 hr. Slides were then washed in PBST (3 x 20 min) and mounted with aqueous medium (Aqua/Polymount; Polysciences). All images were obtained with a confocal microscope (TCS SP2 AOBS; Leica, Deerfield, IL), at 512 x 512-pixel resolution. Images were maximum projections of z-stacks.
Statistical methods. One-way analysis of variance (ANOVA) with the Bonferroni post test and t-tests analysis were performed using GraphPad Prism 4.0 software, with statistical significance considered if $p < 0.05$.

2.3 Results

Insulin predominantly activates Akt-3 in R28 retinal neurons. Inhibition of Akt has been demonstrated in diabetic rat and mouse retinas [50, 51]. Akt is important in regulating cellular survival, so dysregulated Akt signaling may be causal to increased neuronal apoptosis observed in diabetic retinopathy. Initially, Akt isotype localization within the rat retina was characterized (Fig. 2.1A). By immunohistochemistry, Akt-1 demonstrates strongest immunoreactivity within the photoreceptor outer segments, the ganglion cell layer, the inner plexiform, the inner nuclear and the outer plexiform layers. Akt-2 shows a similar localization, but with greater immunoreactivity in the inner nuclear layers and some punctuate staining in the ganglion cell layer that suggests a more nuclear localization. Lastly, Akt-3 also demonstrates strong immunoreactivity in the photoreceptor outer segments and demonstrates strong immunoreactivity in the plexiform layers. Akt3 also demonstrates the strongest immunoreactivity in the outer nuclear layer of the three Akt isotypes. Therefore, dysregulated Akt signaling in diabetes may occur in neurons.

To characterize Akt in cultured rat retinal neurons, R28 cells were stimulated with insulin to characterize Akt isotype activation. Isotype-specific kinase activities reveal that Akt-3 demonstrated the most robust increase in activation ($p < 0.01$), with Akt-1
demonstrating a slight, but significant increase (p < 0.05). In contrast, no increases in Akt-2 were detected (Fig. 2.1B).

PKC activation inhibits Akt phosphorylation. PKCs have gained much attention for the ability to contribute to insulin resistance in diabetes. To determine if activated PKCs can influence insulin signaling in retinal neurons, the PKC activator PMA was utilized. Pretreating R28 cells with PMA prior to insulin stimulation, leads to significant inhibition of Akt activation (p < 0.05), whereas the inactive analog of PMA, 4α-PMA had no effect on insulin stimulation of Akt (p > 0.05). (Fig. 2.2A). In contrast, when the

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**Fig. 2.1:** Akt isoforms are expressed in the retina and demonstrate differences in activation in cultured R28 retinal neurons. A. Normal rat retina sections (10 micron) were subjected to immunohistochemistry with Akt isotype specific antibodies and imaged by confocal microscopy. B. R28 cells were differentiated onto culture plates. These cells were then serum-starved and stimulated with insulin (10nM) for 15 min. Akt isotypes were immunoprecipitated and subjected to kinase activity assays as described in Materials and Methods. Representative pictures of two separate n=3 experiments.
PKC inhibitor bisindolylmaleimide-I (BimI) is utilized prior to insulin-stimulation, there is a marked increase in insulin-stimulated Akt (p < 0.01). Similarly to PMA, when exogenous diacylglycerides are applied, insulin-stimulated Akt is also inhibited (Fig. 2.2B) (p < 0.01). Therefore, PKCs can negatively regulate insulin-stimulated Akt in R28 retinal neurons.

PKC stimulation activates mTOR signaling (shown in chapter 3) and mTOR activation has been demonstrated to negatively regulate Akt activation (feedback inhibition) [83, 84], so we determined if PKC inhibition of Akt can occur in the presence

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**Fig. 2.2: PKC activation negatively regulates Akt phosphorylation.** A. R28 cells were differentiated onto culture plates. Subsequently, these cells were serum-starved and then stimulated with either PMA (100nM), 4α-PMA (100nM) for 30 minutes with or without further stimulation with insulin (10nM) for an additional 15 min. The phosphorylation state and total expression of Akt was then assessed by western blotting. B. Similarly, R28 cells were pretreated with 50µM DAG for 15 min with successive stimulation with 10nM insulin for 15 min and Akt phosphorylation was analyzed. (These are representative n = 3 experiments. * = p < 0.05.)
of the mTOR inhibitor rapamycin. We still observe inhibition of insulin-stimulated Akt, in the presence of rapamycin (Fig. 2.3A). Thus, this PKC inhibition of Akt is mTOR independent. Crosstalk between MAPK cascades and Akt signaling has also been demonstrated [313, 314]. In the presence of the Mek inhibitor, PD98059 (Fig. 2.3A), the SAPK inhibitor SP600125, or the p38 inhibitor SB202190 (Fig. 2.3B), we still observe PMA-inhibition of Akt. Interestingly, the p38 inhibitor blocked insulin-stimulated Akt (p < 0.001).

The role of PP2A, a phosphatase that can dephosphorylate the Ser\textsuperscript{473} site on Akt was also assessed [315, 316]. Utilizing the PP1/PP2A inhibitor okadaic acid, we observe increased Ser\textsuperscript{473} phosphorylation upon insulin treatment (p < 0.05); but PMA treatment still impairs Akt activation (Fig. 2.3C). Therefore, PKC-activation is detrimental to Akt activation through mechanisms independent of mTOR, MAPK cascades and PP1/PP2A activities.
Protein Kinase C and the retina. To study PKCs in the retina, the determination of PKC isotype expression was determined by immunoblotting. We observe the expression of the classical isotypes, $\alpha$, $\beta_I$, $\beta_{II}$, but not $\gamma$. Expression of the novel PKC isotype $\epsilon$, but not $\delta$ and the atypical isotype $\zeta$ were also detectable (Fig. 2.4A). We next determined the localization of these isotypes in the retina by immunohistochemistry (Fig. 2.4B). As shown, PKCs are widely distributed in the retina, with different isoforms having discrete staining patterns, underscoring the importance of focusing, not just on PKC in retinal vasculature, to gain a clear understanding of the role of PKCs in retinal pathophysiology.

**Fig. 2.3:** PKC inhibition of Akt is independent of mTOR, Mek, p38 and SAPK. A. Serum-starved, differentiated R28 cells were pretreated with rapamycin (A), PD98096 (A), SB202190 (B), SP600125 (B), okadaic acid (OA) (C) for 30min prior to stimulation with PMA and/or insulin as described in Fig. 2.2. Akt phosphorylation was then assessed by western blotting. Figures 2.3B and 2.3C are normalized to total Akt expression. All experiments were done at least $n = 3$ times, * $p < 0.05$. 
PKC expression and membrane localizations are unaltered in the diabetic rat retina. We next determined if PKC expression is changed in the diabetic retina (Fig. 2.5A). By western blotting we demonstrate that the expression levels of all PKC isoforms are expressed in the normal retina. Enucleated retinas from Sprague-dawley rats were examined for PKC isoform expression by western blotting with isotype specific antibodies. Immunohistochemistry was performed on sectioned rat eye tissue for PKC isotypes and analyzed by confocal microscopy.
isotypes examined are unaltered by stz-induced diabetes after 4-weeks. Therefore, if PKCs are involved in the pathophysiology of diabetic retinopathy, it is unlikely to be a consequence of altered protein expression levels.

As PKC typically translocate from the cytosol to the membrane upon activation, the membrane localization of various isoforms of PKC in the diabetic retina was analyzed (Fig. 2.5B). We demonstrate that all PKC isotypes demonstrate some basal membrane-associated PKC, but no significant differences were observed between control and diabetic retinas, suggesting no global PKC activation within the diabetic retina.

**PKC localization within low-buoyant membranes is unaltered in diabetic retinas.** PKCs have been demonstrated to become active without any overt cytosolic/membraneous translocation [180-183], so we also examined putative transmembrane movement to low-buoyant lipid microdomains. Implementing discontinuous gradients to isolate these low-buoyant domains, we again demonstrate no significant differences between control and diabetic retinas (Fig. 2.6A). In contrast, when R28 cells are stimulated with PMA, but not insulin, significant increases in the localization of classical and novel PKCs, but not the atypical PKCζ, are observed in fractions 4 and 5, which are the low-buoyant domains (Fig. 2.6B).
Some scaffolding proteins are altered in the diabetic retina. Several scaffolding proteins have been demonstrated to regulate PKCs [184-187]. Thus, membrane or low-buoyant localized PKCs may still be further activated by alterations in proteins that regulate them. One such scaffolding protein is caveolin. After 4-weeks of stz-induced
diabetes a significant 40% decrease in caveolin-1 expression was observed and this decrease is partially restored by insulin treatment (Fig. 2.7A). Another scaffold protein, flotillin-1, was not altered in terms of expression. However, a mobility shift was observed, suggesting posttranslational modification (Fig. 2.7A). Preliminary data from

Fig. 2.6: PMA alters PKC isoform localization in lipid microdomains. A. PKC localization within low buoyant fractions of control and diabetic retinas was assessed by discontinuous sucrose gradient to isolate these domains (n = 3). Equivalent aliquots of each fraction were assessed for the presence of PKC isotypes by western blotting. B. Likewise, PKC localization was also assessed in R28 cells after insulin or PMA treatment (n = 3).
the examination of retinal caveolin-1 expression from 24 week diabetic Ins2\textsuperscript{Akita} mice, another model of type I diabetes [317], compared to sibling controls also demonstrated decreased caveolin-1 expression (Fig. 2.7B). Furthermore, by immunohistochemistry diminished caveolin-1 immunoreactivity is observed in the retina from stz-induced diabetes (Fig. 2.7C). Therefore, diminished or altered low-buoyant associated scaffolding proteins in diabetes may have an effect on PKC activation.

![Image](image.png)

**Fig. 2.7:** Caveolin-1 expression is diminished in the diabetic retina. A. By western blotting caveolin-1 and flotillin-1 expression was compared between 4-wk control and diabetic retinas (n = 6). B. Similarly, caveolin-1 expression was assessed after 6 months of diabetes in the Ins2\textsuperscript{Akita} model. C. Caveolin-1 localization was also assessed between control and diabetic retinas by immunohistochemistry.
PKC activation appears unchanged in the diabetic retina. PKC translocation and low-buoyant fraction localization appeared unchanged in the diabetic retina, so we also assessed the activation state of PKCs by a phospho-specific antibody to MARCKS (myristoylated alanine-rich C kinase substrate), a major in vivo substrate of PKC. Here, we observed no alterations in MARCKS phosphorylation after 1 month of diabetes compared to age-matched controls (Fig. 2.8A). To validate this, when R28 cells are stimulated with PMA, but not insulin, considerable phosphorylation of MARCKS is observed (Fig. 2.8B).

![Fig. 2.8: PKCs do not appear activated in the diabetic retina. A. Retinal homogenates from 3mo diabetic rats and age-matched controls were subjected to western blotting for phosphorylated MARCKS. B. Serum-starved, differentiated R28 cells were stimulated with the indicated concentrations of insulin or PMA for 15 minutes. Homogenates were subjected to immunoblotting for phosphorylated MARCKS. C. Retinal homogenates from 3mo diabetic rats and age-matched controls were immunoblotted for phosphorylation of the hydrophobic motif of PKCs or D. phosphorylated Thr⁴¹⁰ of PKCζ. n=8 controls, n=6 diabetic, p > 0.05 for all control and diabetes comparisons.](image-url)
The phosphorylation state of the conserved phosphorylation hydrophobic motif site of PKCs was also assessed. This site is regulated by either autophosphorylation or by upstream kinases, depending on the PKC isotype [107]. We again observed no notable differences between control and diabetic retinas (Fig. 2.8C). Lastly, we assessed the phosphorylation state of PKCζ on Thr410, a PDK-1 regulatory site. We and others have seen increased phosphorylation of this site on PKCζ upon increases in or exogenous application of ceramide (unpublished observations) [153]. Again, no notable changes were observed between control and diabetic animals (Fig. 2.8D). Accordingly, these data do not support the concept of PKC activation in the diabetic rat retina.

2.4 Discussion

We report that PKCs are able to inhibit insulin-signaling in vitro upon exogenous applications of the PKC activators, PMA or DAG (Fig 2.2). The mechanism by which this occurs is still undefined, but does not appear to involve the activation of PP2A, mTOR or MAPK signaling cascades. There is ample evidence that PKCs can directly phosphorylate and inactivate IRS-1 [151, 152] or the insulin receptor directly [150]. Such mechanisms require further investigation, though preliminary evidences suggest no alterations in IRS-2 tyrosine phosphorylation upon PMA treatment.

Despite a clear in vitro effect, we do not observe any overt activation of PKC upon the onset of diabetes (Fig. 2.4-2.6, 2.8). These findings are in contrast to previously published reports of increased PKC expression, translocation and/or activation in the diabetic retina [109, 318, 319]. These differences may be due to duration of diabetes or
differences in diet. For example, the diet of the animals used in these studies were fed a diet that contains very little unsaturated omega 3 fatty acids compared to what animals are fed in Robert Anderson’s laboratory at the University of Oklahoma (personal communication), which may be important since diacylglycerides with unsaturated fatty acids are more potent activators of PKC. There may, in fact, be increased PKC activation, but the methods utilized herein may not be sensitive enough to detect them. Strategies that further examine the phosphorylation of PKC substrates, suborganelle localization and methods to examine specific subtypes of retinal cells are needed.

While insulin is unable to stimulate translocation of PKCs to these low-buoyant fractions or stimulate MARCKS phosphorylation, surprisingly, when R28 cells are treated with the cholesterol depleting compound, methyl-β-cyclodextrin, we observed insulin-stimulated MARCKS phosphorylation (see next chapter for data). This may suggest that PKCs are bound in an inactive state by associating with scaffolding proteins, such as caveolin. Thus, in diabetes, PKC activity may be increased through decreased caveolin-1 expression (Fig. 2.7).

Regardless of these new data, some of the original findings demonstrating increased PKCs are suspect. PKC translocation does not always yield activation and activatable PKCs may already be bound to the membrane [180-183]. It is important to understand the basic mechanism of PKC activation and measurement of PKC activity. Unlike many kinases, PKC is only active when bound to its cofactors. When cells are lysed in detergent, activity is lost. Activity is regained when cofactors are added back to the kinase activity assay. Therefore, PKC activity is defined as the phosphatidylserine/diacylglycerol-stimulated activity (or just phosphatidylserine for the
atypical PKCs). These cofactors must be present in the assay and thus there is inherent artificial activation in these assays.

For PKC activation, phosphorylation by PDK-1 is a constitutive process required for its maturation, especially for classical PKC isotypes. PDK-1 phosphorylation primes the classical PKCs for activation, but does not actually activate them. Once classical PKCs are phosphorylated they undergo an autoinhibitory conformation change. Upon cofactor binding, this autoinhibition is relieved and consequently the kinase activated. In contrast, unphosphorylated Akt is in an autoinhibited state. The binding of Akt to the lipid-derived second messenger PIP$_3$, allows PDK-1 to interact with and phosphorylate Akt. This phosphorylated Akt no longer requires cofactor binding [88, 107]. Thus, classical PKCs (including PKC$_{\beta}^{II}$) are already fully primed by phosphorylation and any reported changes in activation must be questioned or may be a consequence of any observed increases in PKC expression. However, we have not observed any appreciable alterations in protein expression and further supporting this, a recent microarray experiment and some quantitative RT-PCR of the PKC$_{\beta}$ isoform has not detected any significant alterations in most PKC mRNA expression. However, the microarray analysis reported diminished PKC$_{\alpha}$ mRNA expression, which is currently being validated by quantitative RT-PCR (unpublished data, Genomic Core).

However, for novel and atypical PKCs, it is possible that the specific activity will change in the context of diseases (i.e. diabetes) due to changes in the phosphorylation by its upstream kinase PDK-1. However, a lack of increase in kinase activity does not mean there is no activation. Due to these facts, the many reports utilizing kinase assays that
have reported increases in PKC activity and that suggest/imply a role of PKCβ, are questionable and the novel and atypical PKC isotypes warrant more investigation. Additionally, studies that investigate the tyrosine phosphorylation state of PKCs are also needed, as kinases such as Syk, regulate PKC ability to activate downstream targets such as the Ras/Erk cascades [320]. There is a report of increased H-Ras and Raf-1 in the diabetic retina [321], though our studies demonstrate that downstream effectors, Erk [50] and p90 RSK (unpublished observations), do not appear to be altered in the diabetic retina. However, additional evidence by others suggests that at least in some cell types hyperglycemia can activate Ras signaling [322].

The lack of overt alterations in PKCs is further validated by human clinical trials, which have demonstrated less than ideal therapeutic efficacy of ruboxistaurin (PKC inhibitor) for diabetic retinal complications. Two large, long-term multicenter clinical trials, the PKC-DMES (PKC-Diabetic Macular Edema Study) and the PKC-DRS (PKC-Diabetic Retinopathy Study) have been undertaken. The PKC-DMES recently reported that ruboxistaurin did not result in a significant effect on the progression of diabetic macular edema. Furthermore, ruboxistaurin did not delay the use of laser photocoagulation for treatment [117]. However, another report suggested some utility of this drug in patients that demonstrate a higher baseline of vascular leakage (macula edema) [118]. The PKC-DRS demonstrated that ruboxistaurin did not reduce the progression of severe nonproliferative to proliferative diabetic retinopathy. They did however report a significant reduction in delayed occurrence of moderate visual loss [119]. This could potentially be due to an effect of ruboxistaurin on neuronal and glial cells, not just the retinal vasculature. A more recent publication revealed that this PKC
inhibitor inhibited diabetes-induced changes of retinal hemodynamic properties [120]. In the future, PKC β inhibition may prove useful as a possible adjunct to current therapy for diabetic complications, but further investigations into PKC isotypes are needed. Regardless, these modest effects must be weighed against the uncertain risk of long-term adverse effects from chronic doses of, and subsequent long-term inhibition of PKCs, on normal physiological processes. As an alternative to altered PKC regulation, increased DAG metabolism, a lipid cofactor of PKC, may contribute to the pathology diabetic retinopathy. As shown in the next chapter, increasing DAG flux to phosphatidic acid may limit diabetic complications, including neuronal cell death.
Chapter 3

**Phosphatidic acid and insulin activate mTOR signaling through a cholesterol-sensitive mechanism to synergistically limit inflammatory cytokine-induced retinal neuronal cell death**

3.1 Introduction

Retinal degeneration due to neuronal apoptosis is an underlying cause of many visual diseases including retinitis pigmentosa, macular degeneration and diabetic retinopathy. We and others have shown that in diabetic models as well as in patients with diabetic retinopathy there is an increased rate of neuronal apoptosis [3, 4, 18, 19]. Therefore, understanding the regulation of pro-survival signaling cascades may identify therapeutic targets to treat such retinal neurodegenerative diseases.

One potential regulator of survival is mTOR, a pro-translational/pro-mitogenic/pro-survival effector, which is altered in multiple diseases including cancer, atherosclerosis and diabetes among others [323]. Whereas the conventional thought about mTOR is that it mediates amino acid and growth factor-induced translation, there is increasing evidence for the role of mTOR and its downstream effectors in the regulation of cellular survival. It has been demonstrated that mTOR-dependent suppression of protein phosphatase 2A is important for survival [324]. Furthermore, mTOR signaling suppresses apoptosis through positive regulation of protein phosphatase 5 activity and suppression of cellular stress [325]. We have recently shown that p70 S6K, a downstream effector of mTOR, is an important factor in mediating insulin-induced survival of retinal
neurons [75]. Another study also supported the role of p70 S6K in regulating cell survival by inactivating the pro-apoptotic protein BAD via phosphorylation at Ser$^{136}$ [76].

Of more clinical importance, we have demonstrated that mTOR-p70 S6K signaling is suppressed in the diabetic retina [50]. Therefore, understanding the mechanisms by which mTOR signaling is regulated could have therapeutic implications for inhibiting retinal neurodegeneration. One putative neurodegenerative factor is interleukin-1β (IL-1β). IL-1β has been demonstrated to be increased in the diabetic retina [35-37] and has an important role in mediating neurodegeneration caused by ischemic and excitotoxic conditions in the retina and brain [326-328]. In fact, cerebral ischemia suppresses protein synthesis and p70 S6K phosphorylation [329].

Hypoxic stress, ischemia, inflammatory cytokines and diabetes all have been shown to stimulate phospholipid catabolism and generation of lipid-derived second messangers. Yet, the roles of these lipid-derived second messangers to modulate mTOR/p70 S6K activity in stress-induced states have not been investigated in retinal neurons. In particular, phosphatidic acid (PA), which is typically generated through the phosphorylation of diglycerides by diglyceride kinases or the activation of phospholipase D (PLD), has been demonstrated to positively regulate mTOR signaling [330-332].

The regulation, organization and coordination of signaling cascades have been shown to be controlled within lipid rafts, also called lipid microdomains which include caveolae. It is thought that lipid rafts serve as signalosome assembly platforms to coordinate the interactions between scaffold and anchoring proteins with kinases for efficient downstream signaling [333]. Our work herein further examines the role of cholesterol-
enriched lipid microdomains in the activation of mTOR signaling and the ability to diminish IL-1β induced neuronal cell death.

3.2 Materials and Methods

Materials. Bovine insulin was purchased from Sigma (St. Louis, MO). Laminin and cell permeable cAMP were from BD Biosciences (Franklin Lakes, NJ) and MP Biomedicals (Irvine, CA) respectively. Phorbol 12-myristate 13-acetate (PMA) and 4α-phorbol 12-myristate 13-acetate (4α-PMA) were purchased from Promega (Madison, WI). The ³H-PMA was obtained from American Radiolabeled Company (St. Louis, MO) and NBD-PA, PA and DAG from Avanti Polar Lipids (Alabaster, AL). Polyclonal rabbit anti-phospho-p42/44 MAPK (Thr²⁰²/Tyr²⁰⁴), rabbit anti-p42/44 MAPK, anti-phospho-p70 S6K Thr³⁸⁹, anti-p70 S6K, anti-p90 RSK Ser³⁸⁰, anti-MARCKS Ser¹⁵²/¹⁵⁶, anti-RXRXXpS/T and anti-tuberin Thr¹⁴⁶² antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-rabbit IgG-horseradish peroxidase was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The PKC inhibitor bisindolylmaleimide-I (BimI) and the mTOR inhibitor rapamycin were from EMD Biosciences (San Diego, CA). The MEK inhibitors, PD98059 and U0126, were purchased from Biomol (Plymouth Meeting, PA).

Cell Culture. R28 cells were a generous gift from Dr. Gail M. Seigel, State University of New York, Buffalo [308]. They were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 5.5 mM glucose supplemented with 10% newborn calf serum (Hyclone, Logan, UT). The cells were differentiated to neurons on laminin-
coated plates or coverslips with addition of 25µM cell-permeable cAMP as previously described [309].

**Western Blot Analysis.** Western blot analyses were performed essentially as described previously [309]. Briefly, treated R28 cells were washed in ice-cold Dulbecco's phosphate-buffered saline solution and lysis buffer [50 mM HEPES, 137 mM NaCl, 5 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM NaVO₄, 1% NP-40, Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN)] was added. Cell lysates were cleared by centrifugation, and the Bio-Rad DC protein assay was utilized to determine protein concentration. Typically 30 µg of protein lysate per sample was separated on a 4-12% NuPAGE gels (Invitrogen, Carlsbad, CA) and transferred to Hybond nitrocellulose membranes (GE Healthcare, Piscataway, NJ). The membranes were subsequently blocked in 5% BSA (1 hour) and incubated with the primary overnight (1:1000 dilution). Membranes were subsequently washed 3 x 10 min in TBST and incubated with an HRP-conjugated secondary for 2 hours (1:5000 dilution), washed in TBST (3 x 10 min) and visualized by ECL (GE Healthcare, Piscataway, NJ). Bands were quantified using ImageQuant (Molecular Dynamics, Sunnyvale, CA), or GeneTools SynGene (K&R Technology, Frederick, MD) software. For immunoprecipitation, 2µg of anti-tuberin or anti-insulin receptor β antibody was incubated with 300ug of protein lysate overnight. The next day, protein G was added for 1hour and the complex was subsequently washed twice with lysis buffer and analyzed by western blotting.

**Cellular fractionation.** R28 cells were treated as described in the text and a detergent-free method of isolating lipid rafts was performed as described by others [310]. Briefly, cells were harvested in sodium carbonate buffer (pH 11.0) and homogenized.
Homogenates were adjusted to 4 ml with 90% sucrose in MES-buffered saline (MBS) (25 mM Mes, pH 4.5, 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM NaF, protease inhibitor tablet) to a final concentration of 45% sucrose. Samples were layered at the bottom of a 14 × 89 mm tube containing 4 ml each of 35% and 5% sucrose in sodium carbonate/MBS and centrifuged in a Beckman SW41ti rotor at 200,000g for 16-20 h at 4°C. Serial fractions of 1 ml were removed and analyzed by western blotting. For the ³H-PMA study, 1µCi of ³H-PMA was added to each 100mm dish used along with cold PMA to give 100nM final concentration of PMA. Fractions were then subjected to scintillation counting. Alternatively, when fluorescent lipid analogs were utilized, 200µl of each fraction were added to a microtiter plate in triplicate and the fluorescence was measured with a Molecular Devices SpectraMax Gemini XS plate reader.

*Cellular death quantification.* R28 cells were cultured on glass coverslips and treated as described in the text. After treatment, cells were washed in PBS and fixed in 2% paraformaldehyde for 10 minutes before staining/mounting with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Five randomly sampled fields from each coverslip were observed to determine the percent of pyknotic nuclei as described previously [309].

*Statistical methods.* One-way analysis of variance (ANOVA) with Bonferroni multiple comparison post test and t-tests analysis were performed using GraphPad Prism 4.0 software, with statistical significance considered if p < 0.05.
3.3 Results

Stimulation of Protein Kinase C activates p70 S6K. Several reports have demonstrated that the PKC activator phorbol myristate acid (PMA) induces the activation of mTOR signaling cascades which includes p70 S6K [334-337]. To further evaluate the role of PKC in the activation of p70 S6K, R28 cells, a model of retinal neurons [308, 309], were treated with or without PMA and/or insulin to determine the effects on the activation of p70 S6K. Insulin, as previously reported [75], induced an increase in p70 S6K Thr\textsuperscript{389} phosphorylation (Fig. 3.1A). Additionally, PMA alone activated p70 S6K, and post-treatment with insulin was able to synergistically increase p70 S6K phosphorylation. Furthermore, 4α-PMA, an inactive analog of PMA, had no effect on p70 S6K activation with or without insulin treatment. Phorbol esters can activate signaling cascades independently of PKCs, so we utilized the PKC inhibitor bisindolylmaleimide I (BimI) prior to PMA or insulin treatment. Blockade of PKC activation led to decreased PMA-induced p70 S6K activation, but not insulin-induced p70 S6K activation.

Since the MEKK-ERK-p90 RSK1 pathway has been implicated in the activation of p70 S6K in response to phorbol esters [334] we also explored the phosphorylation state of p42 and p44 (Erk1/2) (Fig. 3.1B). Insulin was unable to significantly activate Erk1/2 in these cells, which is similar to what we have observed in ex vivo and in vivo retinas stimulated with insulin [41], whereas PMA induced a strong increase in the phosphorylation of Erk1/2, which was blocked by the PKC inhibitor, BimI. Similarly to p70 S6K, 4α-PMA had no effect on the phosphorylation of Erk1/2.
To determine the exclusivity of phorbol ester-induced activation of p70 S6K, we examined if another PKC activator, 1,2-Dioctanoyl-sn-Glycerol (DAG), a cell-permeable diglyceride, could also activate this kinase. Here, DAG treatment leads to increased p70 S6K phosphorylation (Fig. 3.1C). Furthermore, this same dose and time also led to increased phosphorylation of Erk1/2. Thus, both lipid and non-lipid PKC activators can stimulate the phosphorylation of p70 S6K.

**Fig. 3.1: PKC activation stimulates p70 S6K and ERK phosphorylation.** R28 cells were serum-starved, pretreated with BimI for 30 min and then stimulated with either insulin (10nM) or PMA (100nM) for an additional 15 min. Total cell lysates were immunoblotted for phosphorylated (Thr\textsuperscript{389}) or total p70 S6K (A) or phosphorylated (Tyr\textsuperscript{204}) or total ERK (B). Serum-starved R28 cells were also stimulated with 50µM of DAG for 30 min and immunoblotted for phosphorylated p70 S6K, ERK and β-actin (C). All studies were performed a minimum of n = 3 times.
_PMA activated p70 S6K is dependent on MEK and Phospholipase D signaling._ To validate a role of MEK signaling in PMA-induced p70 S6K activation in R28 cells, we utilized two inhibitors of MEK, PD98059 and U0126. After inhibitor treatment, R28 cells were stimulated with either insulin or PMA and the phosphorylation state of p70 S6K was assessed (Fig. 3.2A). The presence of either MEK inhibitor had no significant effect on insulin-stimulated p70 S6K, but partially diminished PMA-stimulated p70 S6K. This is in contrast to phosphorylated Erk1/2, where these inhibitors diminished basal phosphorylation and blocked PMA-induced phosphorylation. The mTOR inhibitor rapamycin was able to completely block both insulin and PMA-stimulated p70 S6K, but not PMA-stimulated Erk1/2. Thus, while mTOR is necessary for both insulin and PMA-stimulated p70 S6K, MEK signaling is only partially necessary for PMA-stimulated p70 S6K, but not for insulin-induced activation.

MEK-inhibition only partially blocked the phosphorylation of p70 S6K by PMA, so we sought to determine alternative mechanisms by which PKC may mediate this activation. Recent data supports a role of phosphatidic acid (PA), which can be generated through the activation of diacylglycerol kinases or phospholipase D, in the regulation of mTOR signaling cascades [330-332]. To explore this possibility, we utilized 1-butanol, an inhibitor of phospholipase D (PLD)-mediated PA formation through a transphosphatidylation reaction. When R28 cells were pretreated with 1-butanol prior to stimulation with PMA, we observed complete inhibition of p70 S6K phosphorylation (Fig. 3.2B). This 1-butanol treatment however, had only a minute effect on insulin stimulated p70 S6K. As 1-butanol has been shown to have a nonspecific effect diminishing PI3K and thus PI3-lipids, we also assessed the phosphorylation state of Akt,
which, when stimulated with insulin, is activated in a PI3K-dependent mechanism in R28 cells [309]. Whereas PMA does not increase Akt phosphorylation, 1-butanol treatment did not impair insulin-induced increases in Ser\textsuperscript{473} phosphorylation of Akt. Lastly, the phosphorylation state of Erk1/2 was unaffected by 1-butanol treatment. Therefore, PMA activates p70 S6K, but not Erk1/2 in a PLD-dependent manner.

To further examine a role of PA in p70 S6K activation, we next determined if exogenous PA is able to activate this kinase (Fig. 3.2C). When R28 cells are stimulated with PA, there is an increase in the phosphorylation of p70 S6K. This increase in phosphorylation is blocked by the mTOR inhibitor rapamycin, but only partially blocked by the MEK inhibitor U0126. We also assessed the phosphorylation state of p90 RSK1, which was recently demonstrated to be responsible for PMA-induced activation of mTOR signaling cascades. Exogenous PA elicited an increase in p90 phosphorylation/mobility shift, which was unaffected by rapamycin, but completely blocked by U0126. Thus, these data along with Figure 2A support the notion that inputs from the MEK pathway as well as PA are required for increasing p70 S6K phosphorylation in response to PMA in R28 retinal neurons.
Fig. 3.2: **MEK and PLD are mediators of PMA-activated p70 S6K.** (A) Serum-starved R28 cells were pretreated with either the MEK inhibitor PD98059 or U0126 for 30 minutes. The mTOR inhibitor rapamycin (100nM) was also used in some cases, prior to stimulation with insulin (10nM) or PMA (100nM) for 15 min. Cellular lysate was again immunoblotted for phosphorylated p70 S6K or ERK. (B) The role of PLD was assessed by pretreatment of R28 cells with 0.4% 1-butanol for 30 min, prior to stimulation with insulin (10nM) or PMA (100nM) for 15 min. All experiments were repeated a minimum of n=3 times.
**Activation of p70 S6K, but not MAPKs depends on cholesterol-enriched lipid microdomains** - Lipid microdomains dynamically associate to form platforms important for membrane protein sorting and construction of signaling complexes to allow for efficient downstream signaling in response to many ligands [333]. To explore the role of lipid microdomains in p70 S6K regulation, we examined the consequence of lipid raft disruption by cholesterol depletion utilizing methyl-β-cyclodextrin, which can extract cholesterol from membranes [338]. A short-term 60 min treatment with 1% methyl-β-cyclodextrin (MβCD) did not change the gross morphology of cultured cells at these concentrations (data not shown). When subsequently treated with PMA and/or insulin, PMA and insulin activation of p70 S6K was drastically reduced in the presence of MβCD (Fig. 3.3A). This inhibition occurs despite the ability of PMA to still induce Erk1/2 phosphorylation (Fig. 3.3A). In fact, cholesterol depletion led to a slight increase in p42/p44 phosphorylation.

PKCs mediate the bulk of the responses of PMA, so we next examined PKC activation via an indirect method of examining the phosphorylation state of MARCKS, a substrate of PKCs (Fig. 3.3B). Under basal conditions, MβCD caused a slight, but significant increase in MARCKS phosphorylation. This may, in part, explain the slight basal increase of ERK (Fig. 3A). As expected, PMA induced a large increase in MARCKS phosphorylation that was unaffected by MβCD treatment. While insulin is a poor activator of PKCs in R28 cells (Fig. 3.3B and data not shown), surprisingly cholesterol depletion now allows insulin to activate PKCs, though other insulin signaling cascades are impaired (Fig. 3.3A).
The effects of cholesterol depletion on PA stimulation were also examined (Fig. 3.3C). Here, PA and PMA are able to stimulate p90 RSK1, a downstream target of Erk1/2, even in the presence of MβCD, whereas insulin does not. These results are similar to what was observed with the upstream kinase Erk1/2. In fact MβCD treatment also increases p90 RSK phosphorylation on its own. In contrast, cholesterol depletion prevents p70 S6K activation by PA. Therefore, activation of the mTOR effector p70 S6K is cholesterol sensitive.

**Fig. 3.3:** **Cholesterol dependency of signaling cascades.** (A) R28 cells were pretreated with MβCD and subsequently treated with insulin and/or PMA. Western blots were again utilized to assess the phosphorylation state of p70 S6K and ERK. (B) R28 cells were treated as in (A), and MARCKS phosphorylation state was determined. (C) The phosphorylation state of p90 RSK was also assessed as a function of cholesterol depletion. Representative blots of an n=3 experiment.
Cholesterol depletion alters insulin receptor activation, but not PMA and PA localization. To further examine the role of cholesterol-enriched microdomains on insulin signaling, we examined the effect of cholesterol depletion on the insulin receptor itself (Fig. 3.4A). Phosphotyrosine analysis of the immunoprecipitated IRβ subunit revealed that cholesterol depletion significantly diminishes insulin-induced activation of the insulin receptor.

We also explored if in fact PMA and/or PA localize to low buoyant fractions, consistent with lipid microdomains. When R28 cells are treated with 3H-PMA, and low-buoyant fractions are isolated we show that 70% of the radiolabeled PMA localizes to fractions 4 and 5 (Fig. 3.4B), consistent with lipid-raft enriched fractions as evidenced by strong localization of the lipid raft marker caveolin-1 to these fractions (data not shown). MβCD treatment had no significant effect on the distribution of PMA within fractions 4 and 5 (p=0.225).

Similar to PMA, the localization of PA was also determined utilizing an fluorescent NBD-labeled PA. Here, 22% of exogenously added PA localizes to fractions 4 and 5 (Fig. 3.4C). Furthermore, as observed for PMA, cholesterol depletion did not significantly alter this distribution (p = 0.59). The lack of effect of cholesterol depletion on PMA and PA localization may explain the ability of these agonists to still stimulate MEKK signaling cascades (Fig. 3.3A and 3.3C).
Insulin, PMA and PA differentially phosphorylate tuberin. To further explore the mechanisms by which lipid and non-lipid growth factors activate mTOR signaling, we analyzed the phosphorylation state of tuberin. Tuberin and hamartin form the tuberous sclerosis complex, which negatively regulates mTOR signaling. This negative regulation

Fig. 3.4: Cholesterol disrupts insulin receptor activation, but not the low-density localization of PMA or PA. (A) R28 cells were treated with insulin after prior treatment with 1% MβCD for 60min. The IRβ was immunoprecipitated and its phosphorylation state assessed by a phosphotyrosine (PY) antibody. (B) R28 cells were treated with a ³H-labeled PMA prior to the isolation of low-buoyant fractions (lipid rafts), were isolated on a discontinuous sucrose gradient. Radioactivity was assessed in each fraction by scintillation counting. (C) Similar to (B), R28 cells were treated with an NBD-labeled PA, fractionated, and fluorescence assessed by fluorescent spectrometry. (n=3 for each treatment).
can be abrogated through phosphorylation of tuberin by upstream kinases. After treatment of R28 cells with insulin, PMA, or PA, tuberin was immunoprecipitated and its phosphorylation state was assessed with two different antibodies by immunoblotting (Fig. 3.5). The first of which is an RXRXXpS/T phospho-motif antibody. Increased immunoreactivity was observed in PMA and PA-treated, but not vehicle or insulin-treated cells. In MβCD treated cells, we observed increased basal phosphorylation at this site which did not augment with either PMA or PA. It has recently been demonstrated that Ser\textsuperscript{1798}, which lies within a RXRXXS/T motif, is a major phosphorylation target of PMA stimulation. In contrast, when we utilized a phospho-specific antibody to tuberin for site Thr\textsuperscript{1462}, we observed a marked increase in phosphorylation in cells stimulated with insulin, which was inhibited by pretreatment with MβCD. This inhibition is most likely due to diminished upstream insulin signaling. It is interesting that PMA and PA, when compared to insulin, differentially phosphorylate/regulate tuberin and this may represent the convergence of these two pathways to augment p70 S6K phosphorylation. Furthermore, PMA and PA still lead to phosphorylation of tuberin in cholesterol-depleted cells, suggesting that the cholesterol-dependent regulation of p70 S6K phosphorylation occurs downstream of tuberin. Additionally, these differences in tuberin phosphorylation may be exploited to synergistically promote survival in retinal neurons.
Insulin and PA rescue R28 cells from IL-1β-induced death. The proinflammatory cytokine IL-1β, has been demonstrated to be upregulated in the diabetic retina [35-37] and can be a mediator of neurodegeneration [326-328]. We therefore determined if the ability of insulin and PA to positively regulate prosurvival pathways could prevent IL-1β-induced cellular death. When R28 cells were treated with IL-1β, the remaining attached cells demonstrated 26% pyknosis (Fig. 3.6). PA significantly reduced this apoptosis in half to 13%, whereas insulin significantly reduced apoptosis to 18% in the presence of IL-1β. When R28 cells were treated with both PA and insulin in the presence of IL-1β, apoptosis was further reduced to 8% which was significantly less compared to PA or insulin alone in the presence of IL-1β, suggesting synergism. However, in the presence of the mTOR inhibitor rapamycin, the ability of PA, insulin and both insulin and PA together to inhibit IL-1β-induced cellular death was significantly diminished. Therefore,
mTOR signaling is an important mediator to limit IL-1β–induced cellular death. This implies that lipid-derived mediators, such as PA, could serve as therapeutic agents to promote survival of retinal neurons under inflammatory or diabetic conditions.

Fig. 3.6: PA and insulin rescue retinal neurons from IL-1β-induced cell death. R28 cells were treated with vehicle, insulin (10nM), PA (50µM) or both insulin and PA in the presence of absence of IL-1β (10ng/mL) and/or rapamycin (100nM) for 24 hours. The number of pyknotic nuclei was determined by DAPI staining, then counted and expressed as a percentage of the total number of cells. (* p < 0.05; n = 3 coverslips, ◆ significantly different from PA + IL-1β and Ins + IL-1β)
3.4 Discussion

Neuronal apoptosis in diabetic retinopathy is an early pathological feature that precedes vascular occlusion. This neuronal cell death has been characterized in both humans afflicted with diabetes and in animal models and may underlie vision loss [3, 4, 18, 19]. Cellular death can arise as a consequence of dysregulated signaling cascades. We have previously characterized that the rat retina is an insulin-responsive tissue that demonstrates reduced prosurvival insulin receptor, PI3K-Akt and p70 S6K signaling when diabetes is induced [41, 50]. Therefore, further understanding how these pathways are regulated may provide insights into therapeutic interventions to restore or augment diminished insulin signaling.

Here, we demonstrate that insulin and PMA/PKC act through distinct mechanisms to activate mTOR signaling and subsequently p70 S6K. We show that insulin and PMA induce the activation of p70 S6K (Fig. 3.1) and that PMA does this through the activation of the MAPK cascade and PLD-generated PA (Fig. 3.2). Insulin and PMA/PA lead to increased, but differential tuberin phosphorylation, which putatively will turn off the tuberous sclerosis complex and increase mTOR activation (Fig. 3.5). Furthermore, regardless of the agonist used in this study, the activation of p70 S6K, but not ERK or p90 RSK1, depends on cholesterol-enriched microdomains (Fig 3.3). In fact, PA and PMA demonstrate some localization to cholesterol-resistant microdomains, whereas cholesterol-depletion disrupts insulin receptor activation (Fig. 3.4). These distinct mechanisms for activation of mTOR signaling contribute to increased retinal neuronal cell survival after IL-1β treatment (Fig. 3.6), as summarized in (Fig. 3.7). Thus,
increasing endogenous or exogenous PA could be therapeutic for retina neurodegenerative diseases by restoring the critical balance of prosurvival and proapoptotic signaling cascades.

Fig. 3.7: Cholesterol-sensitive regulation of mTOR signaling. PMA and PA signal through a cholesterol-insensitive manner to phosphorylate/inactivate tuberin, whereas insulin signals through a cholesterol-sensitive mechanism. However, mTOR-p70 S6K activation is cholesterol/raft sensitive regardless of the agonist.

Altering DAG metabolism or the use of exogenous PA could diminish the putatively deleterious effects of DAGs in diabetes. It has been demonstrated that inhibition of diacylglycerol kinase, another enzyme that produces PA, mimics diabetes-induced abnormal retinal hemodynamics [112]. Furthermore, d-alpha-tocopherol (Vitamin E) can hinder hyperglycemic-induced DAG-PKC activation by enhancing DAG kinase activity [339]. Additional evidence supporting a putative therapeutic modality of PA for the retina is seen in RNA interference studies that have demonstrated that knockdown of the DAG
kinase α or β isoforms increase apoptosis [340]. Thus, altering DAG flux to form PA may be therapeutically desirable. However, we have not ruled out the possible metabolism of PA to other prosurvival lipid species such as lysophosphatidic acid.

We also demonstrate that control of sterols and lipid microdomains has an impact on the signaling capabilities of insulin, PMA and PA. Analyses of the role of cholesterol and cholesterol-enriched microdomains have revealed several findings. We found that treatment with MβCD on its own stimulated the activation of ERK1/2 and p90 RSK, a finding that has been observed before with ERK1/2. Recent data supports a role of an oxysterol binding protein (OSBP) to inhibit the phosphatase activity on ERK which inhibition is lost upon cholesterol depletion [341]. Alternatively, as lipid raft disruption has been demonstrated to stimulate PLD activity [342], PLD generated-PA could also potentially lead to activation of ERK [343]. Our data measuring MARCKS phosphorylation, an indirect marker of PKC activation, opens up the possibility that increased PKC activation may also contribute to cholesterol depletion induced-ERK1/2 and p90 RSK1 phosphorylation. Yet, PKCs have multiple pleiotrophic effects and in fact our observation that insulin activates MARCKS phosphorylation after cholesterol depletion might suggest that PKC-dependent insulin resistance, which is observed in diabetes, is a function of altered lipid metabolism. It is not surprising that cholesterol depletion had no impact on the localization of PMA and PA to the low-buoyant fractions as cholesterol depletion has been demonstrated to not have a significant effect on the localization of various sphingolipids within these low-buoyant fractions [344] (and unpublished observations).
We also observed a decrease in IRβ tyrosine phosphorylation in response to insulin in the presence of MβCD, a finding that contradicts recent reports [345, 346]. Though we have not elucidated the mechanism by which it occurs, it may be a cell-type specific effect, and could possibly be mediated though increased endocytosis which has been described as a consequence of cholesterol depletion [347]. Such endocytosis could theoretically diminish IR at the plasma membrane and decrease interaction with its ligands. This is important in light of our observed alterations of sphingolipid metabolism and decreased caveolin-1 content within the diabetic retina (unpublished observations), which may regulate the insulin receptor in these low-buoyant fractions [251, 348, 349]. Thus altered raft structure may be important for insulin resistance within the retinal neurons in diabetes. Additionally, this may also suggest that the lipid composition of neuronal membranes may contribute to the high basal levels of IR activity in the retina [41, 50].

Interestingly, while PMA, PA and insulin all lead to p70 S6K activation through different mechanisms, the pathways appear to converge at the level of the TSC complex of tuberin and hamartin. The TSC complex has been demonstrated to impair mTOR signaling and phosphorylation of tuberin has been demonstrated to relieve this inhibition [337, 350, 351]. We demonstrated that these agonists differentially phosphorylate tuberin, with insulin increasing the phosphorylation of Thr^{1462} whereas PMA and PA had no appreciable effect at this site. In contrast, PMA and PA lead to increased phosphorylation detected by a motif-specific antibody, presumably Thr^{1798} as identified by the Blenis laboratory [350], though insulin did not have the same effect. Thus, the synergism observed in PA and insulin inhibition of IL-1β-induced cellular death may be mediated
through this differential phosphorylation of tuberin to augment downstream mTOR signaling. Synergistic mTOR signaling may have the capability to compensate for the diminished insulin sensitivity observed in diabetes. Overall, restoring insulin signaling and/or shunting DAG metabolism to PA or by increasing PA through exogenous application may prevent neuronal apoptosis in diabetic retinopathy.
Vision loss from diabetic retinopathy results from the cellular dysfunction of multiple cell types of the retina. This multifaceted alteration affects the vascular, glia (micro and macro) and neurons of the retina [33]. The effect of neuronal apoptosis, which occurs early and is chronic in diabetes, is just now being fully appreciated. We and others have reported that the neurons of the retina undergo apoptosis in both human and experimental diabetic models [3, 4, 18, 19, 30]. However, the direct and indirect causes of neuronal dysfunction remain poorly defined. We recently demonstrated that the prosurvival signaling cascades, PI3K-Akt and mTOR-p70 S6K [50], are impaired in the diabetic retina, which may underlie the neuronal apoptosis that occurs.

In addition to loss of neurotrophic input, metabolic stresses may also be important. Sphingolipid metabolites have been demonstrated to regulate cellular stress and fate via a balance between pro-apoptotic/growth arresting lipids and pro-survival/proliferative lipids and their resulting effect on signaling pathways [352]. Ceramides are generally considered proapoptotic sphingolipids that accumulate in response to stress responses and proapoptotic stimuli, such as IL-1β and TNFα. Ceramides contribute to apoptosis/growth arrest at the biochemical level, by inhibiting
PI3K/Akt [213, 353] and ERK [93] signaling cascades and at biophysical levels by regulating mitochondrial permeability [354] and Golgi fragmentation [214].

Glycosphingolipids are metabolites of ceramide that have been implicated in cellular immunity, inflammation and multidrug resistance to cancer [355]. Simple glycosphingolipids, such as glucosyl and galactosylceramide (cerebrosides or monohexosylceramides) serve as building blocks for more complex glycosphingolipids including sulfatides, globosides and gangliosides. Recent reports suggest that some of these complex glycosphingolipids mediate apoptosis, insulin resistance and cellular stress. For instance, the ganglioside GD3 has been implicated in causing apoptosis in response to activation of the surface receptor CD95 [281, 284] and in response to TNFα [356]. Additionally, another ganglioside, GM3, impairs insulin signaling [250, 251, 280] and a GM3 knockout mouse has increased insulin sensitivity. Lastly, in neuronal tissues, glycosphingolipids have been implicated in increasing sensitivity to neurotoxic agents such as the excitatory amino acid neurotransmitter, glutamate [294], a potential contributor to diabetic retinopathy [292, 293].

Alterations in sphingolipid and glycosphingolipid metabolism have been implicated in the pathogenesis of several retinal diseases. Lysosomal storage diseases, which often are a consequence of dysregulated sphingolipid flux, are associated with retinal impairment. As examples, patients with Farber’s disease (acid ceramidase), Tay-Sachs/Sandhoff (hexosaminidase A or B), Gaucher’s (glucosylceramidase), Krabbe’s (galactosylceramidase) and Niemann Pick disease (sphingomyelinase) lose vision due to retinal neuronal cell death. Furthermore, overexpression of a neutral ceramidase gene in Drosophila abrogated retinal degeneration [207]. Thus, understanding the roles that
sphingolipid enzymes and their metabolites have in the retina will offer new targets for retinal diseases. Herein, we investigate the potential roles of ceramides and glycoconjugated ceramide metabolites in diabetic retinopathy.

4.2 Materials and Methods

**Materials.** Bovine insulin was purchased from Sigma (St. Louis, MO). Laminin and cell permeable cAMP were purchased from BD Biosciences (Franklin Lakes, NJ) and MP Biomedicals (Irvine, CA), respectively. Anti-phospho-p70 S6K (Thr^{389}) and total p70 S6K were obtained from Cell Signaling Technology (Beverly, MA). Anti-GRP78 was purchased from Assay Designs (Ann Arbor, MI). Glucosylceramide synthase rabbit antisera was a generous gift from Drs. R.E. Pagano and D.L. Marks, Mayo Clinic and Foundation (Rochester, MN) [357]. Anti-rabbit and anti-mouse IgG-horseradish peroxidase was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). C₈-glucosylceramide and C₈-galactosylceramide were from Avanti Polar Lipids (Alabaster, AL). Gangliosides were purchased from Matreya (Pleasant Gap, PA). N-Butyldeoxygalactonojirimycin was purchased from Toronto Research Chemicals (North York, On) and DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) from Biomol (Plymouth Meeting, PA). Interleukin-1β (IL-1β) was purchased from Promega (Madison, WI).

**Cell Culture.** R28 cells, an E1A immortalized model of retinal neurons, were a generous gift from Dr. Gail M. Seigel, State University of New York, Buffalo [308]. These cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 5 mM glucose supplemented with 10% newborn calf serum (Hyclone, Logan, UT) and
were differentiated on laminin-coated plates or coverslips with addition of 25µM cell-
permeable cAMP as described previously [309]. Cells were treated as described in the
text.

*Animals.* Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were fasted overnight and given a single intraperitoneal injection of streptozotocin (65 mg/kg; Sigma, St. Louis, MO), freshly dissolved in 10 mM sodium citrate buffer (pH 4.5). Diabetes was confirmed 6 days later by blood glucose greater than 250 mg/dL (Lifescan, Milpitas, CA). Age-matched control and diabetic rats were monitored regularly by weight and blood glucose tests. Rats were housed in accordance with the Institutional Animal Care and Use Committee guidelines, and the study protocol adhered to the ARVO Statement for the Use Animals in Ophthalmic and Vision Research. Rats were maintained by the Juvenile Diabetes Research Foundation Animal Core Facility at Penn State University and group housed in solid plastic bottom cages with bedding as well as *ad libitum* food (Teklad Global 18% Protein Rodent Diet) and water under a normal 12-hour light-dark schedule. In some cases, groups of animals were fasted for 16 hours prior to their sacrifice. Another group of animals received subcutaneous insulin injections twice daily for 3 days prior to their sacrifice with 5U Humulin® Regular/5U Humulin® Ultralente as described previously [306]. Retinas were dissected [307] at the indicated times and snap frozen in liquid N₂. The average weight and blood glucose of the animals on the days of sacrifice are indicated in Table 1.
Table I: Average weight and blood glucose on day of sacrifice

<table>
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<tr>
<th>Study</th>
<th>Group</th>
<th>n</th>
<th>Weight (g)</th>
<th>Blood Glucose (mg/dL)</th>
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<td>Fig. 1A</td>
<td>Control (2wk)</td>
<td>6</td>
<td>288.7 ± 4.6</td>
<td>109.1 ± 3.2</td>
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<tr>
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<td>236.7 ± 5.6</td>
<td>440.8 ± 16.4</td>
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<tr>
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<td>107.7 ± 14.9</td>
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<tr>
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<td>371.2 ± 6.5</td>
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<td>517 ± 18.5</td>
<td>89 ± 1.6</td>
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<tr>
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<td>303.6 ± 9.6</td>
<td>409.3 ± 17.6</td>
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Ceramide and Sphingolipid Metabolites Quantification. To measure sphingolipid metabolites, whole retinas were homogenized in 10mM Tris pH 7.2 and after lipid extraction these metabolites were detected by mass spectrometry and normalized to total protein by methods described by the authors [358, 359]. Briefly, homogenized retinas were mixed with methanol:chloroform (2:1) and 0.5 nmol of the following internal
standards were mixed with each sample: ceramide (d18:1/12:0-Cer), sphingomyelin (d18:1/12:0-SM), glucosylceramide (d18:1/12:0-GlcCer), C17-sphingosine, C17-sphinganine, C17-sphingosine 1-phosphate, and C17-sphinganine 1-phosphate. Lipids were then allowed to extract overnight at 48°C. Samples were then subjected to alkaline hydrolysis to remove most of the interfering glycerolipids. Samples were divided in half. Half of the sample was subjected to reverse-phase liquid chromatography and the other half to normal-phase liquid chromatography and then subjected to tandem mass spectrometry.

_Fatty Acid Derivatization and Gas–Liquid Chromatography._ Retinas were homogenized and total lipids were extracted for fatty acid analysis as described previously [305]. To summarize, total lipid extracts were converted to methyl esters by addition of 2% H2SO4 in methanol and heated at 100°C for 65 minutes after a mixture of pentadecanoic acid (15:0), heptadecanoic acid (17:0), and heneicosanoic acid (21:0) were added as internal standards. Tubes were cooled on ice, and fatty acid methyl esters were extracted three times with hexane, dried under N2, and dissolved in nonane. The fatty acid compositions were determined by injecting 3 μL of each sample at 250°C with the split ratio set to 20:1 using a DB-225 capillary column (30 m x 0.53 mm inner diameter; J&W Scientific, Folsom, CA) in a gas-liquid chromatograph (model 6890N; Agilent Technologies, Wilmington, DE) and an autosampler (model 7683; Agilent Technologies). The column temperature was programmed to hold at 160°C for 1 minute, then increased to 220°C at 1°C/min, and held at 220°C for 10 minutes. Helium carrier gas flowed at 4.2 mL/min. The hydrogen flame ionization detector temperature was set to 270°C. The chromatographic peaks were integrated and processed on computer (ChemStation
software; Agilent Technologies). Fatty acid methyl esters were identified by comparison of their relative retention times with authentic standards, and the relative mole percentages were calculated.

**Western Blot Analysis.** Western blot analyses were performed essentially as described previously. Briefly, treated R28 cells were washed in ice-cold Dulbecco's phosphate-buffered saline solution and lysis buffer [50 mM Hepes, 137 mM NaCl, 5 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM NaVO₄, 1% NP-40, Protease Inhibitor Cocktail (Roche)] was added. Cell lysates were cleared by centrifugation, and the Bio-Rad DC protein assay was utilized to determine protein concentration. Typically, 30 µg of protein lysate per sample was separated on a 4-12% NuPAGE gels (Invitrogen, Carlsbad, CA) and transferred to Hybond nitrocellulose membranes (Amersham). The membranes were blocked in 5% nonfat milk in tris-buffered saline with 0.1% tween-20 (TBST) for 1 h and then incubated with the primary antibodies (1:1000 dilution in 5% nonfat milk TBST or 5% BSA in TBST) for 2 h at room temperature or overnight at 4°C. After incubation, the membranes were washed three times with TBST for 10 min each. The blots were then incubated with secondary horseradish peroxidase-conjugated antibodies at a 1:5000 dilution in 5% nonfat milk in TBST for 2 h at room temperature. The membranes were then washed three times with TBST. The bands were visualized by ECL and quantified using ImageQuant (Molecular Dynamics, Sunnyvale, CA), or GeneTools SynGene (K&R Technology, Frederick, MD) software.

**Immunohistochemistry and Confocal Microscopy.** Retina sections (10µm) on glass slides were fixed in 2% paraformaldehyde for 10 min at room temperature and subsequently rinsed in PBS (2 x 10 min). Slides were blocked in 10% donkey serum
(Jackson ImmunoResearch, West Grove, PA) for 1 hour prior to incubation with anti-GCS antibody (1:100) overnight at 4°C. Slides were subsequently washed with PBS containing 0.1% triton-x (3 x 20 min). The slides were then incubated with the secondary antibody (Rhodamine Red-X-conjugated donkey anti-rabbit (1:1000; Jackson ImmunoResearch) and nuclear stain Hoechst (0.5µg/ml) for 1 hr. Slides were then washed in PBST (3 x 20 min) and mounted with aqueous medium (Aqua/Polymount; Polysciences). All images were obtained with a confocal microscope (TCS SP2 AOBS; Leica, Deerfield, IL), at 512 x 512-pixel resolution. Images were maximum projections of z-stacks.

Cell viability/death assays. R28 cells were cultured on glass coverslips and treated as described in the text. After treatment, cells were washed in PBS and fixed in 2% paraformaldehyde for 10 minutes before staining/mounting with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Five randomly sampled fields from each coverslip were observed to determine the percent of pyknotic nuclei as described previously [309]. In other cases, total cell counts were used to determine viability. A Cytotoxicity Detection Kit (LDH) (Roche) was also utilized to determine cellular viability. R28 cells were seeded at a concentration of 5 x 10³ cells/well in a 96 well microtiter plate. After 24 hours, R28 cells were treated with various glycosphingolipids as described in the text and LDH was measured following the manufacturer’s protocol.

Statistical methods. One-way analysis of variance (ANOVA) with Bonferroni multiple comparison post test and t-tests analysis were performed using GraphPad Prism 4.0 software, with statistical significance considered if p < 0.05.
4.3 Results

Diabetes decreases total ceramide content in retinas of streptozotocin-treated diabetic rats. It has been documented that pro-inflammatory cytokines, such as IL-1β and TNFα are increased in diabetic rat retinas [35-37]. These cytokines have been demonstrated to increase ceramide content through the activation of the de novo or salvage pathways, so we hypothesized that ceramide content would be increased in these retinas. Furthermore, ceramide content is increased in the insulin resistant skeletal muscle of rats [191, 192] and humans with diabetes [193, 194]. Ceramide content was measured after 2, 4 and 8 weeks of stz-induced diabetes by ESI/MS/MS. The total of all ceramide molecular species is shown in Fig. 4.1A. After 2 weeks of stz-induced diabetes, ceramide levels remained constant (p = 0.84), however surprisingly after 4 and 8 weeks of diabetes there was a ~30% decrease in total ceramide content (p = 0.006 and p = 0.01 respectively). We have shown previously that acute insulin therapy in diabetic rats can restore or partially restore impaired signaling in this model [50, 306], so we determined if 72 hours of insulin therapy would restore ceramide levels to control levels. We again observed that after 4 weeks of diabetes ceramide mass was decreased ~30% (p < 0.05), but insulin, did not restore the level of ceramides to basal levels (p > 0.05) (Fig. 4.1B). These data suggest that retinal ceramide metabolism is diminished by diabetes.
Diabetes does not alter the fatty acid composition of the retina. In light of a 30% reduction in total ceramide content, we next analyzed the different molecular species of ceramides (Fig. 4.2A). In the normal retina, 79% of the ceramides contained long chain C16:0 or C18:0 fatty acids, whereas only 21% were of the very-long chain species (C20:0 or longer) and this ratio was unaltered after 4 weeks of diabetes. Since the ceramide pool was enriched in palmitic and stearic acid, we next analyzed the total lipid fatty acid composition in the retina (Fig. 4.2B). Similar to ceramide, the total lipid pool contained a larger percentage of palmitic and stearic acid, which equaled to 70%. Additionally, no significant differences were observed between control and diabetic animals with any particular fatty acid chain. These results are in contrast to the liver (Fig. 2C) and plasma (data not shown) of these animals, where palmitic and stearic acid comprised 30-35% of the total lipid composition. Furthermore, in the liver we observed selective reductions of C16:1 and C18:1 lipids and increases in C18:0 and C18:2n6 after 4 weeks of diabetes.
Therefore, the retina is resistant to the fatty acid alterations compared to the liver in diabetes.

Diabetes increases total cerebroside content in the retinas of streptozotocin-treated diabetic rats. A decrease in ceramide mass suggests an alteration in sphingolipid metabolism, so additional sphingolipids were also measured. After 4 weeks of stz-

![Retina - Ceramide Fatty Acid Composition](image1)

![Retina Fatty Acid Composition](image2)

![Liver Fatty Acid Composition](image3)

Fig. 4.2: Retinal fatty acid composition is unaltered in diabetes. A. Ceramide fatty acid species after 4wks of diabetes was assessed by ESI/MS/MS. Furthermore, fatty acid species of total lipid extracts were assessed by gas chromatography (see Materials and Methods) from 4-wk control and diabetic retinas (B) and livers (C). (*p<0.05)
induced diabetes, sphingomyelin, sphinganine and sphingosine mass as measured by mass spectrometry revealed no significant changes compared to age-matched control retinas (Fig. 4.3A). Phosphorylated sphingoid metabolites were present in small amounts, so while no overt alterations were observed, comparisons between control and rats with diabetes are not conclusive (data not shown). Increased glycosphingolipid metabolism has been implicated in renal complications of diabetes [252], so cerebroside content was also assessed. We observed a significant increase of approximately 30% in cerebroside levels in fed diabetic rats compared to their age-matched controls (Fig. 4.3B). After fasting rats for 16 hrs, which diminished the blood glucose concentration of the diabetic animals by 55.6% (see Table 1), the levels of cerebrosides in the retinas of fasted-diabetic animals were at the same levels as their fed-diabetic counterparts, still 30% above basal. Thus, a short-term decrease in glucose concentration did not diminish the higher levels of cerebrosides. We also assessed the fatty acid composition of the cerebrosides (Fig. 4.3C). Similar to ceramide, the majority of cerebrosides contain saturated palmitic and stearic acid fatty acids. These C16:0 and C18:0 molecular species of cerebrosides are significantly increased in diabetes (p < 0.01) and comprise between 63 and 70% of the total cerebroside species. We conclude that the metabolism of ceramide in diabetic rat retinas generate glycosphingolipids, but not other sphingoid derivatives.
Fig. 4.3: Retinal sphingolipid metabolites were characterized by ESI/MS/MS. A. The levels of sphinganine, sphingosine, sphingomyelin was analyzed from 4wk control and diabetic retinas. B. Cerebrosides were also assessed from 4wk control and diabetic animals under fed and fasted conditions. C. The fatty acid composition of the cerebrosides from Fig. 4.3B. (*p<0.05)
Glucosylceramide synthase and the retina. The first committed step to the formation of most glycosphingolipids is catalyzed by glucosylceramide synthase, which forms glucosylceramide through the addition of glucose from UDP-glucose onto ceramide [355]. By immunohistochemistry, we demonstrated that the retina expressed glucosylceramide synthase, with strongest immunoreactivity observed within the neuronal plexiform layers and the outer segments of the photoreceptors (Fig. 4.4). This result demonstrates that the intact rat retina has the capability of metabolizing ceramide to glucosylceramide metabolites.

Inhibition of glycosphingolipid metabolism increases insulin sensitivity in retinal neurons. To investigate potential causality of this increase in cerebrosides, an in vitro model of retinal neurons were utilized to determine the putative effects of altering glycosphingolipid metabolism on insulin signaling. R28 cells were pretreated with N-butyldeoxygalactonojirimycin (NB-DGJ), a selective imino sugar inhibitor of glucosylceramide synthase [360, 361] or DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), for 24 hours. Then R28 cells were treated with high
glucose conditions or glucosamine for an additional 24 hours still in the presence of these inhibitors, with the last two hours under serum-free conditions. R28 cells were subsequently treated with insulin and the phosphorylation of p70 S6K, an insulin-responsive, prosurvival enzyme whose phosphorylation and activity are decreased in the diabetic retina [50], was analyzed by western blotting (Fig. 4.5). Serum-starved R28 cells demonstrate negligible phosphorylation of p70 S6K inase under serum-free conditions. In the absence of insulin, these glucosylceramide synthase inhibitors caused a modest increase in basal phosphorylation of p70 S6K (note that the left portion is more exposed than the insulin-stimulated portion of Fig. 4.5). When R28 cells are stimulated with insulin (10nM for 15 minutes), there is a marked increase in Thr 389 phosphorylation of this enzyme. Pretreatment with NB-DGJ or PPMP induced a significant increase in insulin-stimulated phosphorylation of p70 S6K. Also of importance, high glucose and glucosamine suppressed insulin-stimulated p70 S6K. In the presence of NB-DGJ or PPMP, insulin further stimulated p70 S6K phosphorylation in the presence of inhibiting concentrations of high glucose or glucosamine. Thus, inhibition of glycosphingolipid metabolism increases insulin sensitivity in retinal neurons.

**Fig. 4.5:** Inhibition of glycosphingolipid metabolism increases insulin sensitivity in retinal neurons. R28 cells, a model of retinal neurons, were pretreated with glucosylceramide synthase inhibitors, NB-DGJ (100mM) or PPMP (1mM) for 24 hours before treatments with high glucose (HG, 30µM) or glucosamine (Gln, 5µM) for an additional 24 hours. Cells were then serum-starved for 2 hours under the same conditions and treated with vehicle or insulin (10nM) for 15 minutes. Western blots were then performed and the phosphorylation state of p70 S6K, a kinase whose activity is diminished in the diabetic retina, was assessed. (n=3)
Glycosphingolipids activate the ER stress response in retinal neurons. Diabetes induced stress and insulin resistance have been associated with induction of the endoplasmic reticulum (ER) stress response [287, 289-291]. Stress-induced abnormal protein processing and folding, known as the untranslated protein response (UPR) is associated with neurodegeneration [362, 363]. We investigated if glycosphingolipid metabolites could induce ER stress in cultured R28 cells. Cerebrosides, GD3 and GT1b, but not lactosylceramide or GM3 induced the expression of glucose-related protein 78 (GRP78), a chaperone protein that is a marker of ER stress (Fig. 4.6). Moreover, we utilized examples of GD(di) and GT(tri)- glycosphingolipid classes which we and others have demonstrated to be produced preferentially in the rat retina [364, 365](unpublished observations) as compared to GM(mono)-glycosphingolipid classes. These data suggest that glycoconjugated ceramide metabolites may contribute to ER stress responses in retinal neurons.

![Western blotting](image)

**Fig. 4.6: Exogenous glycosphingolipids induce expression of GRP78.** R28 cells were treated with the indicated glycosphingolipids for 24 hours under serum-free conditions at a dose of 10µM (GluCer, GalCer, LacCer) or 50µM (GM3, GD3, GT1b). Cellular lysate was subjected to western blotting and analyzed for the expression of GRP78 or β-actin.

Glycosphingolipids regulate inflammatory cytokine- and glucosamine--induced cell death in retinal neurons. We initially assessed the effects of exogenous
glycosphingolipids on R28 cells by measuring LDH release (Fig. 4.7A). The relatively high basal activity, a consequence of serum-starvation, was significantly elevated by exogenous C₆-ceramide or GT1b (p < 0.05), but not other exogenous lipids.

Treatment of retinal pericytes with glucosamine resulted in increased glycosphingolipid formation, which suppressed pericyte proliferation [273]. We therefore set out to determine if glucosamine-induced R28 retinal neuronal cell death [366] can be inhibited by suppressing glycosphingolipid metabolism. Treatment of R28 cells with glucosamine for 24 hours under serum-free conditions we observed 25% pyknosis of the remaining adhered cells, which was significantly higher than basal cell death (p < 0.001) (Fig. 4.7B). This apoptosis was suppressed to 14% for cells treated with either NB-DGJ (p < 0.001) or PPMP (p < 0.001).

Similarly, the inflammatory cytokine TNFα, has been demonstrated to mediate its apoptotic effects through altered glycosphingolipid metabolism [279, 356]. R28 cells are unresponsive to TNFα (unpublished observations), so we determined if inhibition of glycosphingolipid flux could suppress IL-1β induced-death in R28 cells. We observed that IL-1β significantly reduced cellular viability to 30% of untreated cells (p < 0.001). Importantly, both NB-DGJ and PPMP increased R28 cell viability in the presence of IL-1β (p < 0.001 and p < 0.05 respectively). Therefore, we conclude that inhibition of glycosphingolipid flux inhibits the apoptotic effects of factors (hexosamine pathway and inflammatory cytokines) thought to be involved in the pathogenesis of diabetic complications.
4.4 Discussion

In this study, we demonstrate for the first time that increased glycosphingolipid flux through cerebrosides may contribute to cell death in diabetic retinopathy. In the diabetic rat retina, cellular ceramide levels are decreased (Fig 4.1) with a corresponding increase in monohexosylceramides (cerebrosides) (Fig 4.3). This increase in glycosphingolipids may mediate ER stress responses (Fig 4.6) and/or insulin resistance.
(Fig. 4.5), which may contribute to the pathogenesis of diabetic retinopathy. Furthermore, decreases in glycosphingolipid metabolites reduce glucosamine- and IL-1β-induced cellular death (Fig 4.7).

Another interesting finding is the high proportion of the saturated palmitic acid and stearic acid containing lipids in the retina compared to the liver (Fig.4.2). Photoreceptor outer segments are enriched in the polyunsaturated docohexanoic acid containing phospholipids [367], however this new evidence suggests that the entire retina is enriched in saturated phospholipids. While it is unclear as to what this means, such saturated lipids would increase the rigidity (decreased fluidity) of membranes. This is further supported by evidence that the inner retina contains more cholesterol than photoreceptors [365]. The coordinate regulation of signaling cascades within areas of membrane rigidity (lipid microdomains/rafts), including the insulin receptor [333], as well as the localization of rafts within synapses [368] suggest that this may be important for maintaining a homeostatic environment that is unique from other tissues.

Many factors including inflammatory cytokines, increased metabolism through the hexosamine pathway and diminished insulin signaling have been implicated in contributing to neuronal apoptosis in the diabetic retina. Our data supports observations by others demonstrating that cerebrosides and their higher order glycosphingolipid metabolites can contribute to these characteristics of diabetic retinopathy. Though we have yet to fully describe the exact mechanisms by which increases in glycosphingolipids contribute to diabetic retinopathy, the pathogenesis of diabetic retinopathy mirrors many aspects of neuropathic glycosphingolipid disorders. In GM1 and GM2 (Tay-Sachs and Sandhoff disease) gangliosidosis there is a progressive neurodegenerative course in the
brain [369], which is characteristic of what we have observed in diabetic rat retinas with increased levels of apoptosis compared to age-matched controls and subsequent decreases in retinal thickness [3]. In humans and mouse models of GM1 and GM2 gangliosidosis there is an increase in microglia activation and macrophage infiltration in the brains of these animals and the extent of inflammation correlated with disease progression [369]. Similarly, in patients with diabetes and mouse and rat models of diabetes there is also increased inflammation characterized by activation of microglia and subsequent neurophagy and increased leukocyte attachment in the retina [5, 11, 14, 37, 370]. Furthermore, accumulation of glycosphingolipids perturbs neuronal calcium homeostasis in Gaucher, Sandhoff and Niemann-Pick A diseases resulting in elevated cytosolic calcium levels [371, 372]. Such increases in calcium may enhance sensitivity of the neurons to stress. For instance, upon depletion of ER Ca2+ stores, cells can enter a form of ER stress, the ‘unfolded protein response’ (UPR), which causes suppression of global protein synthesis, activation of stress gene expression, and induction of apoptosis which have all been implicated or suggested to be involved in diabetic complications including diabetic retinopathy [287, 289, 291]. We have now demonstrated that exogenous cerebrosides induce ER stress in cultured retinal neurons. Though glycosphingolipids are unlikely to be the sole contributor to these pathological conditions in diabetic retinopathy, the similarities to glycosphingolipids disorders are intriguing.

While GCS/GalT produced monohexosylceramide is the first committed step to the formation of glycosphingolipids, further studies are under investigation to determine if specific types of gangliosides, globosides and/or sulfatides are altered and their functional consequence. Of particular interest is the monosialosyl-lactosyl-ceramide
GM3, which has been implicated in insulin resistance in response to TNFα [251]. Mice deficient in GM3 synthase, and thus GM3 and its metabolites, exhibit greater insulin sensitivity [280]. Also of interest are GD3, which has been implicated in TNFα and CD95 induced apoptosis, and GT1b which has been demonstrated to inhibit Akt activation [285]. Our findings suggest that the sequential glycosphingolipid metabolites GT1b and GD3 augment expression of GRP78. However, precursors of these metabolites (LacCer, GM3) did not exhibit this ability. This might suggest that only di-, tri-glycosphingolipid species exert ER responses in retinal neuronal cell culture.

As the retina is largely neuronal and glial, we have emphasized the possible mechanisms by which cerebrosides may affect the neurons. We are cognizant of the fact that the effects of this increase may be manifested through other cell types and even other mechanisms within the neurons themselves in the retina. One characteristic of DR is pericyte dropout. El Bawab’s laboratory has recently demonstrated that glucosamine-induced pericyte growth inhibition is mediated through increased ganglioside production [273]. Glycosphingolipids have been demonstrated to activate microglial cell lines to produce the inflammatory mediators iNOS, TNFα, and IL-1β through protein kinase C and NADPH oxidase [276] and PKA [277]. Lastly, glycosphingolipids can stimulate VEGF production [272], which in turn could possibly modulate vascular permeability and/or angiogenesis. Interestingly, VEGF can be produced under ER stress conditions [297].

The mechanism of increased glycosphingolipid metabolism is unclear, but may be a consequence of hyperglycemia. Excess glucose could lead to increased metabolism through the pentose pathway to form UDP-glucose, a necessary substrate for
glycosphingolipid formation. In fact, stz-induced diabetic rats demonstrate elevated UDP-glucose levels in liver [252]. Excess glucose may also lead to accumulation of hexosamine pathway products, which serve as substrates for more complex glycosphingolipids. The hexosamine pathway can also serve as precursors for the formation of sialic acid, a component of gangliosides. Regardless, ample evidence validates a significant role of glycosphingolipids in diabetes. Autoantibodies to sulphatides [259], GT3 [260], GD3 [261] and GM2-1 [262, 263] have been identified in the sera of patients with type I diabetes. Circulating antibodies to GD3 have been observed in 52% of patients with diabetes [261]. Since GD3 is the most abundant ganglioside in the retina, defining the putative relationship between serum anti-GD3 antibodies and progression of retinopathy deserves further investigation. It can be envisioned that elevated anti-GD3 antibodies may be a biomarker for diabetic retinopathy. Another mechanism potentially leading to increases in glycosphingolipids may be modification of sphingolipid metabolizing enzymes by advanced-glycation end products, which are elevated in renal hypertrophy/diabetic nephropathy [252, 253] and the diabetic liver [257, 258]. Taken together, these studies suggest that the targeting of glycosphingolipids may be therapeutic for diabetic complications including retinopathy.
Chapter 5

Conclusions, Clinical Potential and Future Directions

The major conclusions presented in this document demonstrate that:

1. Insulin signaling is impaired in diabetic retinas and cultured retinal neurons.

2. PKC activation is not the prevailing mechanism regulating impaired insulin signaling.
   a. No major alterations in PKC isotype expression or activation between control and diabetic retinas were observed.
   b. The pathological role of increased diglyceride concentration in diabetes may be independent of PKC.

3. Altered lipid flux may be a contributor to metabolic stress and resultant retinal neuronal pathology observed in diabetes.
   a. Sphingolipid metabolism is shunted in the diabetic retina to the formation of glycosphingolipids at the expense of ceramide.
   b. Increased glycosphingolipids may contribute to diabetic retinopathy through the inhibition of:
      i. insulin signaling
      ii. regulation of stress responses and/or
      iii. mediating inflammatory cytokine or hexosamine pathway induced cellular death.
4. Modifying lipid flux may be a therapeutic modality to diminish diabetic pathology.
   
a. Inhibiting glucosylceramide synthase may be effective in inhibiting glycosphingolipid-mediated retinal cell death.

b. Augmenting diglyceride flux to generate phosphatidic acid may be beneficial for retinal neuronal survival.
   
i. Phosphatidic acid and insulin can synergistically activate mTOR signaling a mediator of retina cell survival.

   ii. Phosphatidic acid can augment insulin signaling via site-specific phosphorylation of tuberin.

   iii. Phosphatidic acid and insulin synergistically limits inflammatory cytokine-induced retinal neuronal cell death.

5. The implications and clinical significance of these findings and conclusions are discussed below.

The identification of protein kinase C as a putative therapeutic target for diabetic complications has led to the development of PKC inhibitors for treatment. A large body of work suggests that PKC inhibitors have beneficial outcomes in rodent models of diabetes and human clinical trials of ruboxistaurin demonstrate some beneficial effects on visual acuity and macular edema [117-120]. Still, as DAGs have been demonstrated to be increased in the diabetic retina [109], this is a suitable target of further investigations. The elucidation of the types of DAG generated in terms of saturated versus polyunsaturated and ether-linked versus ester-linked diacylglycerides, which all have different
physiological outcomes, will allow us to speculate further on the role of PKCs. Inhibition of DAG formation in diabetes may thus be a more beneficial target to address, possibly through the inhibition of phosphatidate phosphohydrolase.

Alternatively, shunting increased DAG synthesis to its phosphorylated form, phosphatidic acid, possibly through the activation of DAGK may also be a favorable therapy. As already mentioned, several studies have demonstrated that DAG kinase is important for cellular viability [340] and that activation of DAGK may be beneficial to some of the vascular alterations in diabetic retinopathy [112, 339]. We demonstrate now that PA promoted neuronal cell survival in response to the proinflammatory IL-1β. Furthermore, PA synergized with insulin to further suppress cytokine-induced apoptosis. We have demonstrated that these effects occur partially through the stimulation of mTOR signaling, possibly at the level of the tuberous sclerosis complex in a cholesterol dependent manner. Additional work needs to be done in vivo to understand any alterations of DAG-PA-LPA as well as phosphatidylcholine and phosphoinositol flux. These studies would help validate shunting such altered lipid metabolites as a potential therapeutic.

Alternatively, we have shown that modulation of sphingolipid metabolism may also be beneficial for diabetic complications. The novel finding that ceramide metabolism is shunted into glycosphingolipid metabolism in the retina offers the possibility that several FDA approved drugs and additional drugs approved for use in Europe may have application for diabetes. Such drugs, Cerezyme (recombinant glucosylceramidase) and Zavesca (N-butyldeoxynojirimycin), currently in use for Gaucher’s disease, diminish glycosphingolipid metabolism and potentially restore the critical balance of the various
sphingolipids. *In vitro*, inhibition of glycosphingolipid metabolism increased insulin sensitivity and diminished glucosamine and IL-1β-induced death. Work by Futerman’s laboratory demonstrated the ability of accumulated glycosphingolipids to increase sensitivity of neurons to apoptosis in response to glutamate [294]. Furthermore, Futerman’s group has also demonstrated that inhibition of glycosphingolipid metabolism suppressed thapsigargin-induced apoptosis, suggesting a role of glycosphingolipids in calcium and ER stress-induced death [373]. Reports of altered glycosphingolipid metabolism in multiple diabetic tissues, and autoantibodies to glycosphingolipids in diabetes (see Chapter 1), suggest that systemic and/or local delivery of drugs, genes or siRNA to inhibit glycosphingolipid flux may be a viable therapeutic approach. At least in the kidneys, inhibition of glycosphingolipid flux has already proven beneficial in an animal model of diabetes [252].

Strategies to modify altered lipid metabolism in the retina will depend upon optimal drug delivery and targeting to test efficacy. One strategy may be to utilize nanoliposomal vehicles to deliver therapeutic agents at high dosages. Such approaches allow for delivery of hydrophobic drugs, DNA, siRNA as well as cell-specific targeting. Pegylated formulations also increase the bioavailability of drugs [374]. We have demonstrated the ability of such peglyated liposomes to diminish corneal inflammation (data not shown), so similar strategies could be utilized to diminish retinal inflammation in diabetes. In fact, several publications have demonstrated the ability of liposomes to target the retina [375-379]. Other strategies could further explore the feasibility of polymer-drug conjugates, antibody/affibody-conjugated therapeutics, nano-colloids, fusion proteins, and implantable infusion devices to more effectively deliver such
therapeutics. In the case of inhibitors of glycosphingolipid metabolism, Cerezyme does not cross the blood-brain barrier, thus ocular delivery strategies are needed. Zavesca, however does cross the blood-brain barrier, but drug delivery strategies could putatively improve pharmacokinetics and efficacy of the drug within the retina.

Alternatively, work by Jean Bennett and William Hauswirth and others have demonstrated the feasibility of gene therapy by viral transfer in the retina to treat retinal diseases [380-382]. We too have demonstrated the capability of overexpressing green fluorescent protein (GFP) in the retina (Fig. 5.1). Consequently, overexpression of glucosylceramidase would be another suitable approach to diminish glycosphingolipid flux. A similar strategy could be utilized to diminish enhanced diacylglycerol flux.

Taken together, this body of work supports the contention that altered lipid flux (sphingolipid, phospholipid) may contribute to the pathogenesis of diabetic retinopathy. Thus, strategies to alter or diminish lipid flux and resultant bioactive lipid metabolites are a viable therapeutic approach for diabetic retinopathy. In fact, therapeutic strategies to manipulate lipid metabolites are of prime relevance in multiple disease models. Reliable and consistent methods to measure clinically relevant endpoints are a necessity to validate these therapeutic approaches that regulate lipid metabolism.
Fig. 5.1: Sprague-Dawley rat eyes were injected intravitreally with AAV containing the GFP gene encoded by the β-actin promoter generously provided by Dr. William Hauswirth (University of Florida). After 6 weeks, retinas were removed, fixed in 2% paraformaldehyde and flat mounted. Fluorescence was assessed by confocal microscopy. Eyes that were not injected with AAV did not demonstrate fluorescence (not shown).
Bibliography


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Fox, T.E., Gardner, T.W., Kester, M. Phosphatidic acid and Insulin activate mTOR signaling in a cholesterol sensitive mechanism to synergistically limit inflammatory cytokine-induced retinal neuronal cell death. Submitted.


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