CONSTITUTIVE INSULIN RECEPTOR SIGNALING IN RETINA AND
CHANGES INDUCED BY DIABETES

A Thesis in
Cellular and Molecular Physiology
by
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ABSTRACT

Diabetes is a growing epidemic in Western society which afflicts over 17 million Americans and over 151 million people worldwide. People with diabetes are at a greater risk of developing vascular complications such as atherosclerosis, stroke, and heart failure, as well as complications in specific tissues such as nephropathy and kidney failure, neuropathy and amputation of extremities, and retinopathy and blindness.

Diabetic retinopathy (DR) is the leading cause of blindness in working age adults, and it is diagnosed by visual inspection of vascular alterations in the retina. Over time, the retinal blood vessels become leaky and undergo neovascularization. This leaking leads to macular edema, hemorrhaging, and build-up of opaque exudates, and the neovascularization causes severe loss of vision due to vitreous hemorrhages and/or retinal detachment. Currently, the only treatment for DR is laser ablation of the growing and leaky vessels. The procedure temporarily slows the abnormal blood vessel growth, but this “amputation” procedure also damages the neural retina, and patients often require numerous treatments. In addition to vascular abnormalities in DR, the neural retina also is affected by diabetes, and apoptosis is increased in the neural retina of diabetic rats and humans. Clearly, alternative treatments are necessary to slow and prevent DR to preserve vision.

In a general sense, diabetes is a disease of improper insulin action leading to hyperglycemia. Insulin causes many cell-signaling events to occur through the
insulin receptor (IR) to promote glucose uptake, but insulin also generates a cellular survival signal which is mediated by the protein kinase Akt. While hyperglycemia has been assumed to be the major factor that promotes diabetic complications, and despite the current knowledge of insulin signal transduction on tissues that directly mediate nutrient storage, relatively little is known about insulin physiology and the effects of diabetes in complication-prone tissues, such as the retina. Therefore, the focus of this project is to elucidate proximal IR signaling and its regulation of Akt in retina tissue, and to test the hypothesis that the retina, similar to other classical insulin target tissues, has insulin signaling defects with diabetes. This work provides a better understanding of mechanisms which may contribute to apoptosis in the diseased retina.

The first specific aim of this project was to determine the early IR signaling events, and to test the hypothesis that retinal insulin receptors are active \emph{in vivo}. When insulin was injected systemically into fasting rats, muscle insulin receptor $\beta$ subunit (IR$\beta$) autophosphorylation on tyrosine residues significantly increased within five minutes, but retinal IR$\beta$ autophosphorylation did not increase until 30 minutes after the injection. Furthermore, the insulin injection increased Akt phosphorylation on serine 473 in retina. Interestingly, it was determined that the basal IR and Akt-1 kinase activities were elevated in retina compared to liver and muscle, and retinal IR kinase activity, in contrast to IRs in liver, did not decrease in fasted rats. Using \emph{ex vivo} retina preparations, 10 nM insulin increased IR$\beta$ autophosphorylation and phosphotyrosine (PY) content in insulin receptor substrate (IRS) 2, but not IRS-1.
Akt serine 473 phosphorylation was increased in the nuclear layers of the retina \textit{ex vivo} with insulin treatment. Of the three Akt isoforms, only Akt-1 kinase activity was increased with insulin in this model. Together, these results suggest that the retina is an insulin-sensitive tissue, the steady-state kinase activity of the IR and Akt are constitutively elevated compared to other insulin-responsive tissues, and exogenous insulin further stimulates serine 473 phosphorylation and kinase activity of the pro-survival kinase Akt.

To test the hypothesis that the constitutive retinal insulin signal is altered in diabetes, the streptozotocin (STZ)-induced rat model was used. The basal level of IRβ and IRS-2 PY content and Akt serine 473 phosphorylation in retina remained constant after 4 weeks of STZ diabetes, but IR and Akt-1 kinase activities were significantly reduced. A similar reduction in IR kinase activity was also found in retinas of diabetic Ins2\textsuperscript{Akita} mice. Likewise, phosphatidylinositol-3 kinase (PI3K, a key enzyme linking signal transduction from IRS to Akt) activity was also reduced. Additionally, serine 9 phosphorylation on GSK3β, a downstream substrate for Akt, was also significantly reduced in retinas of diabetic rats. When whole retinas from STZ rats were cultured in normoglycemic media and treated with 10 nM insulin \textit{ex vivo}, IRβ autophosphorylation was significantly greater than control retinas, similar to other insulin responsive tissues in the STZ rat when challenged with an insulin bolus. These data suggest that the constitutive protein kinase activity of insulin signaling proteins in retina is reduced by insulin deficient diabetes. Furthermore, the
mechanism by which this occurs may be different from known protein phosphorylation events.

The third specific aim of this project is to examine the mechanism of the constitutive autophosphorylation of the retinal IR. The first approach utilized intravenous somatostatin (SST) infusion to lower basal circulating insulin in normal fasted rats. This significantly reduced liver IRβ autophosphorylation, but had no effect on retinal IRβ autophosphorylation. The second approach utilized vitreal injection of neutralizing antibodies against IR ligands: αinsulin, αinsulin-like growth factor I (IGF-I), and αIGF-II. Surprisingly, only αIGF-II antibody reduced the constitutive IRβ and IGF-1Rβ autophosphorylation. Together, these results suggest that novel mechanisms, such as alternative non-insulin ligands, namely IGF-II, regulate constitutive IR autophosphorylation and downstream pro-survival signaling in retina.

Collectively, the data contained in this thesis suggests the importance of insulin receptor signaling in retina tissue and will potentially lead to novel therapies for treating DR or other neurodegenerative diseases of the retina.
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<th>Full Form</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ARVO</td>
<td>Association for Research in Vision and Ophthalmology</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BGV</td>
<td>blood glucose value</td>
</tr>
<tr>
<td>β-ME</td>
<td>β (2) mercaptoethanol</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BRB</td>
<td>blood retinal barrier</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>centigrade</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>caspase</td>
<td>cysteine-aspartic acid protease</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanine monophosphate</td>
</tr>
<tr>
<td>CHO-IR</td>
<td>Chinese hamster ovary cell, insulin receptor overexpressing</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>cRNA</td>
<td>complimentary ribonucleic acid</td>
</tr>
<tr>
<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interface contrast</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>DMEM</td>
<td>Dubelco’s modified Eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DR</td>
<td>diabetic retinopathy</td>
</tr>
<tr>
<td>dpm</td>
<td>disintegrations per minute</td>
</tr>
<tr>
<td>ECF</td>
<td>enhanced chemifluorescence</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine-N, N’, N”-tetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether) N, N’, N”-tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERG</td>
<td>electroretinogram</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular regulated kinase</td>
</tr>
<tr>
<td>FGF2</td>
<td>fibroblast growth factor 2</td>
</tr>
<tr>
<td>FoxO</td>
<td>Forkhead box-containing protein, O-subfamily</td>
</tr>
<tr>
<td>FRAP</td>
<td>FKBP12 rapamycin associated protein</td>
</tr>
<tr>
<td>G/A</td>
<td>ganglion/astrocyte cell layer</td>
</tr>
<tr>
<td>Gab-1</td>
<td>Grb associated binder 1</td>
</tr>
<tr>
<td>GABA</td>
<td>γ aminobutyric acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFAT</td>
<td>glutamine:fructose-6-phosphate aminotransferase</td>
</tr>
<tr>
<td>GLUT</td>
<td>glucose transporter</td>
</tr>
<tr>
<td>Grb-2</td>
<td>growth factor receptor bound 2</td>
</tr>
</tbody>
</table>
GSK3β  glycogen synthase kinase 3 β
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF  hypoxia inducible factor
hIR  human insulin receptor
HBP  hexoseamine biosynthetic pathway
IDDM  insulin-dependent diabetes mellitus
IGFBP  insulin-like growth factor binding protein
IGF-I  insulin-like growth factor I
IGF-II  insulin-like growth factor II
IGF-IIR/M6P  insulin-like growth factor II receptor/mannose 6-phosphate receptor
IGF-IR  insulin-like growth factor I receptor
IKK  IκB kinase
INL  inner nuclear layer
IP  intraperitoneal
IPB  immunoprecipitation buffer
IPL  inner plexiform layer
IR  insulin receptor
IR-A  insulin receptor A isoform
IR-B  insulin receptor B isoform
IRI  immunoreactive insulin
IRS  insulin receptor substrate
IV  intravenous
MALDI-TOF  matrix assisted laser desorption and ionization-time of flight
MAPK  mitogen activated protein kinase
MEK  MAPK/Erk kinase
MEM  modified Eagle’s medium
mIR  murine insulin receptor
mRNA  messenger ribonucleic acid
mTOR  mammalian target of rapamycin
NGF  nerve growth factor
NFM  non-fat milk
NF-κB  nuclear factor κ B
NP-40  nonylphenyl polyethylene glycol
OCT  optimal cutting temperature
ONL  outer nuclear layer
OPL  outer plexiform layer
OS  outer segments
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate buffered saline
PBST  phosphate buffered saline/0.1% Triton X-100
PCA  perchloric acid
PCR  polymerase chain reaction
PDZ  phloridzin
PH  pleckstrin homology
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3’-OH kinase</td>
</tr>
<tr>
<td>PI3P</td>
<td>phosphatidylinositol 3-phosphate</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PP</td>
<td>pancreatic polypeptide</td>
</tr>
<tr>
<td>PR</td>
<td>photoreceptor</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PY</td>
<td>phosphotyrosine</td>
</tr>
<tr>
<td>RAC</td>
<td>related to A and C kinase</td>
</tr>
<tr>
<td>RAGE</td>
<td>receptor for advanced glycated endproducts</td>
</tr>
<tr>
<td>rd</td>
<td>retinal degeneration</td>
</tr>
<tr>
<td>RPE</td>
<td>retinal pigmented epithelium</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SB</td>
<td>sample buffer</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium chloride sodium citrate buffer</td>
</tr>
<tr>
<td>SST</td>
<td>somatostatin</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
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<tr>
<td>TTR</td>
<td>transthryretin</td>
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</table>
VEGF  vascular endothelial growth factor
ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

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1.1 Introduction to the Insulin Signaling Pathway

Insulin is a potent anabolic hormone that stimulates the uptake and storage of carbohydrates, fatty acids, and amino acids into glycogen, fat, and protein, respectively. The signal transduction pathway (Fig. 1, pg. 41) by which insulin action affects various cells to accomplish their biosynthetic roles has been extensively investigated, and this investigation has increased our understanding of metabolism, physiology, and pathophysiology of disease. Insulin signaling is also the subject of numerous excellent reviews (1-3). Insulin action begins with the synthesis of the mature peptide in the β cells of the pancreas. The β cells are constituents of a pancreatic “organelle” termed the islets of Langerhans which also consist of α, δ, and PP cells. These islets, ~2% of the pancreatic mass, secrete the hormones glucagon, somatostatin, and pancreatic polypeptide, respectively, and are organized into ovoid structures dispersed throughout the pancreas. Thus, the islets are organized to produce the hormones that have opposing actions on blood glucose levels.

The single insulin gene in humans encodes a 110 amino acid peptide, preproinsulin. The “pre-” portion is a 24 amino acid signal sequence that directs synthesis of the peptide into the endoplasmic reticulum (ER), where it is cleaved. This remaining peptide is termed proinsulin. In the ER, proinsulin is folded and three disulfide bonds stabilize the insulin A and B chains of 21 and 30 amino acids, respectively. Proinsulin is then stored in secretory granules within the β cell until the β cell receives signals to release the contents into circulation. While in the secretory
granule, the C (connecting) chain (35 amino acids) that links the A and B chains together is removed to form the mature insulin molecule. Insulin, C peptide, and uncleaved proinsulin are all secreted into circulation via the portal vein in response to a variety of stimuli—most notably increased plasma glucose (4, 5). In the liver, insulin works reciprocally with glucagon to control the balance of glucose and lipid metabolism.

Insulin action at the cellular level begins with the insertion of the insulin receptor (IR) into plasma membranes. The IR is a heterotetramer comprised of two extracellular α subunits and two transcellular β subunits linked by disulfide bonds. The diverse effects of insulin commence when the receptor binds ligand on the α subunits, which induces a conformational change and activates the intrinsic tyrosine kinase activity in the β subunits in the cytoplasm. The IR autophosphorylates its β subunit on tyrosine residues within its tyrosine kinase domain and initiates a cascade of phosphorylation, dephosphorylation, and translocation events within the cell. Immediate protein substrates of the IR kinase include, among others, the insulin receptor substrates (IRS 1-4), Grb-2 associated binder (Gab-1), Shc, and Cbl, which in turn recruit other adapter and enzymatic proteins to propagate the signal and produce a biological effect. Specifically, the IR→IRS→phosphatidylinositol 3’-OH kinase (PI3K)→Akt pathway has received much attention as it is well conserved (Fig. 1, pg. 41), and plays a role in many cellular processes including glucose uptake, glycogen synthesis, protein synthesis, and cellular survival.
The IRS proteins interact with the IR β subunit and are phosphorylated on numerous residues with insulin stimulation (6, 7). Four IRS proteins derived from four independent genes, and a similar protein, Gab-1, have been described. These molecules all contain similar domains and numerous sites for both tyrosine and serine phosphorylation. IRS and Gab proteins possess no catalytic activity before or after phosphorylation, but they serve as docking proteins to concentrate other signaling molecules at the plasma membrane by the binding of their pleckstrin homology (PH) domain to the inner leaflet. At various phosphorylated tyrosine residues, proteins that contain Src homology 2 (SH2) domains bind and are recruited to the plasma membrane. The tissue distribution and function of the IRS proteins are not fully redundant despite their similarities. This is illustrated in genetic ablation studies in which IRS-1−/− mice show significantly reduced growth, while in IRS-2−/− mice, growth is only diminished by 10%, but the IRS-2−/− mice develop overt diabetes leading to death (8-10). It is currently believed that IRS-2 may play a more prominent role in brain function, which may also be mediated by other growth factors that signal through IRS-2 (7).

The regulatory subunit of class IA PI3K contains two SH2 domains, which flanks their p110 interaction domain, and binds to IRS proteins following insulin stimulation. Like IRS proteins, these proteins also act as docking molecules, and five proteins are in this class: p85α, p50α, AS53 (p55α), p85β, and p55γ. These proteins recruit the catalytic subunits of PI3K: p110α, p110β, or p110δ (11, 12). The primary function of p110 proteins is to phosphorylate the 3′-OH position of inositol rings of
the lipids within the plasma membrane to form phosphatidylinositol 3-phosphate (PI3P), and they also contain serine kinase activity. Membrane regions rich in PI3P aid in the recruitment of PH-domain containing proteins.

Further along in the signaling sequence, Akt-1, -2, and -3 (or PKB-α, -β, and -γ, respectively), via their PH domains, are recruited from the cytosol to plasma membranes enriched in 3’ phosphorylated inositol rings where the kinase is maximally activated by phosphorylation on conserved threonine and serine residues. The Akt kinases were also termed RAC kinase for Related to A and C kinase because of structural similarities among the three (13). A consensus amino acid sequence has been identified for efficient phosphorylation of substrates by Akt, but there is significant overlap among other kinases for the same substrate. Also, structural, cellular localization, and tissue-specific variations must be considered in determining whether a protein is a substrate of the Akt isoforms. Akt’s are involved in numerous insulin-induced cellular events. For example, the glucose transporter 4 (GLUT4) is the insulin-responsive transporter in skeletal muscle and adipose tissue; in response to insulin, intracellular vesicles containing GLUT4 translocate and fuse with the plasma membrane. A dominant negative form of Akt, when overexpressed in adipocytes, blocks insulin-stimulated GLUT4 translocation, and overexpression of a dominant active form of Akt is sufficient for GLUT4 exocytosis in the absence of insulin (14, 15). The targets of Akt which promote GLUT4 translocation have yet to be identified. Akt also phosphorylates and inhibits glycogen synthase kinase 3β (GSK-3β), which allows glycogen synthesis and storage to occur in liver and muscle as
glycogen synthase is not phosphorylated by GSK-3β and, therefore, remains active (16). It has been postulated that Akt regulates protein synthesis. Various proteins of the protein synthesis initiation factor complex are phosphorylated by Akt, as well as mTOR/FRAP and p70 S6K. Together, mTOR/FRAP and p70 S6K further regulate the initiation rates of protein synthesis by modulating the activity of the mRNA cap-binding complex and S6 ribosomal protein phosphorylation (17).

Akt has also been shown to exert a strong anti-apoptotic effect on cells. The viral form of Akt, v-akt, has transforming effects on cells, suggesting an overabundance of Akt signaling may override apoptotic signals within cells, contributing to an oncogenic phenotype (18, 19). Numerous targets of Akt signaling have been identified that contribute to the prosurvival effect of insulin. Caspases (cysteine-aspartic acid proteases) are responsible for cleavage of cellular proteins resulting in apoptosis of the cell. Caspases are cleaved and activated by numerous cellular signals and other caspases in a cascading manner (20). The phosphorylation of caspase 9 by Akt inhibits this activation to promote cellular survival (21). The Bcl family member protein, Bad, has also been implicated in regulating apoptosis in an Akt-dependent manner (22). When unphosphorylated, Bad antagonizes pro-survival Bcl proteins such as Bcl-2 and Bcl-XL in mitochondria, but when phosphorylated by Akt, Bad is sequestered and inactivated by chaperone 14-3-3 proteins in the cytosol. Bad phosphorylation is believed to maintain the integrity and functionality of the mitochondria for the production of ATP when Bcl-2 and Bcl-XL are allowed to dimerize. It is also hypothesized that the tumor suppressor, p53, is also a target of
Akt activity (23). Rat hippocampal neurons that were exposed to hypoxic conditions or nitric oxide toxicity to induce apoptosis were rescued by IGF-1 treatment and Akt activation, which was also dependent on p53 expression (23). Clearly other factors, such as HIF (hypoxia inducible factor) proteins, can also mediate a protective effect against hypoxia, and this observation is consistent with another report (24) that Akt translocates to the nucleus where it can regulate nuclear proteins. FoxO family transcription factors are also targeted by Akt in the nucleus (25). Upon phosphorylation by Akt, FoxO transcription factors are transported out of the nucleus where they are sequestered in the cytoplasm by 14-3-3 proteins in an inactive state. FoxO family proteins regulate expression of genes that induce apoptosis such as FasL; conversely, Akt may regulate pro-survival genes by influencing other transcription factors such as NF-κB and CREB (26). This function of Akt may be a key point of distinction between the classically insulin-responsive tissues and retina, or neurons in general, in which Akt plays a greater role in promoting cellular survival (27) and insulin-mediated synapse integrity (28) than in nutrient storage. Further specificity may involve specific Akt isoform expression in the retina, which has not yet been fully elucidated.

In general, the current understanding of insulin action is derived from studies involving the classically “insulin-responsive” tissues or cells and cell lines derived from them. The isoforms of the IR, IRS proteins, PI3K subunits, and Akt vary by tissue and cell type. These expression patterns probably confer locally specific responses. It has been demonstrated that the IR is expressed abundantly in brain,
retina, and other neurons, and retinal IR function probably differs from its functions observed in previously studied systems (29-31). Thus, further study is required to understand the similarities and differences of IR signaling in the central nervous system and retina compared to other insulin-sensitive tissues.
1.2 Properties of Retinal Insulin Receptors

Insulin binding characteristics have been described in the mammalian brain and other regions of the central nervous system (CNS), including the retina (31). However, our understanding of insulin action in the retina and CNS is minimal compared to other insulin responsive tissues in which insulin signaling is perturbed by diseases such as diabetes and polycystic ovarian syndrome (3, 32). Comprehensive studies by Waldbillig and Rodrigues et al answered many questions about basic mammalian retinal IR characteristics, which are summarized within Table 1 [pg. 42, (33-37)]. They demonstrated specific insulin binding in purified bovine rod outer segments, which was specifically competed with excess insulin as opposed to 100-fold greater amounts of proinsulin. Similar to brain and liver, 1 nM insulin stimulated phosphate incorporation into the IRβ subunit (33). Furthermore, the IR from bovine rod outersegments, when stimulated with insulin, possesses tyrosine kinase activity towards an exogenous substrate (34). Despite similarities among IRs in vitro, retinal IRs may function in a cell-specific manner in vivo.

We have recently demonstrated that the retinal IR kinase activity, similar to brain IR activity, is not diminished with fasting (30). In contrast, the tyrosine phosphorylation and kinase activity of the IR from liver and skeletal muscle fluctuate with circulating insulin levels during periods of fasting and feeding. This then influences the physiology of downstream mediators of insulin signaling. Within 90 minutes of re-feeding fasted rats, IRβ and IRS-1 tyrosine phosphorylation and p85
association with IRS-1 are elevated in liver concomitant with a rise in plasma insulin (38). Likewise, PI3K activity associated with phosphotyrosine-containing proteins or Shc is reduced with fasting in chicken liver and restored upon refeeding (39). Our observations in retina are consistent with those on brain IR kinetic activity (40) in which retinal IR tyrosine phosphorylation is unaltered between fasted and fed rats. Likewise, the kinase activity of the IR in retina mirrors brain IR kinase activity and is resistant to changes associated with fasting. Furthermore, in fasted rats, this tonic state of retinal and brain IR kinase activity was greater than liver IR kinase activity (30). Taken together with the above studies by Waldbillig and Rodrigues, the IR in retina behaves similarly to liver IR in vitro, while retinal IR activity is more similar to brain IR activity in vivo where the IR is maintained in a tonic state of activity. This difference may result from how circulating insulin is delivered to tissues of the body, and suggests that the blood-retinal barrier (BRB) minimizes changes of insulin access to the retina.

One major structural difference between IRs expressed in retina/neurons and IRs expressed in liver, skeletal muscle, or adipose is the extent of glycosylation. Both α and β subunits are glycosylated, but in general, the extent of glycosylation of IRs expressed on neuronal cells is less than IRs expressed in liver and skeletal muscle, and can vary among different neurons (41-43). 125I-insulin cross-linking studies by Waldbillig et al (33) demonstrated further similarities between brain and retinal IRα in which the ligand-receptor complex migrates ~10 kDa further on SDS-PAGE than liver IRα. Terminally-linked sialic acid residues comprise most of the IR
glycosylation motifs in adipocytes, as shown by neuramidase sensitivity, since that glycosylation motif is absent in brain IRs (44). Four potential N-linked sites are on the extracellular portion of the IRβ, which also contains O-linked oligosaccharides (45). Like the IRα, the IRβ subunit in retina and brain also migrates slightly faster on SDS-PAGE compared to liver IR (30). Enzymatically catalyzed IR glycosylation is important for normal IR function as demonstrated by site-directed mutagenesis of the four N-glycosylation sites on the IRβ (46). This N-glycosylation-deficient mutant IRβ subunit is able to undergo autophosphorylation in response to insulin in vitro, but the IR loses all kinase activity towards an exogenous substrate. This alteration prevents any effect of insulin in cells that overexpressed the mutant IR on either glucose uptake or thymidine incorporation into DNA. Although glycosylation is required for IR functionality, an explanation for variability of the glycosylated IR among tissues currently remains elusive. However, one might speculate that the decreased glycosylation of neural IRs influences tonic IR activity. Further analysis of IR glycosylation by mass spectroscopy may help to clarify structure-function relationships.

The α and β subunits of the IR are transcribed and translated as a single peptide from one gene on human chromosome 19 before assembly into the mature α₂β₂ form. A second difference that affects IRs in the CNS involves exon 11 expression on the α subunit, which is the only sequence variance in the mature peptide (47, 48). This splice variant is differentially expressed in various tissues. Brain and retina exclusively expresses the IR lacking exon 11 (type A; IR-A), but the
majority of liver IRs express exon 11 (type B; IR-B) (49-51). The tissue-specific
mechanism that regulates exon 11 expression of the IR has not been determined, but,
interestingly, the affinities of IR-A and IR-B differ with respect to insulin and other
growth factors such that growth factors other than insulin (namely as IGF-I and IGF-
II) may influence IR signaling in retina and brain (52).

Consistent with the “housekeeping” nature of the IR promoter, with a GC-rich
promoter and the lack of a TATA box such that the rate of transcription remains fairly
stable (53), the IR has been shown to be expressed constitutively and broadly
throughout the retina on neuronal, glial, endothelial, and retinal pigmented epithelial
(RPE) cells. Using B10 anti-sera, a human anti-IRα sera from a patient with severe
insulin resistance, Rodrigues et al (37) localized the IR to the outer segments and
retinal nuclear layers of bovine, monkey, and human retina. Interestingly, only rod
outer segment disks in monkey, and human Müller glial cells were also
immunoreactive for the IR with this antibody. IRα structural heterogeneity may have
contributed to the variable results with this IRα antibody. Therefore, Naeser (54) and
Gosbell et al (51) extended those observations by measuring IR immunoreactivity in
all retinal layers of human and bovine retina, respectively, using polyclonal
antibodies against intracellular portions of the IRβ that are not differentially
glycosylated. Retinal endothelial cells and pericytes both display specific binding for
insulin. Treatment of these cells in vitro rapidly stimulated thymidine incorporation
into DNA suggesting a strong mitogenic effect (55). Interestingly, it was also
observed that the retinal vascular cells are more sensitive to the growth promoting
effect of insulin than aortic endothelial and smooth muscle cells. The IR expressed in RPE cells has also been investigated, and it was found that RPE cells expresses the IR that is similar to the peripheral form and not the neural form. Compared to liver, the IR in RPE cells also exhibits neuraminidase sensitivity, and overall specific binding of insulin is on the same order as liver IRs (36). Furthermore, the IR in RPE cells localized exclusively to the basolateral surface of the cell, suggesting a possible role in unidirectional insulin transport from the choroidal circulation to the photoreceptors (56).

Lastly, it has been reported that IR/IGF-IR hybrid receptors exist, and their expression appears to be widely distributed, including in neurons (57, 58). That is, an $\alpha\beta$ segment of the IR is crosslinked to an $\alpha\beta$ segment of the homologous IGF-IR, and this heterodimer has the potential to bind either insulin or IGF-I. The function of these receptors is not completely understood, but their existence may be a mechanism to downregulate a signal from one receptor, or to add greater specificity of signaling by either insulin, IGF-I, or IGF-II. Hybrid IR/IGF-IR expression may also change in disease states such as hyperinsulinemia (59). Our laboratory has detected this complex in immunoprecipitates of the IR followed by immunoblot analysis for the IGF-IR, and vice versa, in retina (not shown). Further investigation is required to determine the functions of IR/IGF-IR hybrids in retina and in other tissues alike.

In summary, because the retina, and other regions of the CNS, expresses the IR at levels similar to other classically insulin-responsive tissues, it suggests an important biological function for the IR in neuronal tissues.
1.3 Insulin in the Retina

1.3.1 Insulin and the Blood-Retinal Barrier

The blood-retina-barrier (BRB) exquisitely regulates the exchange of material between the plasma and the neural retina. This partition gives the retina immune privilege as in other regions of the body such as the brain, testes, and anterior chamber of the eye (60). The BRB selectivity functions in stark contrast to the endothelium of other tissues which contain numerous fenestrations, and larger gaps, to allow for the free diffusion of molecules between the interstitial space and endothelial lumen. This is the case for liver, skeletal muscle, or adipose tissue which rapidly metabolizes nutrients as they are absorbed from the gut, and insulin is released into circulation. Therefore, the relatively rapid passage of insulin from the blood to some tissues is necessary, and it is reasonable to hypothesize that insulin must be transported across the BRB for insulin to act on the neural retina. Some comparisons can be drawn from studies on insulin transport across the blood-brain barrier (BBB). In the brain, accumulation of exogenous insulin begins after 60 minutes of insulin infusion and is thought to occur by a saturable, receptor-mediated transport process (61-63). This is in contrast to transendothelial insulin transport in hind limb muscle which may not be saturable or occur by an IR-mediated process (64). After 60 minutes, insulin accumulation in brain proceeds in a linear fashion despite constant levels of insulin in circulation. This results in relatively constant
levels of insulin in the cerebrospinal fluid. The mechanism of transport across the BBB is also thought to be more efficient at physiological levels of insulin, as rates of insulin accumulation in brain decreases with infusion of pharmacological levels of insulin (62).

As stated above, the IR is expressed throughout the retinal endothelial network. The retinal vasculature expresses IRs which resemble those expressed in liver, rather than the less-glycosylated neural form of the IR, as assessed by insulin cross-linking and SDS-PAGE autoradiography (65, 66). Investigations lead by King et al (67) spearheaded our understanding of endothelial cell transport of insulin. Using an aortic endothelial cell monolayer separating two compartments as their model, they demonstrated that insulin is carried unidirectionally across the monolayer. The transport of insulin was mediated specifically by the IR, ~80% of the transported insulin remained intact, and pre-treatment with anti-IR antibodies blocked the transport. This finding strongly suggests that endothelial cells do not degrade significant amounts of insulin, as compared to other growth factors, which may be reflected in a lower expression of insulin degrading enzymes in retina and vascular cells (68). These data suggest that a major function of IRs on endothelial cells is to regulate transport of insulin to a specific tissue. The specific pathway that endothelial cells utilize to transport insulin has not been fully described. Incubating endothelial cells with leupeptin, a lysosomal protease inhibitor, had no effect on insulin transport suggesting those cellular compartments are not involved. Monensin, a proton ionophore which raises endocytic vesicle pH, reduced insulin transport. This result
suggests that endocytic vesicle pH must be regulated for proper IR/insulin
dissociation and insulin transport (69).

Using electron microscopy to detect gold-labeled insulin, transcellular flux of
insulin was also described in cultures of retinal vascular cells (70). Within two
minutes of insulin binding, IRs with bound insulin were clustered into clathrin-
coated pits—the beginning of the well-described receptor-mediated endocytosis
pathway (71). After 10 minutes, the insulin/IR/clathrin-coated pit complex had
invaginated and localized to the interior of vacuole-like compartments, which the
authors identified as endosomes. Following 20 minutes of insulin stimulation, the
labeled insulin was associated with what the investigators termed a multivesicular
body, and the labeled insulin again was localized to the interior of this electron-dense,
highly membranous structure. After 60 minutes, the majority of labeled insulin
resided in secondary lysosomal structures, while as much as ~23% of the labeled
insulin had been transported to the basal plasma membrane or released to the
extracellular space. These experiments in vitro demonstrate an IR-mediated transport
of insulin across retinal endothelial cells, and that circulating insulin has the potential
to be transported intact to the neural retina by retinal endothelial cells.

Similar transport results have been reported in post-mortem bovine eyes that
were reperfused with bovine blood and $^{125}$I-insulin (72). In time course experiments
lasting up to 60 minutes, insulin was localized to the basal surface of endothelial cells
and also associated with pericytes. However, no labeled insulin was observed
associated within the neurons or glia in the retina, which may suggest either more
time is required for insulin transport in bovine eyes, or the vascular, neural, and glial functions were compromised in eyes of exsanguinated animals contributing to reductions in insulin transport rates. By contrast, Shires et al (73) showed that $^{125}$I-insulin injected into the superior vena cava of anesthetized rats accumulated in the vitreous of normal rats within two hours. This accumulation is also thought to be due to a saturable transport process.

The tight junctions that form in retinal endothelial cells promote a greater resistance across the barrier and may restrict retinal insulin permeability compared to other tissues. When cultures of aortic endothelial cells were directly compared to retinal endothelial cell cultures, the electrical resistance across the monolayer was ~4-fold greater in retinal endothelial cells (74). The permeability of insulin in the retinal endothelial cell cultures was significantly less than the aortic endothelial cell cultures, indicating that expression of specific barrier proteins contribute to the permeability characteristics of retinal cells.

The results presented above and observations from our laboratory illustrate the substantial differences between the tighter BRB and the endothelium of other tissues. Administering superphysiological amounts of insulin intraportally to fasted rats induces significant phosphorylation of the IRβ, IRS, and IRS-1 associated PI3K activity in muscle within 90 seconds (75). By contrast, we found no effect on retinal IR phosphorylation until 30 minutes post injection using a similar model (30). These results are consistent with other studies of brain and retina insulin uptake (61, 73, 76). Collectively, these studies confirm the difference between the endothelium of retina
and different insulin-sensitive tissues, and that the transport of insulin across the BRB is significantly slower than across other vascular beds. This suggests the neural retina is not likely a major target of insulin to regulate immediate nutrient metabolism as is skeletal muscle, but the steady-state transport of insulin to the neural retina may provide a stable trophic signal as opposed to insulin’s better known rapid metabolic functions.

Insulin signaling characteristics in endothelial cells have been investigated and described as in muscle, liver, adipose, and IR-overexpressing cells, but some of the final biological outputs of endothelial cells in response to insulin are unique. As demonstrated by King and others (55, 67, 77), retinal vascular endothelium contain IRs that, when stimulated, promote cell division. Furthermore, endothelial IRs also transport insulin unidirectionally (70). In general, the IR signaling characteristics in endothelial cells resembles what has been described in other systems, and the Akt pathway is activated in response to insulin. In human endothelial cell cultures induced to die with TNF-α treatment, insulin restores Akt phosphorylation and reduces rates of apoptosis (78). This mechanism also appeared to be dependent on Akt’s ability to inhibit caspase 9 activity, while Bad may not be a target of Akt in endothelial cells. Insulin’s ability to stimulate production of other autocrine growth factors, such as VEGF, may also play a role in endothelial cell survival. Insulin and VEGF may have combined effects in promoting the formation of endothelial tubes in vitro (79). Glucose uptake in retinal endothelial cells is also not affected acutely by insulin, consistent with a lack of expression of the GLUT4 transporter in retinal
endothelial cells (80-82) as well as other structures of the eye (83). Together, insulin promotes both mitogenic and survival effects on endothelial cells. In terms of diabetic retinopathy, these growth promoting effects may produce temporary detrimental consequences on the retinal vasculature as discussed below.

1.3.2 Retinal Insulin Production

An interesting observation by Havrankova et al (84) added considerable controversy concerning the role of insulin in neuronal cells. They detected insulin immunoreactivity in brain tissue, and they put forth the hypothesis that insulin may be synthesized de novo by mammalian tissues other than pancreatic β cells. It was discovered that insulin concentrations were at least 10-fold greater in brain than in plasma, but others have disputed those results (85). Therefore, brain may either have mechanisms in which to concentrate insulin or synthesize the peptide. Devaskar et al (86) furthered those observations by investigating whole brain and cultured primary neurons and glial cells from rabbits. The insulin transcript was detected using sensitive techniques, such as nuclear run-on assays and reverse-transcription PCR, but not by Northern blot analysis, suggesting insulin mRNA in these tissues is a rare transcript. When neonatal rabbit neurons and glial cells were cultured, it was demonstrated that insulin continued to accumulate in the culture media over six days in culture, supporting the hypothesis that neurons synthesize insulin de novo. Further in vivo evidence of brain-derived insulin came from mRNA in situ hybridization
which showed significant binding to neuronal cells over various regions of the brain. However, insulin antisera was unable to detect specific insulin immunoreactivity as it had in previous studies, which may be due to the inability of insulin antibodies to react across different species. This evidence for de novo synthesis was strengthened with electron microscopic analysis of the rat CNS demonstrating the presence of both insulin I and II mRNAs and insulin protein in the ER and Golgi apparatus (87).

Although the amount of insulin in brain is probably considerably lower than that expressed in β cells, it is intriguing to speculate that neuronal insulin may provide a local trophic or developmental signal, act as a neurotransmitter, or regulate nutrient metabolism in neurons. A lower concentration would be predicted on the basis that pancreatic β cells concentrate insulin in granules, but the brain does not (see Table 1, pg. 42).

The evidence for neuronal insulin production has been extended to retina tissue. Using insulin antiserum, insulin immunoreactivity was detected in human retinal tissue and in the optic nerve of mice (88). Insulin was reported to be located in the ganglion cell layer, the inner nuclear layer, and the inner and outer plexiform layers, but it was completely absent from the photoreceptors. Glial cells from the optic nerve also stained positive for insulin. A human retinoblastoma cell line, Y79, also was positive for insulin immunoreactivity, mRNA, and binding sites, although this may be an artifact of the transformed nature of the cells, as insulin and IR expression may be altered (89, 90). Using cultured primary rat Müller glial cells, the Das group showed both insulin protein and mRNA within this specific subset of
retinal cells (91). Given the supportive nature of glial cells in general, this suggests the Müller cells may secrete the insulin for retinal neurons as a paracrine hormone. Furthermore, intact retina tissue was shown to express the mRNA for preproinsulin in rats (92). In embryonic chickens, the amount of insulin expression in retina was considerably less than pancreatic insulin expression, as a more sensitive analysis using RT-PCR was required for insulin mRNA detection (93).

Given the extensive evidence that retina and numerous other extra-pancreatic tissues express insulin-like molecules (94), insulin may have the potential to modulate neuronal functions. The observations in chick retina, as observed in other species, suggests that retinal (or neuronal) expression of insulin is not purely a mammalian phenomenon, and that insulin, or other insulin-related peptides in other species, may have an evolutionarily conserved role in general neuronal development. Retinal development is sculpted by coordinated differentiation and apoptosis, and Diaz et al have established that retinal cell survival in chicks depends on insulin produced by the retina (95). Using antibodies to inhibit insulin action in developing chick retina, they observed a significant elevation in apoptosis; ganglion cells were particularly sensitive to the induction of apoptosis when insulin signaling was blocked. Lastly, consistent with our observations in mammalian retina, chick retina responds to insulin in vitro with significant elevations in Akt phosphorylation, while the Ras/Raf/MEK/ERK pathway is relatively insensitive to insulin stimulation (95). This supports the hypothesis that retinal insulin serves as a trophic factor in either an autocrine or paracrine manner. However, developing chick retina stimulated with
insulin exhibits increased $^3$H-thymidine-DNA incorporation and protein synthesis, suggesting insulin may play a role in mitogenesis and differentiation during embryonic stages (96). These results suggest insulin signaling maybe important in the adult retina as well, and it also plays a major role in development. In fact, insulin binding in the retina is greater in fetal compared to adult chick retina (36).

1.3.3 Insulin signaling in Caenorhabditis elegans and Drosophila melanogaster

Recent studies of the insulin-like signaling in worms and flies have provided important insights into insulin physiology from mice to humans. A particularly interesting line of study in the nematode worm, *Caenorhabditis elegans*, has revealed a neuron-specific insulin-like signaling system that controls the worm’s lifespan (97). This signaling system closely mirrors what has been established in mammalian cells and described above. The IR homolog in *C. elegans*, DAF-2, signals to AGE-1 (homolog to mammalian PI3K), AKT-1 and -2, and DAF-16 (homolog to mammalian FoxO transcription factors, Fig. 1, pg. 41) (98). In wild-type *C. elegans*, when nutrients are abundant, the worms’ life-span is ~12 days, but in the absence of nutrients, their growth becomes arrested and they enter a dauer stage in response to a dauer-inducing pheromone. This is a form of “hibernation” that allows the worm to prolong its life span until its environment becomes more favorable for reproduction.
Experiments by Ruvkun and co-workers (98) have established the IR-like signaling of DAF-2 in *C. elegans* to be intimately linked to regulation of life-span.

Genetic sequencing of the DAF-2 gene revealed that it is an IR homolog in *C. elegans* (97). It is 36% identical to the human IR within its ligand binding domain (35% identical to the IGF-1R), and 50% identical with 70% amino acid similarity within the tyrosine kinase domain. The genetic organization is also quite similar as it has a proform, a proteolytic site, and a transmembrane domain. *C. elegans* carrying a mutation in DAF-2 displayed arrested growth and a rise in fat accumulation that is associated with dauer formation, despite a favorable environment for survival and reproduction. This evidence suggests a strong evolutionary link in IR function in which the insulin signaling pathway regulates metabolism.

Since that seminal report established a significant role of insulin-like signaling in *C. elegans*, an entire cascade homologous proteins have been described (98). Comprehensive genetic studies of *C. elegans* mutants have established a pathway from DAF-2 to DAF-16, the FoxO homolog, which regulates life-span in the worm. For example, essentially all viable worms with DAF-2 mutations alone enter the dauer stage and have a prolonged life span, as discussed above. A complementary mutation in DAF-16 will reestablish a normal phenotype (99). Therefore, activity of the FoxO transcription factor, DAF-16, is required for dauer formation, and signaling from DAF-2 directly antagonizes such activity, as the IR affects FoxO activity in mammalian cells. Using that strategy, other mediators of the DAF-2 signaling pathway were linked. *C. elegans* with AGE-1 mutations, like DAF-2 mutations, enter
the dauer stage, and normal life-span is restored when an activating mutant of AKT-1 is also expressed. Furthermore, the gene products of AKT-1 and AKT-2 in *C. elegans* directly antagonize DAF-16 as is the case in mammalian cells (98).

Now the question becomes, again as in mammalian systems, what is the role of the IR-like DAF-2 signaling system in neurons? Using tissue specific promoters, Wolkow et al (100) elegantly demonstrated in *C. elegans* that restoring the insulin-like signaling system to DAF-2 and AGE-1 mutants in neuronal cells regulates longevity. This was in contrast to restoring DAF-2 signaling to muscle and gut cells of *C. elegans*, which did not restore normal life-span. However, a functional divergence for insulin-like signaling emerged from their study. It was discovered that restoring DAF-2 to the muscle and intestinal cells reduced fat accumulation and restored normal metabolic processes, but did not alter life-span. This signaling dichotomy in lower organisms suggests it may also be conserved in mammals. Furthermore, 37 insulin peptide-like genes have been identified in *C. elegans*, and deletion of one of them does not effect life-span, so there is probably functional redundancy (101). Interestingly, the INS genes were expressed by neurons suggesting a possible neuroendocrine role for insulin conserved throughout evolution.

The fruit fly, *Drosophila melanogaster*, also possesses an insulin-like signaling cascade with many similarities to *C. elegans* and mammalian systems with mutations that effect fly growth. Comparisons among the conserved insulin signaling pathways can be seen in Figure 1 [pg. 41, (102)]. In *D. melanogaster*, evidence suggests that the primary role for the insulin-like signaling is to control growth by
regulating cell size and number, and mutations within the Dinr gene result in
abnormal development (103, 104). Like C. elegans, D. melanogaster express
multiple insulin-like peptides, three of which localize to neurosecretory cells in the
brain. When one of these peptides is overexpressed, it increases both cell size and
number. When the Dinr gene is also overexpressed, the eye (comprised mostly of
photoreceptor cells) displays an overgrowth, while Dinr mutations result in reduced
body weight and fewer ommatidia, the fly photoreceptor units. Genetic analysis of
these mutants suggests that the insulin-like peptides are ligands for the DINR as the
eye phenotypes are corrected in insulin-like peptide overexpressing mutants with
Dinr mutations (103). The DINR peptide is unique in that it expresses a ~60 kDa C-
terminal extension that is proteolytically cleaved in some cells, and this extension is
hypothesized to give the receptor different functions in different cells. A similar
function of the IR may occur in mammalian cells; that is, some functions of the IR
may depend on the cell type expressing the IR rather than a proteolytic event which
modifies the receptor. Given the conserved nature of the IR throughout evolution, the
function of the IR remains very similar in promoting anabolic effects, yet other
modifications or effector molecules remain to be discovered. Glycosylation and exon
11 expression are two other modifications of the IR that also may be contributing to
differential IR activity and regulation in mammalian cells. Taken together, the
conservation of insulin-like peptide expression in neurons or neurosecretory cells
from lower species to mammals suggest a specialized function of insulin and the IR,
and that neuronal cells have evolved to utilize insulin signaling differently. In terms
of the retina, this function may be in place to regulate proper embryonic or adult
development and cell growth, maintain cellular survival, metabolism, or regulate
phototransduction, all of which require further investigation.
1.4 Retinal Insulin Signaling

The physiological outcomes of IR activation in retina and other neurons of the CNS are beginning to be elucidated. It has been established that IRs are expressed on different neurons and glia, and co-localization studies have determined that the IR, IRS-1, PI3K, and phosphotyrosine motifs topographically overlap in neurons (105, 106). Hence, an insulin signaling pathway could be functional in those cells since it resembles IR signaling in other systems, but the physiological role has received little attention.

Observations from Dr. Gardner’s laboratory extended previous observations and have begun to establish a possible function of insulin signaling in the retina (30). Slow *in vivo* phosphorylation of the IR and Akt in retina with systemic insulin administration was observed. *In vitro*, there was no significant change in IRS-1 tyrosine phosphorylation in response to insulin, but IRS-2 was rapidly phosphorylated and dephosphorylated. Retina expresses mRNA for all three Akt isoforms, but insulin stimulates only Akt-1 activity. This suggests tissue specific signaling in the retina tissue. Consistent with the findings of Diaz et al (107), the ERK 1/2 pathway stimulation was relatively unaffected compared to the Akt pathway with insulin. It can not be ruled out that other proteins mediate the insulin signal in retina, or that subsets of the IR expressed on different parts of the retina may signal differently. In brain, the IR is a component of the synapse, and signals arising there may be mediated by p58/53, a substrate of the IR which is expressed in brain and absent in
liver and skeletal muscle (108, 109). The IR is expressed broadly on single neurons, while IR and p58/53 co-localize in the synapse, suggesting a specialized function for insulin signaling within the same neuron. Indeed, novel mediators of insulin signaling may exist in retina and other neurons that have not been described in other cell types. An overall hypothesis is that the IR→Akt pathway in retina is tonically active and does not fluctuate in response to the organism’s nutritional state, and that this signal is required for proper development and cell survival.

Experiments analyzing systemic insulin in rats have produced interesting results, suggesting that different parts of the brain may be differentially insulin sensitive since insulin transport varied by brain region. Within three minutes of systemic injection, cerebellar IRs were phosphorylated, but there was no effect on those from the frontal cortex (110). Therefore, regions of the brain with low permeability to insulin behave like the retina in which transport is slower, resulting in delayed activation of the IR. A recent report by Niswender et al (76) characterized insulin signaling in hippocampus in response to intraperitoneally administered insulin. In this model, PI3K activity was not significantly elevated until 15 minutes post injection. These results suggest that, like retina, the hippocampus can respond to insulin, and signals to known mediators of IR the signaling pathway. However, the complete physiologic outcome of neuronal insulin signaling in vivo remains to be seen.

Some of the molecular mechanisms of how insulin may reduce apoptosis have been identified by studying retinal neurons in culture (111). Serum starvation-
induced apoptosis of retinal neurons was partially inhibited with insulin treatment, and inhibitors of the PI3K pathway inhibited insulin’s effects. Furthermore, insulin reduced cleavage and activation of caspase 3, suggesting caspase 3 may also be a target of Akt. Interestingly, the mitotic rates of these retinal neurons were unaffected with insulin treatment, in direct contrast to retinal endothelial cells which proliferate in response to insulin (55). Together with our results in vivo, these data strongly point to the Akt-mediated survival pathway as a mediator of insulin-induced retinal cell survival. This, however, does not rule out effects from other growth factors that are present in the retinal milieu. For instance, to promote retinal ganglion cell survival in vitro, combinations of growth factors are required in addition to membrane depolarization, cell-cell contact, and the second-messenger cAMP (112, 113).

Heidenreich et al reported lower rates of glucose uptake in response to insulin in neurons in vitro (29). Moreover, insulin can stimulate amino acid uptake in brain neurons (114). The question of insulin-stimulated substrate uptake has also been addressed in human Y79 retinoblastoma cells (115). Of the amino acids assayed, only glycine uptake was stimulated by both insulin and IGF-I. In this way, insulin could potentially regulate neurotransmission as glycine is a putative neurotransmitter. However, uptake of other neurotransmitters such as GABA and dopamine were unaltered in this cell line.

Other aspects of retinal function have been investigated in response to insulin. Insulin has been reported to mediate differentiation and synaptogenesis (28), and to
regulate acetyltransferase proteins (116, 117). Embryonic chick retinal cells in vitro increased their cholinergic acetyltransferase activity over time in an insulin dose-dependent manner. Holdengreber et al also showed an insulin-mediated increase in choline acetyltransferase protein with short-term exposure to insulin, and that this increase was found in the ganglion and inner nuclear cell layers. Insulin, known to modulate the activity of various transcription factors, may regulate choline acetyltransferase protein by regulating jun (118), but other transcription factors may also be involved. Insulin may also synergize with other trophic factors in retina to promote differentiation. In the postnatal chicken retina, insulin and FGF2 were required to induce waves of proliferation and expression of progenitor markers, such as Pax6, in Müller glia cells (119). Together, insulin appears to promote some aspects of neurogenesis in retina (96).

Acute effects of insulin on retinal electrophysiology have also been studied. For instance, the a- and b- waves of the electroretinogram (ERG) were reduced when insulin was applied to an in vitro system (120). Such an effect on the photoreceptors is consistent with our and other’s observations of IR expression on photoreceptors (37, 51, 54). A potential mechanism by which insulin may alter the ERG was provided by Stella et al when they showed an insulin-dependent reduction of Ca\(^{2+}\) influx in photoreceptors (121). However, these results are in contrast to those of Lansel and Niemeyer in cat retina where, under normoglycemic conditions, the b-wave amplitude of the ERG was unaffected (122). Only when the eye was perfused with hypoglycemic solutions, which reduced the b-wave, did insulin recover retinal
nerve ERG activity. The physiological relevance of these results is questionable because under hypoglycemic conditions, circulating insulin is also usually reduced, but their results also suggest insulin crosses the BRB. So, if the BRB were more permeable to insulin, one may expect vision to change briefly with fluctuations in circulating insulin. Therefore, IRβ phosphorylation in retina may be maintained stably for proper phototransduction and synapse function.

Since the retina is the only neural tissue exposed to light, some questions concerning light-mediated IR activity are beginning to be answered. Rajala et al, found that the IRβ interacts with the regulatory subunit of PI3K, p85, in bovine rod outer segments. In pull down assays, the interaction between the IR and PI3K was dependent on tyrosine phosphorylation of the IRβ, and treatment of the tissue with insulin *in vitro* increased PI3K association and activity associated with the IRβ (123). This suggests some aspects of insulin signaling in retina may occur independently of IRS-regulated PI3K activation, or this IRβ-PI3K interaction may be specific to the rod outer segments. In a second report, the same group (124) showed that light increases IRβ phosphorylation and PI3K activity in rod outer segments associated with the IR in mice and rats. They propose that this phenomenon may be a mechanism to protect the retina from light induced damage. This suggests Akt would also be activated by light since it is immediately downstream of PI3K. Our observations in retina treated *in vitro* with insulin suggest this would be the case as all nuclear layers of the retina, including photoreceptors, had increases in immunoreactivity for the active, phosphorylated form of Akt, and the mRNAs of Akt-
1 and -3 localized to the nuclear layers of the retina (30). Even more interesting is their suggestion that IR activation could be modulated by other ligands and that the intracellular portion of the IRβ may be modulated by novel ligand-independent mechanisms to increase its phosphorylation, or that the IR may play some role in phototransduction.
1.5 Insulin and Insulin Receptors in Retinal Disease

Theories about the pathogenesis of diabetic retinopathy have been plentiful and include increased polyol pathway flux, nonenzymatic glycosylation and RAGE (receptor for advanced glycation endproducts) activation, increased oxidative stress, protein kinase C activation, altered growth factor expression, and hypoxia (125-128). No comprehensive mechanism has yet been established that provides a causal link between the fundamental metabolic abnormalities of diabetes and the initiation and progression of retinopathy that includes an explanation to account for loss of vision. During this period there have been two implicit assumptions underlying most diabetes complications research: first, that retinopathy is solely or primarily a blood vessel disorder; and second, that excess glucose is necessary and sufficient to account for the phenotype (129). Until recently these appeared to be reasonable operative assumptions. The clinical signs of retinopathy are determined by the appearance of hemorrhages, microaneurysms, cotton-wool spots, macular edema, lipid exudates, and neovascularization. These features are visible with ophthalmoscopy because they contain pigment (hemoglobin) or reduce retinal transparency (cotton-wool spots and lipids). However, at least 95% of the retinal volume and mass is comprised of neurons and glial cells that lack pigment and are thus indistinguishable by biomicroscopy, fluorescein angiography, or ultrasound, and only marginally detectable by current optical coherence tomography. Recent work by several investigators now provides evidence that challenges this first assumption. There is
now clear evidence for loss of retinal neurons by apoptosis (130, 131), activation of
glial cells (132, 133), and microglial cells (131, 132) by altered expression of glial
fibrillary acidic protein (GFAP). These changes develop early in the course of
experimental diabetes and precede detectable structural microvascular lesions as
assessed by fluorescein angiography or trypsin digest preparations. In fact, the loss of
retinal neurons was first described 40 years ago (134, 135), and close histologic
examination of eyes with macular edema reveals extensive loss of ganglion cells and
inner nuclear layer neurons (136). These anatomic changes support the well-
recognized functional changes in the ERG, color vision, and contrast sensitivity that
precede vascular changes (137). Collectively, these data strongly support a major if
not direct effect of diabetes on retinal neural function that may or may not depend on
alterations in vascular function (138).

The second assumption regarding the singular causative role of glucose
appears to be based on the observation that insulin does not increase glucose uptake
in retinal endothelium via the GLUT1 glucose transporter. Moreover, the results of
the Diabetes Control and Complications Trial (DCCT) (139) may have been
overstated in that hyperglycemia alone causes diabetic complications. That is, the
results of the DCCT are believed to have proven that chronic hyperglycemia is the
singular causative factor that is responsible for retinopathy and other diabetes-related
complications (5). The major indices of metabolic control in the DCCT were
hemoglobin A1c and blood glucose values; these tests reflect the net carbohydrate
metabolism (intake minus utilization) and the degree of insulin action. Deficient
insulin action (qualitative or quantitative)—rather than hyperglycemia—is the cardinal metabolic feature of diabetes, and the improved control achieved in the DCCT was accomplished via significantly higher insulin administration (140). Thus, the major question relevant to retinopathy research remains: what is the mechanism of the effects observed in the DCCT? Is it from obvious changes such as lowering the glucose burden or higher insulin levels and greater insulin action, or from effects on amino acid or lipid metabolism that are also central to diabetes? At this point the answer remains elusive but it is clearly premature to exclude factors other than glucose. In fact, no single parameter may account for vascular and neural lesions.

With these points in mind, questions that need to be asked are does retinal insulin signaling change in diabetes? If so, are the changes relevant to the genesis of retinopathy? The published work in this area is remarkably limited. Zetterstrom et al (141) found that retinas from rats with 4 weeks of experimental diabetes expressed higher IR levels than controls, and this increase reflected a doubling of the neuronal and a 20% decrease in the peripheral (vascular) IR subtypes. Neuronal insulin receptors in wheat-germ agglutinin-purified cortical synaptosomal membranes were also increased, and the receptors exhibited normal autophosphorylation properties. The authors concluded that retinal and cortical IR are sensitive to circulating insulin and glucose levels. Wheat-germ agglutinin binds to sialic acid residues so the method employed to isolate IRs in this study selected for vascular over non-vascular IR.

It is not possible to investigate retinal IR in intact human eyes, and only one study has examined vitreous insulin levels in patients (142). However, details of the
subjects’ medical history, medications, diabetes type, or duration were not included, making interpretation of the data difficult. Type I diabetic patients may have reduced vitreous insulin, whereas insulin resistant and hyperinsulinemic Type II patients may have normal or elevated vitreous insulin concentrations. Insulin levels have been reported to be reduced by approximately half in the vitreous of rats with 4 weeks of streptozotocin-induced diabetes (73). Thus, it appears that circulating insulin readily gains access to the vitreous cavity in normal and diabetic rats, and that at least part of the vitreous insulin content derives from the pancreas. Unfortunately it is difficult to investigate this point in humans.

While long-term intensive insulin therapy clearly reduces the risk of retinopathy progression, a minority of patients may develop exacerbation of their retinopathy after institution of tight control. In the DCCT, 13% of intensively treated patients versus 7.6% of conventionally treated patients who had retinopathy at baseline developed worsening within 6 months of treatment (143). However, only two patients developed proliferative retinopathy and 3 developed clinically significant macular edema requiring laser treatment. Thus, “early worsening” is uncommon and seldom associated with vision loss. Recently, Poulaki et al (144) found that subcutaneous insulin implants delivering low-dose (2 units) of insulin daily increased blood-retinal barrier permeability and VEGF expression in rats with short-term streptozotocin diabetes. These short term responses may reflect impaired ability of diabetic rat retinas to respond to insulin therapy, but are in contrast to our observations that 3 days of insulin treatment substantially improves glial reactivity
and glutamine synthetase activity without complete restoration of euglycemia (133, 145), or that chronic insulin therapy reduces apoptosis (130). The long-term benefits of intensive insulin therapy are unequivocal, and one might reasonably interpret the DCCT data to represent an insulin dose-response trial (Fig. 2, pg. 43). If so, systemically administered insulin may be a direct pharmacologic intervention for the retina and other complications-prone tissues, including the kidneys and peripheral nerves. Indeed, a direct role for insulin on retinal cell viability is strongly suggested by the observations that in developing chicken retinas, insulin reduces apoptotic cell death (146), and insulin antibodies induce ganglion cell apoptosis (107). These observations have yet to be confirmed in human retinas but the high degree of homology in insulin signaling and retinal development across species suggests an important role for insulin in the maintenance of retinal cell survival as the insulin-signaling pathway is a key determinant of neuronal survival in species from *C. elegans* (147), *Drosophila melanogaster* (103, 148), and mice (149). We have also found that insulin is a potent inhibitor of cell death in rat retinal neurons in culture in response to serum deprivation (111). Thus, the cumulative data support a direct and beneficial role of insulin on retinal cell function and survival during embryologic development and stressful conditions such as nutritional deprivation and hypoxia.

In clinical medicine, exceptional cases often provide opportunities to gain new insights into the specific etiologic factors in disease pathogenesis. Although hyperglycemia is most often considered to be a strong predictor of diabetes complications, more than 15 well-documented cases of diabetic retinopathy and
nephropathy have been described in patients without overt hyperglycemia or glucose intolerance (150-154). These cases suggest that hyperglycemia may not be essential for the development or progression of retinopathy. How could impaired insulin action, and thus diabetes, not alter glucose levels and yet result in retinopathy? Insulin has protean consequences from tissue to tissue and cell to cell that are modified by substrate levels and interactions with other hormones and growth factors. The signaling pathways for insulin are highly complex and divergent, and many probably remain undiscovered. It is conceivable that some patients may have selective impairments in insulin action that affect glucose uptake and metabolism less than other aspects of diabetes such as lipid metabolism as suggested by Chaturvedi (155). That is, specific mutations in the complex signaling pathways of insulin could lead to a phenotype in which lipid or amino acid metabolism is defective with minimal changes in glucose metabolism, so patients may be “diabetic” in terms of overall insulin action without frank hyperglycemia. This concept requires additional investigation.

Further evidence for a role of insulin action derives from observations that insulin sensitivity is an important determinant for the progression of retinopathy in Types I and II diabetes. The European Diabetes Prospective Diabetes Complications Study (EURODIAB-PCS) found that insulin resistance (as measured by body mass index and triglyceride levels) is nearly important a predictor of retinopathy as HBA1c (156). Similar results have also been found in the DCCT cohort (157), and these findings confirm earlier studies showing that acute insulin sensitivity is a strong
predictor of retinopathy and other vascular complications in Type 1 diabetes (158), and that patients with retinopathy are more insulin resistant than those without retinopathy (159). Insulin insensitivity was also found to be a major risk factor for the presence of retinopathy at the time of Type 2 diabetes diagnosis in the United Kingdom Prospective Diabetes Study (UKPDS) (160).

Taken together, these laboratory, epidemiologic, and clinical trial data strongly suggest that altered insulin action, at least at the systemic level, plays an important role in the pathogenesis of diabetic retinopathy. It is now important to determine if retina-specific changes in insulin action cause cell death, vascular permeability, and vision loss. Better understanding of the importance of insulin sensitivity and action will provide the opportunity to develop new drugs that enhance the general or specific actions of insulin and the insulin receptor.
1.6 Introduction of Dissertation Project

1.6.1 Statement of Problem

Diabetes and its associated complications are reaching epidemic proportions world-wide, while the treatment options for patients with DR have remained relatively unchanged for over 50 years. Excess glucose has been thought to be the major factor causing DR, but one’s degree of insulin resistance has also been postulated to be a predictor for development of DR. Therefore, examining insulin action in retina tissue will shed light on the mechanisms which lead to worsening of DR. By gaining a better understanding of how insulin, and other growth factors, function and mediate neuroprotection in the retina, potential new therapies can be developed.

1.6.2 Aims

1. To determine the proximal IR signaling characteristics in intact retina tissue in vivo and ex vivo (Chapter 3).
2. To examine how diabetes affects retinal IR signaling in animal models of diabetes (Chapter 4).
3. To investigate the mechanisms which contribute to constitutive IR autophosphorylation in retina (Chapter 5).
1.7 Figures, Tables, and Legends

![Comparison of the conserved insulin-like signaling pathway among flies, worms, and mammals.](image)

**Figure 1.** Comparison of the conserved insulin-like signaling pathway among flies, worms, and mammals. Insulin-like peptides signal through structurally similar receptor tyrosine kinases, and the signal is perpetuated down a conserved pathway. IRS proteins (termed CHICO in *Drosophila* and unidentified in *C. elegans*) recruit PI3K and lead to Akt activation in response to insulin-like hormones. Common endpoints of this pathway are GSK3 and FoxO (FKHR) transcription factors. PTEN is a lipid phosphatase which negatively regulates PI3K activity by reducing phosphatidylinositol 3’-OH phosphate (PIP3) content in cell membranes, and mutations of PTEN have been described in tumors. Conservation of this central growth-promoting pathway suggests numerous effectors have evolved around it to add specificity between different cell types within one organism. Dashed lines indicate hypothesized interactions. Adapted from Garofalo RS, *Trends in Endocrinology and Metabolism*, 13, 156-162.
Table 1. A summary comparison of β cell, brain, and retina insulin production and IR properties among peripheral/vascular tissues, brain, and retina from mammalian systems.
Figure 2. In individuals with IDDM, the maintenance of retinal function depends on insulin. Adapted from the DCCT trial (N Engl J Med, 329, 977-986), the percentage of new patients developing new retinopathy (y-axis), is significantly reduced in individuals receiving greater amounts of insulin per day (intensive insulin therapy). Similar results were also found in patients with pre-existing mild DR. Therefore, insulin per se may have therapeutic implications in preventing diabetic complications.
CHAPTER II

METHODS AND MATERIALS
Animals

Age-matched male Sprague-Dawley rats (Charles River, MA) were used in all experiments. Rats were housed under a 12:12 h light:dark cycle in wire-bottom cages (maximum five per cage) with free access to a standard rat chow and water at The Pennsylvania State University College of Medicine, Hershey, PA. Where noted, rats were fasted for 18 or 24 h overnight. All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Resolution on the Care and Use of Laboratory Animals.

**Intravenous insulin injection.** For in vivo insulin injection experiments, rats were fasted 18 h prior to being anesthetized with a 10:1 ketamine:xylazine cocktail by intramuscular injection at 53.3 mg/kg for ketamine and 5.33 mg/kg for xylazine. Upon loss of motor reflexes, the abdominal cavity was opened and 50 or 500 µg of bovine insulin (Sigma, St. Louis, MO) or vehicle (0.9% saline) was injected via the portal vein; the abdominal cavity was opened and nothing was injected into sham control rats. For the 60 min time point, a second ketamine:xylazine injection at 50% the first dose was administered after 40 min. Rats were warmed on 37°C heating pads to help maintain body temperature. At the indicated times in the figures, gastrocnemius skeletal muscle and retina were removed and snap-frozen under liquid nitrogen and stored at -80°C until analysis.

**Comparison of constitutive IR signaling in freely fed and fasted rats.** Rats were either fasted 18 h overnight, or had free access to chow and water. Rats were
then anesthetized using sodium pentobarbital at 100 mg/kg, and liver, gastrocnemius, and retina tissue were excised and frozen under liquid nitrogen upon loss of motor reflexes. Tissue samples were stored at -80°C until analysis.

**Ex vivo retina preparation.** For *ex vivo* experiments, rats were anesthetized using sodium pentobarbital at 100 mg/kg, decapitated upon loss of motor reflexes, and their retinas were removed by cutting across the cornea, removing the lens, and squeezing the eyeball with forceps to rapidly extract the retina. Retinas were incubated in MEM (Sigma) supplemented with 5 mM pyruvate and 10 mM HEPES for 15 minutes at 37°C, 5% CO₂, and gentle shaking, essentially as described by us and others for the study of retinal metabolism and effects of growth factors (161-163). Insulin (10 nM, Sigma), IGF-I, IGF-II (both IGFBP-resistant, Upstate Biotechnology, Lake Placid, NY), or vehicle was then added and these conditions were maintained throughout the duration of the experiment. At the indicated times, retinas were snap-frozen in liquid nitrogen and stored at -80°C until analysis or fixed in 2% paraformaldehyde for 10 minutes at room temperature for immunohistochemical analysis (see below).

**Induction of diabetes and insulin therapies.** Diabetes was induced by intravenous (IV) or intraperitoneal (IP) injection of streptozotocin (STZ, 65 mg/kg, Sigma) dissolved in sodium citrate buffer, pH 4.5, and control rats received equivalent volumes of buffer alone as described previously (130, 164). There was no significant difference in severity of diabetes, as assessed by blood-glucose monitoring, between rats receiving STZ IV or IP (S. Bronson, personal...
communication). STZ-injected rats were considered diabetic when exhibiting blood-
sugar levels > 13.9 mmol/L (250 mg/dL) within 5 d after diabetes induction (Lifescan
One-Touch® meter, Milpitas, CA); otherwise, they were excluded from the study.
Rats were housed up to 5 per cage with free access to food and water until sacrifice.
Insulin therapy was begun 5 days after induction of diabetes by implanting a
subcutaneous insulin pellet to deliver ~2 U insulin (bovine)/d [LinShin, Canada Inc.,
Toronto, ON, Canada (130)]. A second implant was given after 4 weeks, and one rat
required a third implant as its blood sugar level rose above 13.9 mmol/L. A shorter-
term insulin therapy was utilized in some cohorts of rats which consisted of two
injections daily of 5U Humulin® Regular/5U Humulin® Ultralente (Lilly,
Indianapolis, IN) at 09.00 and 17.00 for three days prior to sacrifice. On the day of
sacrifice, rats were anesthetized with 100 mg/kg sodium pentobarbital and sacrificed
by decapitation following motor reflex loss for rapid dissection of retina tissue.
Retinas were immediately frozen in liquid nitrogen and stored at -80°C until analysis,
or used immediately for *ex vivo* experiments.

*Somatostatin (SST) infusion.* The SST infusion protocol was performed
according to previously published methods (165). Briefly, catheters were inserted
into the right jugular vein of anesthetized rats [(90 mg/kg ketamine and 9 mg/kg
xylazine administered intramuscular) (165)], sutured in place above the superior vena
cava, and exposed through a dorsal incision at the base of the neck. The rats were
then housed individually, fasted, and allowed to recover for 24 h. A primed constant
infusion of 60 µg + 3 µg · kg⁻¹ · h⁻¹ SST (Bachem; Torrance, CA; n = 10) or
equivalent volumes of vehicle (0.9% saline + 0.2% BSA; n = 10) was begun for 180 min. This time point was chosen as this length of time of SST infusion in humans diminishes basal circulating insulin and alters brain metabolism (166). After 180 min, rats were then anesthetized with sodium pentobarbital through the venous catheter, and blood glucose levels were measured using a One-Touch® Basic blood glucose meter (LifeScan, Inc.). Upon motor reflex loss, liver samples were excised and immediately frozen under liquid nitrogen. The rats were decapitated and trunk blood was collected with EDTA as an anticoagulant (Becton Dickinson, Franklin Lakes, NJ), and excised retinas were immediately frozen under liquid nitrogen and stored at -80°C until analysis.

**Intravitreal antibody injections.** Rats were fasted overnight for 18 h, and anesthetized with a 10:1 ketamine:xylazine cocktail by intramuscular injection at 53.3 mg/kg for ketamine and 5.33 mg/kg for xylazine. A rat received either a 200-fold molar excess [based on published observations of vitreal levels of growth factors (73, 167, 168)] of either α-insulin (3.1 µL, Sigma), αIGF-1 (3.2 µL, Santa Cruz Biotechnology, Santa Cruz, CA), or αIGF-2 (8.25 µL, Upstate Biotechnology), and the contralateral eye received an equal volume of a non-specific linking antibody (rabbit α-sheep, Jackson Immuno Research, West Grove, PA; n = 4 for each antibody treatment). As a positive control for in vivo IR tyrosine phosphorylation, some rats (n = 4) received either vehicle in one eye or 10 nM insulin (5 µL, Sigma, bovine crystalline) in the contralateral eye. To maintain a loss of motor reflexes, a second injection of ketamine:xylazine, half the original dose, was administered after 40 min.
The rats were sacrificed and retinas immediately frozen under liquid nitrogen at 60 min.

**Periocular insulin administration in rats.** Rats were anesthetized with a 10:1 ketamine:xylazine cocktail by intramuscular injection at 53.3 mg/kg for ketamine and 5.33 mg/kg for xylazine. Humulin® Regular, diluted with Humulin® diluent (Lilly) was injected under the subconjunctiva using a standard insulin syringe. After 30 min, blood glucose levels were monitored and the rats were sacrificed by decapitation for analysis of IR and Akt phosphorylation. Retina samples were frozen under liquid nitrogen and stored at -80°C until analysis.

**Ins2Akita mice.** The Ins2Akita mouse has been described elsewhere (169, 170). Briefly, this mouse has a mutation (Cys96Tyr) of the Insulin2 gene on the C57BL/6 background. This mutation induces ER stress in the β cell leading to a loss of β cell function and mass leading to overt diabetes by ~7 weeks of age. For measurement of IR kinase activity, control and diabetic mice were 15 weeks old. Mice were anesthetized with sodium pentobarbital (200 mg/kg) IP prior to sacrifice and retina extraction. Retinas were frozen under liquid nitrogen and stored at -80°C until analysis.

**Genetic ablation of retinal IRs.** This mouse model was kindly provided by Dr. Domineco Accili, Columbia University. This mouse was genetically engineered from IR-/- mice (171) which have a perinatal lethal phenotype from severe diabetic ketoacidosis. To investigate the minimum requirements for IR expression in various tissues to confer survival and prevent diabetes, the human IR (hIR) gene was
introduced into IR\(^{-/-}\) mice under control of the transthyretin (TTR) promoter in the laboratory of Dr. Accili. This induced expression of the hIR in liver, \(\beta\) cell, and choroid plexus of the brain, and the mice survived, were not diabetic, and reproduced normally. The genetic background of these TTR-IR mice were a combination of C57BL/6, 129, and FVB (D. Accili, personal communication).

Because the FVB strain carries a mutation in the Pde6 gene (172), a phosphodiesterase which controls cGMP levels and Ca\(^{2+}\) channel activity to regulate membrane hyperpolarization of photoreceptors in response to light and opsin activation, this results in a retinal degeneration (rd) phenotype in which \(\sim\)100\% of all photoreceptors are lost by apoptosis by P35 (173). As demonstrated in Fig. 3 (pg. 61), the TTR-IR mice express the mutated \textit{pde6} gene.

To overcome this limitation, a breeding strategy was employed to select for the wild-type \textit{pde6} gene, express the TTR-hIR transgene in all mice, and produce mice expressing varying amount of endogenous murine IR (mIR) by crossing the TTR-IR mice with the control strain C57BL/6. As summarized in Fig. 4 (pg. 62), three generations of crosses with C57BL/6 produces the mice expressing wild-type \textit{pde6}, the TTR-IR transgene, and either mIR\(^{+/+}\), mIR\(^{+/-}\), or mIR\(^{-/-}\).

Monitoring the genetic makeup of each mouse was performed by PCR (Titanium Taq PCR kit, Clontech, performed according to the manufacturer’s suggested protocol) of DNA prepared from tail biopsies (\(\sim\) 0.5 cm) for the endogenous mIR, the neomycin negative selection marker for null IR expression (171), the hIR for TTR-IR transgene expression (D. Accili, personal communication),
and the mutated \textit{pde6} gene (174). Tail biopsies were stored at \(-80^\circ\text{C}\) for at least 1 h prior to DNA isolation. Samples were digested with 200 µg proteinase K in 400 µL tail buffer (50 mM Tris pH 7.5, 100 mM EDTA, 125 mM NaCl, and 1% SDS) overnight at 42\(^\circ\text{C}\) with constant agitation. Supernatants were collected after adding 200 µL saturated NaCl, and DNA was spooled onto glass pipettes after adding \(~600\) µL 100% ethanol. After air-drying, the DNA samples were stored in 1X TE buffer (100 mM Tris, 10 mM EDTA) at 4\(^\circ\text{C}\) until PCR analysis. The endogenous mIR was detected using the forward primer 5’ CTG TGC ACT TCC CTG CTC ACA 3’ and reverse primer 5’ TCT TTG CCT GTG CTC CAC TCT 3’ which produced a product \(~200\) bp after separation on 1% agarose gels. The neomycin cassette (null IR allele) was detected using the forward primer 5’ GAT CGG CCA TTG AAC AAG ATG 3’ and reverse primer 5’ CGC CAA GCT CTT CAG CAA TAT 3’ which produced a product \(~700\) bp after separation on 1% agarose gels. The hIR transgene was detected using the forward primer 5’ TAC CCC GGA GAG GTG TGT CCC 3’ and reverse primer 5’ ATG GTC GGG CAA ACT TCC TGG CAG 3’ which produced a product \(~500\) bp after separation on 1% agarose gels. The mutated pde6 gene was detected using the forward primer 5’ GTA AAC AGC AAG AGG CTT TAT TGG GAA C 3’ and reverse primer 5’ TGA CAA TTA CTC CTT TTC CCT CAG TCT G 3’ which produced a \(~550\) bp product after separation on 1% agarose gels if one or both of the mutated \textit{pde6} alleles were present.
R28 cell culture

R28 retinal neurons are a transformed cell line derived from post-natal day 6 rat pups (175), and the experimental methods to culture these cells have been described (111). For all experiments, a confluent T75 flask (75 cm²) of R28 cells were seeded 1:9 on 60-mm laminin-coated plastic dishes in DMEM (Sigma) supplemented with 10% newborn calf serum (HyClone, Logan, UT), MEM vitamin solution (Cellgro, Herndon, VA), 2 mM glutamine (Invitrogen, Carlsbad, CA), essential amino acids solution (Sigma), 0.05 mg/mL gentamicin (Invitrogen), and 250 µM pCPT-cAMP (Sigma), a cell-permeable analogue of cAMP. Upon ~90% confluence, cells were transferred to serum-free media, with the same supplements, for 2 h. Cells were treated with the following doses of growth factor: insulin (Sigma) – 10 nM, IGF-1 (IGFBP-resistant, Upstate Biotechnology) – 1.3 nM (111), brain-derived neurotrophic factor (BDNF, R&D Systems, Minneapolis, MN) – 100 ng/mL, epidermal growth factor (EGF, R&D Systems) – 4 ng/mL, and nerve growth factor (NGF, R&D Systems) – 10 ng/mL. The IR kinase inhibitor, HNMPA-(AM)₃, was purchased from Calbiochem (La Jolla, CA) and used according to their protocol. Length of growth factor treatment is described in the figure legends, and cells were harvested for protein analysis by immunoprecipitation and/or immunoblot by solubilization in buffer as previously described (30, 111), and as described below.
Immunoprecipitation and immunoblotting procedures

Sample preparation. Retina, liver, muscle, and brain tissue lysates were prepared by sonication of frozen samples at 4°C. Liver and muscle samples were previously powdered by compression under liquid nitrogen. The immunoprecipitation buffer (IPB) consisted of 50 mM HEPES pH 7.3, 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 2 mM NaVO₄, 10 mM Na-pyrophosphate, 10 mM NaF, 2 mM EDTA, 2 mM PMSF, 10 mM benzamidine, 10% glycerol, 1% NP-40, and one protease inhibitor tablet (Roche Diagnostics, Mannheim, Germany) per 10 mL. Samples were rocked for 15 min at 4°C, centrifuged at 14,000 rpm for 10 min at 4°C, and protein concentrations were determined on supernatants using the DC protein assay kit (Bio-Rad, Hercules, CA) compared to a BSA standard curve.

Immunoprecipitation. In each experiment, equivalent protein, ranging between 250-500 µg, was subject to immunoprecipitation in 1 mL IPB with the indicated antibody and 30 µL of a 50% slurry of BSA-blocked protein A Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ). The immunoprecipitation reaction was performed for 2 hours to overnight at 4°C with gentle rocking. The immune complex was washed twice with 250 µL IPB, and boiled (100°C) in 30 µL
sample buffer (SB, 10% glycerol, 1% SDS, 50 mM Tris pH 6.8, and 1% β-ME [v/v]) for three min.

_Ponceau S membrane staining, immunoblotting, and stripping membranes._

Equivalent protein, or immune complexes immunoprecipitated from equivalent protein, per lane were resolved by SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose membrane for Ponceau S stain (Sigma) and immunoblotting. Ponceau S, 500 mg, staining solution was prepared with 100 mL 1% acetic acid. Membranes were stained at least 5 min and destained with water for 5 min. Images were scanned into Photoshop (Adobe, San Jose, CA), and the image parameters (brightness and contrast of grayscale images) were adjusted equally. For immunoblotting, membranes were blocked in either 5% non-fat milk (NFM) or 3% BSA. Primary antibody incubations were at room temperature for 2 h or overnight at 4°C. Secondary antibody and tertiary incubations were at room temperature for 1 h. Membranes were washed 3 times for 10 min with TBST between incubations and development. Images were captured using ECF (Amersham) or ECL (Cell Signaling, Beverly, MA) kits according to the manufacturer’s instructions. All images were captured by either film (Kodak X-OMAT Blue XP-1, Rochester, NY), fluorimager (Molecular Dynamics, Sunnyvale, CA), or GeneTools GENOME (K&R Technology, Frederick, MD) software and quantified using NIH Image 1.61, ImageQuant (Molecular Dynamics), or GeneTools SynGene software (K&R Technology). Where noted, membranes were stripped at 50°C for 1 hour in a buffer containing 63 mM
Tris pH 6.8, 2% SDS, and 0.035% 2-mercaptoethanol and confirmed by re-blotting with secondary antibodies and detection before resuming immunoblotting procedures.

*Antibodies used.* All antibodies were used according to the manufacturer’s suggested protocol for immunoprecipitation and immunoblotting. From Upstate Biotechnology: \( \alpha \text{PY}, \alpha \text{IRS}-2, \alpha \text{Gab}-1, \alpha \text{pan p85} \) (also recognizes \( \alpha \text{p50} \) and \( \alpha \text{p55} \)) (176), and \( \alpha \text{phospho-Akt-1 thr308} \). From Santa Cruz Biotechnology: \( \alpha \text{IR}\beta, \alpha \text{IGF-1R}\beta, \) and \( \alpha \text{IRS}-1 \). From Cell Signaling: \( \alpha \text{pan Akt}, \alpha \text{pan phospho-Akt ser473}, \alpha \text{phospho p44/p42 MAPK}, \alpha \text{p44/42 MAPK}, \) and \( \alpha \text{phospho GSK3}\beta \). The \( \alpha \text{PY1158} \) was from Biosource (Camarillo, CA), and \( \alpha \text{actin} \) was from Sigma. All secondary antibodies recognizing rabbit and mouse primary antibodies were from Amersham and conjugated to horseradish peroxidase, alkaline phosphatase, or biotin. Tertiary incubations were with streptavidin-alkaline phosphatase (Invitrogen).

**Kinase assays**

*IR.* Tissue lysates (500 \( \mu \text{g} \)) were immunoprecipitated using anti-IR\( \beta \) antibodies (overnight, 4\( ^\circ \) C) as described above. The immune complex was washed twice in kinase buffer (177), which did not diminish the amount of IR bound as assessed by immunoblot analysis (not shown). The kinase buffer consisted of 50 mM HEPES pH 7.3, 0.05% BSA, 150 mM NaCl, 0.1% Triton X-100, 20 mM MgCl\(_2\), and 2 mM MnCl\(_2\) (177). A mock immunoprecipitate containing no tissue lysate served to blank the assay. After the last aspiration of buffer, the kinase reaction was performed
at room temperature in 250 µl of kinase buffer with 100 µM ATP, 3 mg/ml poly [Glu:Tyr] (Sigma), and 25 µCi/ml ³²P-γ ATP (Amersham) for 45 min with constant mixing. The reaction was stopped by brief centrifugation and spotting 35 µL of supernatant on Whatman p81 phosphocellulose paper. Filter papers were washed three times for five minutes in 0.75% phosphoric acid and once for five minutes in acetone before counting on a Beckman LS 6000SC Scintillation Counter (Beckman Instruments, Fullerton, CA).

**PI3K.** The PI3K assays were performed as described (178) with slight modifications. Retinas were homogenized in ice-cold buffer A consisting of 50 mM Tris-HCl (pH 7.5), 0.1% Triton X-100 (w/v), 1 mM EDTA, 1mM EGTA, 50 mM sodium fluoride, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, a protease inhibitor cocktail (Sigma), 0.1% β-mercaptoethanol (v/v), and 1 mM microcystin. The tissue homogenates were centrifuged at 14,000 rpm for 10 min at 4°C, and supernatants (75 µg protein) were subjected to dual immunoprecipitation by incubation overnight at 4°C with 2 µg each of anti-IRS-1 and IRS-2 (Santa Cruz Biotechnology), which were preconjugated (2 h at 4°C) to Gammabind G Sepharose (Amersham). The immune complexes were washed once with buffer A containing 0.5 M NaCl, once with buffer B [50 mM Tris-HCl (pH 7.5), 0.03% Brij-35 (v/v), 0.1 mM EGTA, and 0.1% β-mercaptoethanol (v/v)], and once with TNE buffer consisting of 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5 mM EGTA, and 0.1 mM sodium orthovanadate. The immune complexes were then
incubated at 35° C for 10 min in 50 µL of TNE buffer (pH 7.4) in the presence of γ-32P ATP (10 µCi/assay) and the substrate, phosphatidylinositol (PI, 20 µg/assay). The reaction was stopped by adding 20 µL of 6 N HCl and 160 µL of CHCl3/CH3OH (1:1). The organic phase was then spotted on a TLC plate and subjected to ascending chromatography using the solvent CHCl3/CH3OH/H2O/NH4OH (60:47:11.3:2). PI 3-phosphate (PI3P) thus resolved was quantified by phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA).

Akt-1, -2, and -3. Akt isoform-specific kinase assays were performed as described (179). The supernatants (75 µg protein) of retinal tissue homogenates were subjected to immunoprecipitation (overnight at 4°C) with 2 µg of anti-Akt-1, -2 (Santa Cruz Biotechnology), or anti-Akt-3 (Upstate Biotechnology) primary antibody, which is initially pre-conjugated (1 hr at 4°C) to Gammabind G Sepharose (Amersham). The immunoprecipitates were washed and incubated in assay buffer [20 mM MOPS (pH 7.4), 25 mM β-glycerophosphate, 1 mM sodium orthovanadate and 1 mM dithiothreitol] at 35° C for 10 min, in the presence of protein kinase A inhibitor peptide (1 µM), crosstide peptide substrate (GRPRTSSFAEG, 30 µM, Upstate Biotechnology) and [γ-32P]ATP (Amersham, 10 µCi/assay). The amount of 32P incorporated into crosstide was determined using p81 phosphocellulose papers (as above). The observed dpm values were corrected for non-specific binding by subtracting the background values (enzyme blank) obtained with mock immunoprecipitates.
ATP assay

Intact retinas were homogenized in 500 µL of ice-cold 6% PCA either immediately after extraction, or after 15 and 45 min of incubation in the explant culture media (above). The assay was performed exactly as described (161, 162, 180, 181), and measured using FluorMeasure software (C&L Instruments, Hummelstown, PA). Amounts of ATP were normalized to total protein in the sample, and expressed as nmol ATP/mg protein.

Akt-1, -2, and -3 in situ hybridization

The synthesis of 35S-labeled riboprobes and RNA in situ hybridization was performed essentially as described (182, 183) on optimal cutting temperature (OCT) embedded cryostat sections, 16 µm thick, of whole eyes from normal rats. Vectors for the sense and antisense riboprobes of Akt-1 (bp 1696-2306), -2 (bp 1632-2229), and -3 (bp 1476-1724) were kindly provided by Dr. Morris Birnbaum, University of Pennsylvania. Hybridization stringency was optimized by applying probes (10⁷ cpm/ml) in formamide hybridization buffer to sections, coverslipping, and incubating in a humidified chamber overnight at 55°C. Slides were washed in 4× SSC to remove the coverslip and buffer, dehydrated, and immersed in 0.3 M NaCl, 50% formamide, 20 mM Tris-Cl, 1 mM EDTA at 60°C for 15 min, followed by RNase A
(20 µg/ml) treatment for 30 min at 37° C. Slides were passed through graded salt solutions and washed in 0.1× SSC for 15 min and 0.05× SSC for 30 min at 60° C. Air-dried slides were exposed to Hyperfilm-βmax (Amersham) for 5-7 d, dipped in Kodak NTB3 photographic emulsion, stored with desiccant at 4° C for 10-14 d, and developed and stained with hematoxylin for evaluation. All sections were processed together to facilitate signal comparisons among the groups. This work was performed in the laboratory of Dr. Ian Simpson, Pennsylvania State University College of Medicine, Department of Neuroscience and Anatomy.

**Immunohistochemistry**

*Ex vivo* retinas (see above) were embedded in OCT compound (optimal cutting temperature, Sakura Finetek USA, Torrance, CA), following fixation in 2% paraformaldehyde and snap-frozen in dry ice-cooled 2-methyl-butane. Sections, 10 µm, from vehicle and 10 nM insulin treated retinas were mounted on the same slide and washed twice for 10 minutes at room temperature in PBS/0.1% Triton X-100 (PBST). The slides were blocked with 10% donkey serum diluted in PBST for 1 hr at room temperature before primary antibody incubations at 4° C overnight. Anti-phospho-Akt<sup>ser473</sup> (IHC-specific, Cell Signaling) was diluted 1:50, and the secondary antibody was diluted 1:1500 and conjugated to rhodamine red-X (Jackson Immunoresearch, West Grove, PA) and incubated for 1 hr at room temperature. Confocal images were captured with a Nikon E800 microscope equipped with a DIC
and a PCM2000 Multi-Line dual laser and visualized using Simple PCI (C Imaging Systems) and Adobe Photoshop software. The digital images were treated identically.

**Insulin ELISA**

Plasma insulin was measured by ELISA with a commercially available kit and performed according to the manufacturer’s protocol (Alpco; Windham, NH).

**Statistics**

Quantified results are reported as mean ± SEM, normalized to the controls. Data were analyzed by Student’s t-test or by one-way ANOVA followed by a multiple comparison post test, as described in the figure legends, using InStat 2.00 software (San Diego, CA). Statistical significance was considered for p < 0.05.
2.1 Figures and Legends

**Figure 3. Mutated pde6 expression in TTR-IR mice.** The TTR-IR mice, produced in part on an FVB background, express the mutant pde6 gene, resulting in an rd phenotype. PCR analysis of DNA from the first TTR mouse received (T) demonstrates the presence of the mutated gene (~500 bp product). Lanes 1-7 are DNA samples from seven mice that were backcrossed once with C57Bl/6 and intercrossed once, demonstrating the presence of the mutant pde6 allele and confirming earlier suspicions of an rd phenotype.
Figure 4. Breeding strategy to select for experimental TTR-IR littermates.
Because the original TTR-hIR+ mIR-/- mice carried the pde6 mutation, crosses to a background strain, C57Bl/6 were carried out and mice were selected for the TTR-hIR transgene. A second round of breeding with C57Bl/6 produced carriers of the TTR-hIR transgene, heterozygous mIR+/-, and wild-type for pde6. Breeding these mice produce experimental littermates that express the TTR-hIR transgene and are either mIR+/-, mIR+/-, or mIR-/- (100%, 50%, or 0% mIR expression in retina).
CHAPTER III

CHARACTERIZATION OF INSULIN SIGNALING IN RAT RETINA IN VIVO AND EX VIVO


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3.1 Abstract

Insulin receptor (IR) signaling cascades have been studied in many tissues, but insulin signaling in retina has received little attention. Retinal IR signaling and activity were investigated in vivo in rats that were freely fed, fasted, or injected with insulin, by phosphotyrosine (PY) immunoblotting and by measuring kinase activity. A retina explant system was utilized to investigate the IR signaling cascade, and immunohistochemistry was used to determine which retinal cell layers respond to insulin. Basal IR activity in the retina was equivalent to that in brain and significantly greater than that of liver, and remained constant between freely fed and fasted rats. Furthermore, IR signaling increased in the retina following portal vein administration of superphysiological doses of insulin. Ex vivo retinas responded to 10 nM insulin with IR β subunit (IRβ) and IRS-2 tyrosine phosphorylation and AktSer473 phosphorylation. The retina expresses mRNA for the three Akt isoforms as determined by in situ hybridization, and insulin specifically increases Akt-1 kinase activity. Phospho-AktSer473 immunoreactivity increases in retinal nuclear cell layers with insulin treatment. These results demonstrate that the retinal IR signaling cascade to Akt-1 possesses constitutive activity, and that exogenous insulin further stimulates this pro-survival pathway. These findings may have implications in understanding normal and dysfunctional retinal physiology.
3.2 Introduction

The IR signaling network induces pleiotropic effects at the cellular and molecular level, and has been under intense investigation in liver, skeletal muscle, and adipose tissue for better understanding of health and disease (1, 184, 185). Upon ligand binding, the IRβ subunit undergoes a conformational change that activates the intrinsic tyrosine kinase leading to receptor autophosphorylation and the IR signaling cascade. Ultimately, insulin stimulates many cellular events including glucose, amino acid, and fatty acid uptake, induces protein, glycogen, and triglyceride synthesis, and promotes cell survival while inhibiting apoptosis. Comparatively, little is understood regarding the role of IR signaling in neural tissue.

The IR expressed in retina has been characterized by its binding capacity, kinase activity, mobility on SDS-PAGE, and immunohistochemistry (31, 33, 34, 37). It is expressed in all retinal layers and is homologous to neuronal IRs in brain that feature less α subunit glycosylation and increased mobility on SDS-PAGE as compared to liver IRs. Retinal and liver IR kinase activities are similar in vitro; in vivo, brain IR kinase activity remains constant through feeding and fasting in contrast to liver IR kinase activity which diminishes in the fasted state (40). Binding studies have also demonstrated the presence of the IR in the retinal microvasculature (77).

In brain, IR, IRS-1, and PY immunoreactivity colocalize, suggesting a role for insulin action in neural tissue metabolism, growth, and/or survival (106, 186, 187). Other studies have revealed that the retina expresses a number of proteins associated
with insulin signaling. Folli et al (105) reported IRS-1 and PI3K immunoreactivity in rat retina. The majority of IRS-1 localized to the ganglion cell layer, but cells of the inner nuclear layer, mostly bipolar and amacrine cells, were also immunoreactive for IRS-1. Gosbell et al extended those observations in rat retina by demonstrating IRS-1 expression in the rod outer segments (50), and Diaz et al demonstrated that Akt and, to a lesser extent, ERK 1/2 phosphorylation increases following superphysiological insulin treatment for 24 h in whole embryonic chick retina (107). The Ras and ERK 1/2 pathway may play an important role in neuroretina development as its activity diminishes upon differentiation. Oncogenic Ras transformation of chick neuroretina cells induces proliferation via the Raf/MEK/ERK pathway (188). Collectively, these reports suggest that the insulin signaling pathway is important in retinal physiology.

Activation of the ser/thr kinase Akt by phosphoinositide 3-kinase (PI3K) is necessary for the pro-survival mechanism of insulin action (189). Akt inhibits apoptosis by phosphorylating a number of apoptosis regulators including caspase-9 (21), Bad (22), GSK-3β (190), IKK (191), and apoptosis signal-regulating kinase-1 (192) in the cytosol. Akt translocates to the nucleus to phosphorylate Forkhead family transcription factors (25, 193). Akt function is particularly important for neuronal cell survival. Insulin-induced Akt activation decreases rates of apoptosis in a PI3K-dependent manner in serum-starved cerebral granular neurons (27), and activated Akt inhibits apoptosis by preventing p53 transcriptional activity in hippocampal neurons (23). Barber et al (111) demonstrated Akt-dependent cell survival in cultured retinal neurons in response to insulin and IGF-1. Treatment of
the retinal neurons with insulin and IGF-1 activated Akt in a PI3K-dependent manner and reduced apoptosis induced by serum starvation. However, the roles of each Akt isoform in the central nervous system and retina remain unclear.

Prior studies have not examined the effect of systemic insulin administration on the retina, so proximal retinal insulin signaling characteristics were examined. Retinal IR activation led to activation of Akt both in vivo and ex vivo. In vivo, the retinal IRβ was tonically phosphorylated and kinetically active. The retinal IR was relatively insensitive to changes in circulating insulin under mildly fasted conditions or with an insulin injection (50 µg). Higher doses (500 µg) increased retinal IRβ tyrosine phosphorylation. The retina retained the ability to respond to further insulin stimulation, and a retinal explant culture system (ex vivo) was used to explore how additional insulin stimulation signals in retina. Physiological insulin concentrations increase IRβ autophosphorylation and Akt^ser473^ phosphorylation in the retinal nuclear layers in ex vivo cultures. Akt 1-3 mRNAs are expressed in retina, and only Akt-1 kinase activity increased with insulin. These results demonstrate that the IR in retina, when stimulated, generates an intracellular signal that is mediated through specific Akt isoforms.
3.3 Results

3.3.1 Rat retina expresses abundant IR

To gain insight into the expression levels of IR in retina, immunoblot analysis of the IR β subunit was performed and compared to liver and brain. Equivalent amounts of protein from tissue lysates (25 µg) were probed with antibodies that recognize the IRβ following SDS-PAGE. The results reveal equivalent expression among retina, brain, and liver (Fig. 5, pg. 82), suggesting an active physiological role for the IR in retina. The IRβ in retina and brain migrates slightly faster on SDS-PAGE compared to liver IRβ, suggesting less extensive glycosylation of retinal IRβ as has been reported for IRα (33, 111).

3.3.2 Intravenous insulin administration increases retinal IRβ tyrosine phosphorylation

In order to determine whether acute elevation in circulating insulin activates retinal IR in vivo as in other tissues, IR autophosphorylation was measured after an insulin injection. A bolus (500 µg or 50 µg) of insulin was administered intraportally to normal fasted rats (weight ~ 250 g). This dose and route of administration has been used to stimulate peripheral IR autophosphorylation (75, 194). At 15, 30, 45, and 60 minutes post injection, gastrocnemius muscle and retina were snap frozen and
analyzed for IRβ PY content by immunoprecipitation and immunoblotting of the tissue lysates. Representative PY immunoblots for the 500 µg dose (Fig. 6A, pg. 83) from the same rats at each time point demonstrated a robust response in muscle and relatively protracted retinal IRβ autophosphorylation. The membranes were stripped and reprobed for IRβ to demonstrate equivalent immunoprecipitations for normalization (not shown). Quantification of the immunoblots and expression of the data in terms of PY/IRβ ratios demonstrated a ~30-fold increase in PY content in muscle IRβ 15 min post-injection which peaks at 30 min (Fig. 6B, pg. 83). Retinal IRβ phosphorylation, unlike muscle, did not increase significantly until 30 min post-injection, and remained only 3-4 fold elevated above vehicle injected controls for 60 min (Fig. 6C, pg. 83). This difference in magnitude of the fold increase of IR autophosphorylation in retina tissue may be due to the higher basal IRβ autophosphorylation in the retinas of vehicle treated rats compared to muscle in the same rats (Fig. 6A, pg. 83), and to the blood-retinal barrier (BRB), which limits diffusion. Retinal lysates were also analyzed for Aktser473 phosphorylation at 45 minutes post-injection, when IRβ phosphorylation was maximal. Insulin induced a 48% increase (p < 0.05) in Aktser473 phosphorylation over vehicle injected controls; there was no statistical difference between sham and vehicle injected controls (Fig. 6D, pg. 83).

The 50 µg dose of insulin elicited no detectable change in the tonic retinal IRβ phosphorylation state after 15 and 45 minutes (Fig 6E). This result is in contrast to
that from skeletal muscle of the same rats in which basal IRβ was barely detectable and increased with the insulin injection. These results indicate that systemic insulin may not cross the intact BRB as rapidly as other peripheral tissues.

Superphysiological insulin further activates the IR signaling cascade above basal states in retina in a dose-dependent manner, but with a different temporal course than muscle, illustrating important differences between retinal insulin signaling compared to other peripheral tissues.

3.3.3 IRβ PY content and kinase activity remain constant in retina between fasted and freely fed rats

Since retinal IRβ autophosphorylation increased in response to acute superphysiologic insulin administration, it was examined whether the tyrosine phosphorylation and kinetic activity of the IRβ in retina diminished in fasted animals as it does in liver and muscle. Simon et al previously demonstrated that brain IRβ kinase activity did not change in fasted chickens (40). Therefore, retina, liver, and gastrocnemius muscle from six freely fed or fasted (18 hrs) rats were homogenized, subjected to immunoprecipitation of the IRβ, and blotted for PY content (Fig 7A and 7B, pg. 85). As demonstrated above, retinal IRβ displayed basal tyrosine phosphorylation, and image analysis of the blots from retina revealed no statistical difference in IRβ phosphorylation between freely fed and fasted rats (Fig. 7B, pg. 85). As expected, IRβ phosphorylation was significantly elevated in skeletal muscle
and liver (p<0.01 and p<0.001, respectively) from freely fed rats compared to fasted controls. Therefore, in contrast to liver and skeletal muscle, the IRβ PY content in retina remains stable despite short-term physiological changes in nutritional status and circulating insulin (Table 2, pg. 87).

IRβ immunoprecipitates were also subjected to autophosphorylation assays with the addition of ATP to the immune complex followed by PY and IRβ immunoblot analysis and quantification. Consistent with the results above, retinal IRβ autophosphorylation rates were not different between fasted and freely fed rats, but in liver and skeletal muscle, IRβ autophosphorylation activity was increased 150% (p<0.01) and 75% (p<0.05), respectively, in the freely fed state (not shown). When IRβ immune complexes were incubated with an IR substrate, poly Glu:Tyr (195), and ATP, the rate of kinetic activity was maintained in fasted rat retina and brain in contrast to liver IRβ which was significantly decreased by 50% (p<0.05, Fig. 7C, pg. 85). Together, these data demonstrate striking differences in insulin signaling physiology within a whole animal. Retinal IR phosphorylation and activity, similar to those in brain, maintain tyrosine phosphorylation and activity during fasting conditions.

3.3.4 The IRβ is autophosphorylated by insulin treatment in ex vivo retina

Despite tonic retinal IRβ tyrosine phosphorylation, activity, and relative stability to circulating insulin levels, the retina possesses a reserve capacity to
respond to further insulin stimulation, as in other insulin-sensitive tissues. We exploited this characteristic to better understand insulin signaling mechanisms in retina, in an *ex vivo* organ culture system. This model mimics brain slice studies and has been used by us (161, 162) and others (163) to investigate retinal metabolism and effects of growth factors on the retina. Following dissection, retinas were treated with either 10 nM insulin or vehicle and analyzed as above. Increased IRβ autophosphorylation was detected with 1 nM insulin (not shown), and 10 nM insulin is sufficient to induce IRβ phosphorylation of cultured retinal neurons (111). The retinal IRβ displayed lower basal tyrosine phosphorylation (Fig 8A, 8C, and 8D; pg. 88), and within two minutes of 10 nM insulin stimulation, the IRβ exhibited nearly 4-fold greater PY immunoreactivity, which remained elevated 3-fold but was diminishing after 30 (Fig. 8A and 8B, pg. 88) and 60 minutes (not shown) of insulin stimulation. Expression of the IR in this model is stable (Fig. 8A, pg. 88). IGF-IRβ PY content remained stable with 10 nM insulin treatment demonstrating no insulin-induced activation of the IGF-IR in this model (Fig 8C, pg. 88). Because IGF-II is also a ligand for the IR (52, 196), and IGF-II mRNA has been detected in adult rat brain (197), a dose response study was performed with IGF-II on retinal IRβ activation in retinal explants (Fig. 8D, pg. 88). IGF-II (10 nM) had no effect, while 100 nM and 1 µM IGF-II maximally stimulated phosphorylation of the IR. Total ATP levels were measured as described (161, 162, 181), and they remained constant throughout the duration of the experiment (always > 9.8 nm ATP per mg of protein) indicating that the tissue remains energetically viable. Therefore, isolated retina
tissue responds to physiological insulin concentrations with IRβ tyrosine phosphorylation, and the BRB, bypassed in this model, may play a significant role in regulating circulating insulin transport and action on the neural retina.

3.3.5 *Retina tissue expresses Akt-1, -2, and -3 mRNA*

To better understand Akt-mediated survival in retina, cRNA *in situ* hybridization was performed to determine which Akt isoforms are expressed in retina. Both sense and anti-sense strands were hybridized to 16 µm cryostat sections, and representative dark-field images are shown in Figure 9 (pg. 90; courtesy of Dr. I. Simpson and M. Klinger, Dept. of Neural and Behavioral Sciences, Penn St. College of Medicine). Akt-1 mRNA localized to every cell layer of the retina, consistent with its broad distribution among different tissues (198). Akt-2 mRNA expression localized mainly to the cells of the inner nuclear layer and, to a lesser extent, to the ganglion and astrocyte cells of the innermost retinal layer and the outer segments of the photoreceptors (outer nuclear layer). Akt-3 mRNA expression follows a similar profile as Akt-1, consistent with reports in human tissue that Akt-3 is highly expressed in neuronal cells (199). Therefore, all Akt isoforms may potentially play a role in maintaining retinal survival and/or metabolism.
3.3.6 Insulin causes isoform-specific Akt activation in retina

Akt regulates glucose and glycogen metabolism in many insulin responsive tissues, and mediates survival in neurons and vascular cells in response to insulin (27, 78, 111). Using the ex vivo retina culture system described above, the characteristics of retinal Akt activation were investigated in response to insulin. Akt was maximally phosphorylated 2-fold above basal conditions (p<0.05) on serine 473 five minutes after the addition of insulin (Fig. 10A and 10B, pg. 91). Akt phosphorylation then declined to basal levels 30 minutes following the addition of insulin, possibly due to active phosphatases, and there was no statistical difference between vehicle and insulin treated retinas after 30 minutes. However, this data was dependent on antibodies that recognize phospho- and total Akt 1-3, and little is known about specific Akt isoform activity in response to different growth factors in retina.

Therefore, Akt kinase assays were performed using isoform-specific antibodies on ex vivo retinas stimulated with insulin for 5 minutes to determine which Akt isoforms were activated (179). This time point was chosen because ser 473 phosphorylation is maximal by immunoblot analysis (Fig. 10A and 10B, pg. 91). In retinal explants, we observed a two-fold increase in activation of Akt-1 in response to insulin (p<0.05), but no change in Akt-2 or Akt-3 activity (Fig. 10C, pg. 91). These results suggest that insulin preferentially activates specific Akt isoforms in retina. These data also suggests that the increase in ser 473 phosphorylation by immunoblot analysis reflects mostly Akt-1 kinetic activity in retina. Comparisons of retinal Akt-1
activity were also made to liver and muscle tissue from fasted (18 hrs) rats. Interestingly, retina tissue exhibited significantly more Akt-1 activity than liver and skeletal muscle (Fig 10D, pg. 91), demonstrating that the retina maintains higher Akt-1 kinase activity than other insulin responsive tissues. Taken together with tonic IRβ phosphorylation in the basal state, the IR→Akt signaling system in retina appears constitutively active compared to other insulin responsive tissues and may play a significant role in cell survival and metabolism.

3.3.7 Phospho-Ser473-Akt immunoreactivity increases in ex vivo retina
nuclear layers

To understand which retinal layers respond to insulin, explanted retinas were fixed following insulin or vehicle treatment, and cross sections were immunostained for Aktser473 phosphorylation. Panel A of Figure 11 (pg. 92) shows a histological section of a vehicle treated retina in which low-level phospho-Aktser473 is observed diffusely throughout the retina. With insulin treatment (Fig. 11, panel B, pg. 92), the immunoreactivity for phospho-Aktser473 is increased in the nuclear layers and the innermost portion of the retina suggesting numerous retinal cell types are insulin responsive.
3.3.8 Insulin stimulates IRS-2 tyrosine phosphorylation in retinal explants

IRS-1 and -2 are substrates for the IRβ kinase, and IRS-1 expression has been observed in rat retina (50, 105). Using the same time course for retinal explants, IRS-1 and -2 phosphorylation were investigated by immunoprecipitation and PY immunoblotting. Basal IRS-1 tyrosine phosphorylation was detected, but 10 nM insulin does not further increase the phosphorylation over the time course (Fig 12A, pg. 93). When normalized to total IRS-1 content and quantified, there is no significant change in IRS-1 tyrosine phosphorylation. IRS-2 tyrosine phosphorylation, however, was substantially increased 125% in response to insulin (Fig. 12B, pg. 93), suggesting that IRS-2 may mediate the insulin signal more than IRS-1. This is the first demonstration that insulin receptor activation induces IRS-2 tyrosine phosphorylation in retina, and the roles of IRS-1 and IRS-2 in retinal insulin signal transduction require further definition.

In addition to Akt, the Ras/ERK signaling pathway was also investigated in the retinal explant system, by immunoblotting for phosphorylated ERK 1/2. Similar to Akt, detection of basal phosphorylation of ERK 1/2 was observed, and there was no change with 10 nM insulin treatment by immunoblot analysis after 30 min (not shown). This implies that the ERK 1/2 pathway is either not a major mediator of IR signaling in adult retina, or ERK 1/2 phosphorylation is already nearly maximal and further stimulation with insulin is not detectable by immunoblot analysis.
3.4 Discussion

The purpose of this study was to examine retinal IR activity and signaling characteristics. The results presented describe proximal insulin signaling physiology in normal rat retina using in vivo and ex vivo approaches. The major findings of the in vivo studies are that the retinal IRβ exhibits constitutive tyrosine phosphorylation and activity that increases with superphysiological insulin administration via the portal vein (Fig. 6, pg. 83), and remains stable during feeding and fasting in normal rats (Fig. 7, pg. 85). Ex vivo studies reveal that IR activation increases IRβ (Fig. 8, pg. 88), IRS-2 (Fig. 12, pg. 93), and Akt phosphorylation, and Akt-1 kinase activity (Fig. 10, pg. 91). IRS-1 (Fig. 12, pg. 93) and ERK 1/2 (not shown) phosphorylation remained stable in response to 10 nM insulin. Furthermore, insulin increased phospho-Aktser473 immunoreactivity in retinal nuclear layers (Fig. 11, pg. 92). This is the first demonstration of physiologic retinal insulin signaling characteristics in rats.

A critical observation in this study is that the IRβ in retina displays constitutive tyrosine phosphorylation and kinase activity despite variable nutritional status and circulating plasma insulin. These results in retina are similar to those described in chicken brain (40). As expected, the kinetics of such activation are delayed when compared to IR activation in skeletal muscle because the transport of insulin across the blood-retinal barrier is slower. Peripherally administered insulin binds to retinal microvasculature (77) and pericytes (72), is detected in the vitreous fluid (73), and crosses the blood-brain barrier of rats (200). This is the first
demonstration of exogenous insulin activating retinal IRs in vivo. Because of the stability in retinal IR phosphorylation and kinase activity compared to muscle, insulin transport and IR activation in retina may not play a major role in short-term nutrient uptake, but may provide a tonic survival signal. The elevated basal activity of retinal IRs may result from a lower rate of receptor internalization or dephosphorylation, increased ligand stabilization, or an undefined mechanism. Additionally, insulin further stimulated IRβ phosphorylation in rats under normal illumination without dark adaptation suggesting that retinal IRs can be activated by both ligand-dependent and –independent mechanisms (124). Budd and colleagues (92) have reported preproinsulin mRNA expression in rat retina, but whether insulin protein is secreted by retinal cells and contributes to the basal tyrosine phosphorylation of retinal IRs in an autocrine or paracrine fashion is unknown. Locally produced IGF-I or IGF-II may also be a source of basal IR phosphorylation in retina. Evidence points to cross-talk among insulin, the IGFs, and their cognate receptors (196), with further specificity derived from coordinated expression of IGF binding proteins. We show retinal IR activation with IGF-II stimulation; although, greater amounts were required than what has been reported in the vitreous cavity (167), but that may not represent local IGF-II within the retinal extracellular space. Further specificity for retinal IR activation may be a result of exon 11 splicing, in that the IR-A form (-exon 11) has a higher affinity for IGF-II than IR-B (+exon 11), even when hybridized to IGF-1Rs (52, 201). Since the retina, like the brain, expresses IR-A exclusively (49, 50), ligands other than insulin may play a role in tonic IR activity. The source of basal retinal IR tyrosine
phosphorylation and activity requires further investigation. Thus, it is hypothesized that retinal insulin signaling is in a constitutively higher steady-state, and the basal phosphorylation and activity of the IR and Akt maintain a tonic cell-survival signal in the metabolically active retina.

Whole retinal explants, similar to classic brain slice studies, provide an ideal model to study insulin signal transduction as the blood retinal-barrier is removed. Insulin rapidly induces IR\(\beta\) phosphorylation in explant retinas, and the IR preferentially phosphorylates IRS-2 to a greater extent than IRS-1. Insulin also appears to activate Akt-1 compared with Akt-2 and Akt-3. Together, insulin activates an IR\(\rightarrow\)IRS-2\(\rightarrow\)Akt-1 signaling cascade in retina that does not further increase ERK 1/2 phosphorylation, yet other signaling molecules may be involved to mediate the retinal response to insulin. The significance of preferential Akt isoform activation with insulin is unclear. Akt-1 has the broadest tissue expression, while Akt-2 is restricted to the classically insulin-responsive tissues and Akt-3 to neuronal cells and testis (198, 202, 203). Akt, like the IR, exhibits basal phosphorylation and activity in retina \textit{in vivo}; but, insulin promoted Akt phosphorylation \textit{in vivo} and increased Akt\textsuperscript{ser473} immunoreactivity in the retinal nuclear layers suggesting numerous cell types, including photoreceptors and ganglion cells, respond to insulin in the intact retina. This is consistent with the finding of broad IR expression in retina tissue (51). Akt nuclear translocation has been reported in hippocampal neurons in a cerebral ischemia reperfusion model (204), suggesting Akt\textsuperscript{ser473} phosphorylation is a critical event promoting neuronal survival, and other proteins, such as Tcl1, may also play a
significant role in neuronal survival by aiding Akt translocation (193). Further investigation is required to determine the nature of retinal insulin signaling in plexiform layers (109) and the function and regulation of each Akt isoform in retina.

IRs are broadly expressed within the central nervous system, and their signals affect whole organism feeding behavior, cellular metabolism, and survival. Based on gene deletion studies, IRS-1 and IRS-2 have distinct functions despite being immediate substrates for the IR (8-10). This and other studies (205, 206) also suggest that IRS-2 is the preferred docking substrate in neurons in response to insulin or IGF-I. Interestingly, brain development and survival is normal in neuron-specific IR knock-out mice. This suggests that IR signaling in brain does not influence development and is most likely complemented by other growth factors (207) because ablation of IRS-2 results in reduced brain growth during development and increased neurodegenerative characteristics such as tau hyperphosphorylation (208). Numerous other receptor tyrosine kinases phosphorylate and signal via IRS-1 or IRS-2 in neurons, such as the Trk receptors (209). Therefore, one may predict IRS-2 gene disruption would disrupt retinal and other neuronal functions to a greater degree than disruption of a single neuronal growth factor receptor if a lack of IRS-2 dampens or terminates the signal of many receptors (210). PI3K is downstream of IRS proteins, and it has been detected immunohistochemically (105) in the same retinal layers that we have described IR signaling action. The expression pattern of all the p85/p110 subunits and isoforms is currently unknown, but there is an association between the
IR and p85/p110α in retinal outer segments (123), and a role for PI3K in retinal development (211).

Numerous studies have shown that retinal insults that induce apoptosis are overcome with growth factor treatment. Axotomized rat ganglion cells die by apoptosis via caspase-3 activation, and IGF-I and BDNF treatment in vivo reduce ganglion cell death (212, 213). Following hypoxia, IGF-I mediates protection of retinal cells (214). In terms of diabetes, increased apoptosis has been demonstrated in retinal neurons cultured in excess hexosamines, experimental animal models, and in humans; with insulin treatment, retinal apoptosis was decreased in cultured retinal neurons and in the retinal neurons of diabetic animals (130, 180, 215). Taken together, retinal neurons and vascular cells that are induced to undergo apoptosis in vitro and in vivo appear to be rescued by insulin and other growth factors and, consequently, Akt pathway activation. Retinal vascular cells, neurons, and pigmented epithelium undergo apoptosis in various retinal disease states such as diabetic retinopathy, macular degeneration, and glaucoma (130, 215-217). Therefore, insulin or other growth factors that activate the anti-apoptotic kinase Akt may prove beneficial in preserving retinal cell survival and function.
3.5 Figures, Tables, and Legends

Figure 5. Immunoblot analysis of IRβ from rat liver (L), retina (R), and brain (B) lysates. Equal protein (25 µg) was separated on 7.5% SDS-PAGE. A representative immunoblot is shown of four experiments. The mean percent ± SEM intensity of the IRβ band is listed below each representative sample (retina set to 100%) demonstrating no statistical difference in IR expression between liver, retina, and brain tissue. Note the slightly faster migrating IRβ in retina tissue, compared to liver, as has been reported in brain probably due to less glycosylation in neurons.
Figure 6. Intraportal insulin activates retinal insulin receptors in vivo. Rats (~250 g) received either nothing (sham), vehicle (0.9% saline), or insulin (500 µg, A-D, or 50 µg, E) via the portal vein. (A) At the indicated times (minutes), gastrocnemius muscle and retina were snap frozen under liquid nitrogen and the tissue lysates were analyzed as described in Methods. A representative PY blot is shown from both muscle and retina in a rat that received either a vehicle (V) or insulin (I, 500 µg) injection. There was no difference in IRβ phosphorylation between vehicle and sham operated rats (not shown). (B) Muscle PY blots were stripped, reprobed for total IRβ, quantified, and the data expressed as fold increase of PY/IRβ ratios with the 0 time point set to 1 (n=5 per time point per treatment). IRβ phosphorylation significantly increased with insulin at all time points examined (** p < 0.01 different from 0 time point by ANOVA and Dunnett’s post test). (C) Retina
PY blots were stripped, reprobed for total IRβ, quantified, and the data expressed as fold increase of PY/IRβ ratios with the 0 time point set to 1 (n=5 per time point per treatment). A significant increase was first observed at 30 minutes after insulin administration. IRβ phosphorylation was maximal after 45 min, and remained elevated for 60 min (* p < 0.05 and ** p < 0.01 different from 0 time point by ANOVA and Dunnett’s post test). IRβ phosphorylation did not change in vehicle injected animals. (D) 45 min after insulin administration, when IRβ phosphorylation was maximal following a 500 µg insulin bolus, retinal lysates were examined for activated Akt. The inset depicts a representative immunoblot of phospho-Akt_{ser473} (top) and total Akt (bottom) from sham (S, n=3), vehicle (V, n=3), and insulin (I, n=4) injected rats. Quantification of the blots and expression in terms of phosphorylated to total Akt ratios (V set to 1.0) demonstrates a significant increase in Akt_{ser473} phosphorylation in retinas of insulin injected rats (* p < 0.05 by ANOVA and Tukey-Kramer post test). (E) Rats received either nothing (sham, S), vehicle (V), or 50µg of insulin (I) via the portal vein and were sacrificed after 15 and 45 min for analysis of skeletal muscle and retinal IRβ phosphorylation as above. As expected, skeletal muscle IRβ phosphorylation increased, but there was no significant change in retinal IRβ phosphorylation among sham, vehicle, and insulin injected rats over the time course, although basal IRβ autophosphorylation was observed in retina. Results are representative of three independent experiments.
Figure 7. Retinal IRβ phosphorylation remains constant in freely fed and fasted rats. Experiments were performed on fasted (18 hrs) or freely fed rats. (A) Tissue lysates were immunoprecipitated and blotted for IRβ tyrosine phosphorylation, which was found to be unaltered in retina, but increases in muscle and liver compared to fasted rats (1-2, retina; 3-4, muscle; 5-6, liver; 1, 3, and 5, fasted rats; 2, 4, and 6, fed rats, n=6 per group). (B) Quantification of the blots in (A) and expressed as PY/IRβ ratios. There was no statistical difference in IRβ phosphorylation in retina tissue between fed and fasted rats. In the freely fed state, muscle and liver IRβ phosphorylation was significantly greater than retina IRβ (** p < 0.01 and *** p < 0.001 by ANOVA and Student-Newman-Keuls post test over retina PY/IRβ ratio.
from fed rats). (C) IR kinase assays were performed on liver, retina, and brain lysates from fasted (18 hr, n=4) and freely fed rats (n=4). Similar to what has been reported in brain, retinal IR kinase activity remains stable (p=0.80), while liver IR kinetic activity is significantly reduced in fasted rats compared to both retina and brain (* p<0.05 by ANOVA and Student-Newman-Keuls post-test).
Table 2. Circulating insulin and glucose were significantly lower in fasted rats (18 hr) compared to freely fed rats. Whole blood was collected at the time of sacrifice for insulin and glucose analysis. Results are mean ± SEM of n=6 rats per group, and the P values were determined by Student’s t-test.

<table>
<thead>
<tr>
<th></th>
<th>Fasted Rats</th>
<th>Fed Rats</th>
<th>P</th>
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<tbody>
<tr>
<td>Insulin, ng/ml</td>
<td>0.32 ± 0.049</td>
<td>1.95 ± 0.36</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>68.8 ± 2.4</td>
<td>95.5 ± 2.6</td>
<td>&lt;0.0001</td>
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Figure 8. The IRβ is tyrosine phosphorylated by insulin in ex vivo retinal explants. An explant culture system was used to study insulin signal transduction in retina without the influence of the blood-retinal barrier. (A) The IRβ was immunoprecipitated from retinal lysates and blotted for PY (top panel) and total IRβ (bottom panel) after the indicated times (min) of treatment with 10 nM insulin. (B) Quantification of the immunoblots reveal a significant increase in IRβ.
phosphorylation at all time points examined when normalized to total IRβ (n = 3 per time point, ** p < 0.01 from 0 minutes by ANOVA and Dunnett’s post test). (C) Insulin (10 nM) does not activate the IGF-IRβ in retinal explants. Retinas were treated with the indicated concentrations of IGF-I or insulin and subjected to immunoprecipitation of the IGF-IRβ followed by PY Western blotting. 100 nM insulin and 1.3 nM IGF-I treatment resulted in equivalent IGF-IRβ phosphorylation. IGF-IRβ immunoprecipitates gave rise to a doublet upon PY immunoblotting; the lower band is the IRβ from hybrid receptors (not shown). Results are representative of three experiments. (D) IGF-II (10 nM) does not activate the IRβ in retinal explants. Retinas were treated with the indicated concentrations of insulin or IGF-II and analyzed for IRβ phosphorylation. 100 nM and 1 µM IGF-II treatment both resulted in maximal phosphorylation of the IR. The IRβ immunoprecipitates gave rise to a doublet upon PY immunoblotting; the upper band is the IGF-IRβ from hybrid receptors (not shown).
Figure 9. Rat retina expresses Akt 1-3 mRNA species. cRNA in situ hybridization was performed for all three Akt isoforms in intact eyes with anti-sense (AS) and sense (S) probes and counterstained with hematoxylin. Representative dark field images are shown. RPE, retinal pigmented epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; G/A, ganglion and astrocyte layer. Akt-1 and -3 mRNA expression have similar profiles among all retinal layers, while Akt-2 mRNA is observed primarily in the INL and G/A layer. Akt 1-3 mRNA is also expressed in the outer segments of the photoreceptors (arrows). Results are representative of duplicate experiments.
Figure 10. Insulin induces isoform-specific activation of retinal Akt. (A) Lysates of retinal explants of untreated (basal, B) retinas and those with vehicle (V) or 10 nM insulin (I) for the indicated times (minutes) were blotted with antibodies against phosphorylated Akt<sup>ser473</sup> (top panels) and total Akt (bottom panels). Akt expression was maintained throughout the time course. A representative immunoblot from one experiment is shown. (B) Quantification of the immunoblots reveal a maximal increase in Akt phosphorylation after 5 minutes (n=4-6 per time point, * p < 0.05 from 0 time point by ANOVA and Dunnett’s post test). This was followed by Akt dephosphorylation to the starting point, which was not significantly different among basal and insulin or vehicle treated retinas for 30 min. (C) Akt-1, -2, and -3 kinase assays performed on retinal explants reveal a nearly two-fold increase in kinetic activity of Akt-1 after five minutes of 10 nM insulin treatment (* p<0.05). There was no significant change in Akt-2 or -3 kinase activity. (D) Akt-1 kinetic activity was compared among equivalent protein immunoprecipitations from retina, muscle, and liver of fasted (18 h) rats. Muscle and liver had 29% and 62%, respectively, of retina Akt-1 activity per mg of protein (* p<0.05 and ** p<0.01 from retina by ANOVA and Tukey-Kramer post test, n=3).
Figure 11. Phospho-Akt<sup>ser<sup>473</sup></sup> immunoreactivity increases in retinal nuclear layers after insulin treatment. Retinal explants were performed as described followed by immunohistochemical analysis of phosphorylated Akt following five minutes of vehicle (panel A) or 10 nM insulin (panel B) treatments. G/A, ganglion and astrocyte cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer (photoreceptor cells). The intact retina is separated from the RPE cell layer in this model. Phospho-Akt<sup>ser<sup>473</sup></sup> is detectable in vehicle treated retinas and is expressed throughout all retinal layers. Insulin increases phospho-Akt<sup>ser<sup>473</sup></sup> immunoreactivity in the ONL, INL, and G/A (arrow) layers.
Figure 12. Insulin stimulates IRS-2 tyrosine phosphorylation in retinal explants. Retinal lysates treated with 10 nM insulin \textit{ex vivo} were analyzed by immunoprecipitating (IP) for IRS-1 and -2, followed by PY immunoblot analysis and normalizing to respective IRS content. (A) IRS-1 tyrosine phosphorylation did not significantly increase over the time course (n=8 per time point). (B) IRS-2 phosphorylation, however, responded robustly to insulin with a 125% increase in tyrosine phosphorylation after two minutes followed by a gradual dephosphorylation to basal levels (n=4 per time point). IRS-1 and -2 expression was constant during the time course.
CHAPTER IV

DIABETES REDUCES CONSTITUTIVE INSULIN RECEPTOR SIGNALING IN RETINA: A POSSIBLE MECHANISM FOR INITIATION OF RETINOPATHY
4.1 Abstract

It has been demonstrated that the retina is insulin-responsive and possesses constitutive insulin receptor (IR) activity in vivo. Hyperglycemia has been associated with the development and progression of diabetic retinopathy (DR), but the role of insulin signaling and the effects of diabetes in the retina has received little attention. Using the streptozotocin (STZ)-induced diabetic rat model, we tested the hypothesis that diabetes diminishes retinal IR signaling within the time frame of increased retinal apoptosis. The expression, phosphorylation status, and/or kinase activity of the IR, insulin receptor substrate- (IRS) 1 and 2, phosphatidylinositol-3 kinase (PI3K), Akt, and glycogen synthase kinase (GSK) 3β proteins were investigated in retinas of age-matched control, diabetic, and insulin-treated diabetic rats. Four weeks of STZ diabetes reduced IR, IRS-1/2-associated PI3K, and Akt-1 kinase activity without altering IR or IRS-1/2 expression and tyrosine phosphorylation or Akt expression and thr308/s473 phosphorylation. Sustained insulin treatment of STZ diabetic rats for eight weeks restored IR kinase activity and GSK3β ser9 phosphorylation. When incubated in normoglycemic media, intact retinas of diabetic rats had increased IRβ autophosphorylation following insulin treatment compared to retinas from normal rats. These results suggest that experimental diabetes impairs the constitutive retinal insulin signaling pathway with features in common with and distinct from those in peripheral tissues. Thus, dysfunctional IR signaling in retina may contribute to the initial stages of DR such as vascular and neuronal apoptosis.
4.2 Introduction

Diabetic retinopathy (DR) has been generally considered as a microvascular disorder that results from consequences of excess glucose. Polyol pathway activation, nonenzymatic glycation, oxidative stress, leukostasis, and protein kinase C activation have been related to its development (218-220). However, a growing body of evidence suggests that the neural retina undergoes significant alterations, including diminished electroretinogram response, color vision, and contrast sensitivity, in human and rodent diabetes prior to the onset of vascular abnormalities (137, 221, 222), and a clinical diagnosis of DR. Thus, deciphering the early changes in retina is paramount to understanding the pathogenesis of DR, and to limit early retinal neuronal and vascular apoptosis (130, 215).

In spite of the recognized importance of peripheral IR signaling in diabetes, a role of retinal insulin deficiency has received little attention because insulin has not been considered to have a significant role in retinal physiology. However, recent evidence has accumulated to suggest that insulin does activate IRs in retina (30). The retina expresses equivalent amounts of the IR protein compared to liver, and retinal IRs are capable of activating downstream kinases (30, 33, 34, 95). Less extensive glycosylation of the IR in the CNS permits the α and β subunits to migrate ~10 and ~2 kDa farther, respectively, on SDS-PAGE (30, 41), and rat retina tissue primarily expresses the IR-A (~exon 11) splice variant (50). For the IRα, a gel shift is observed in retinas from patients with Type 1 diabetes and in STZ-diabetic rats with slightly
greater expression of the neuronal IRα with less glycosylation (141, 223), and we have also reported impaired IR processing in cultures of retinal neurons treated with glucosamine (180). The homologous IGF-IR has also been examined in retinas of diabetic animals. Gerhardinger et al (224) reported decreased IGF-I mRNA expression in retina, but basal IGF-IRβ tyrosine phosphorylation and total Akt<sup>ser473</sup> phosphorylation were maintained. In normal rats, the autophosphorylation and kinase activity of retinal IRs are equivalent to liver IR from fed animals, is elevated compared to liver IRs from fasted rats, and, in contrast to IRs from liver and muscle, does not fluctuate with circulating insulin. The basal activities of PI3K, Akt-1, and p70S6K are also greater in retina than in liver and muscle [(30), Wu and Gardner, manuscript submitted]. Furthermore, exogenous insulin stimulation of whole retina tissue results in activation of the Akt branch of the insulin signaling network with no change in ERK 1/2 phosphorylation (30). In particular, insulin stimulates Akt phosphorylation within the same retinal layers where apoptosis is increased in diabetic rats and humans, suggesting that the loss of insulin and/or excessive metabolic substrates such as hyperglycemia, hyperlipidemia, or elevated amino acids, may perturb retinal function and contribute to retinal dysfunction and cell death (130).

Defects of insulin signaling in the classically insulin-responsive tissues—muscle, fat, and liver—have been extensively investigated in humans and animal models of diabetes. At the level of the IR, reduced insulin-binding, reduced expression, alternative splicing, increased hybrid formation with IGF-IRs, and
diminished autophosphorylation and proreceptor processing are fundamental features of insulin resistance in diabetes and obesity (225-231). The activity of PI3K associated with IRS and PY-containing proteins differs between hyper- and hypoinsulinemic animal models of diabetes. In two insulinopenic states, such as STZ-induced diabetes or a 72 h fast, insulin-stimulated p85α association with IRS-1 is increased in liver and muscle \textit{in vivo} (232, 233). Downstream of PI3K, insulin-stimulated Akt (or PKB) phosphorylation, which contributes to glucose uptake, is reduced in muscle of STZ-diabetic rats and adipose cells of Type II diabetic subjects, but not in normal, fasted rats (233, 234). However, insulin stimulation of the ERK 1/2 pathway remains relatively unaffected by either obesity or diabetes (235). Thus, diminished activity of the IR→Akt signaling pathway in the classically insulin responsive tissues appears to play a central role in insulin resistance, and leads to the cardinal features of diabetes, including hyperglycemia, hyperlipidemia, and reduced protein synthesis with increased protein catabolism. In cerebral and retinal neurons, the IR signaling pathway provides trophic support (27, 111), and defects of the IR disrupt retinal axonal guidance in \textit{Drosophila} (236) and induce ganglion cell apoptosis in chick retina (107). Therefore, the systemic inhibition of insulin signaling in diabetes would be expected to have different effects in retina than in peripheral tissues.

It has previously been demonstrated that insulin is a trophic factor for retinal neurons \textit{in vitro} that depends on the PI3K/Akt signaling pathway (111) and on p70S6K (237). Furthermore, in a model that mimics diabetes, hyperglycemic media
and glucosamine were found to attenuate insulin’s pro-survival effect on retinal neurons (180). To extend these previous observations to retina \textit{in vivo}, the hypothesis that STZ-induced diabetes reduces the constitutive kinase activity of the IR and downstream kinases in retina was tested. The results show that reduction of insulin in the diabetic state significantly reduced constitutive IR kinase activity in retina. Diabetes also reduced retinal IRS-1/2-associated PI3K activity, Akt-1 activity, and GSK3β ser 9 phosphorylation, despite normal expression of the signaling proteins and preservation of Akt thr 473 and ser 308 phosphorylation. Insulin-treated diabetic rats had normal IR kinase activity. Additionally, whole retina tissue from diabetic rats treated \textit{ex vivo} with insulin in physiologic glucose concentrations displayed increased IR autophosphorylation. Together, these results suggest that the constitutive IR→Akt pro-survival signaling pathway in retina is impaired by diabetes. It is likely these changes contribute to early events in the development of diabetic retinopathy, such as death of neurons and vascular cells.
4.3 Results

4.3.1 Diabetic rat characteristics

Weights and non-fasted blood glucose levels of age-matched control and STZ-diabetic rats are shown in Table 3 (pg. 114). The reported blood glucose values were taken from rats prior to sodium pentobarbital injection and sacrifice. There were significant reductions in weight gain and increases in blood-glucose levels in diabetic rats at both time points examined, confirming the diabetic state in this model, and only diabetic rats treated with insulin pellets did not lose weight.

4.3.2 Diabetes reduces constitutive IR kinase activity in retina

Previously, it was shown that the retina has constitutive IR autophosphorylation and kinase activity, similar to avian brain (40), that is 2-fold greater than liver of fasted rats and equal to IRs from brain and liver of fed rats (30). Therefore, the effects of reducing circulating insulin in a model of Type 1 diabetes on retinal IR autophosphorylation and kinase activity was examined. Retinas were examined from non-fasted control and diabetic rats after 4 and 8 weeks duration because it was shown previously that this duration of diabetes increases the rates of retinal cell death (130). Retinal lysates were immunoprecipitated for the IRβ subunit and immunoblotted for PY content and normalized to total IRβ expression. This
method isolates all retinal IRs irrespective of the extent of glycosylation. Tyrosine autophosphorylation of the IRβ in retina was unchanged after 4 weeks of diabetes, and retinal IR expression, assessed by immunoblot analysis of the immunoprecipitates, revealed equivalent expression of the IRβ subunit between control and diabetic rats. (Fig. 13A, pg. 115). Reprobing the membranes for the IGF-IRβ subunit revealed the presence of IR/IGF-IR hybrid receptors in retina, but quantification of IR/IGF-IR hybrid expression revealed no change in expression or autophosphorylation with diabetes (not shown), consistent with Gerhardinger et al (224) in which IGF-IRβ expression and phosphorylation is unchanged in retinas of diabetic rats.

The extent of tyrosine phosphorylation of the IRβ subunit is an indirect measure of kinase activity, but there are other modifications, cell-specific conditions, and IR heterogeneity that can alter total IR kinase activity (238). Although the constitutive tyrosine phosphorylation of the retinal IRβ is maintained after 4 weeks of diabetes (Fig. 13A, pg. 115), we assayed the phosphotransferase activity of the retinal IR following 4 weeks of diabetes by measuring phosphorylation of the exogenous substrate poly-(4Glu:1Tyr) (195). Interestingly, the kinetic rate of phosphorylation of an exogenous substrate was reduced 26% (p < 0.01) in retinas after just 4 weeks of diabetes, and 25% (p < 0.05) after 8 weeks (Fig. 13B, pg. 115), time points which coincide with increased cell death and vascular permeability in the STZ-diabetic rat model (130). Subcutaneous insulin pellets implanted into diabetic rats recovered the diminished IR kinase activity (Fig. 13B, pg. 115). These results imply that the
functionality of the constitutively active IR in retina tissue is impaired by diabetes *in vivo*.

4.3.3 *Retinal expression of proximal insulin signal transduction proteins are unchanged after 4 and 8 weeks of diabetes*

The IRS and Gab-1 proteins transduce a significant portion of the insulin signal and bind the regulatory subunit, p85, of PI3K (6). These proteins and their association were examined by immunoprecipitation and immunoblot analysis in retinas of normal and diabetic rats. Expression of IRS-1, IRS-2, and Gab-1 were unchanged in retinas between normal and diabetic rats (8 weeks of diabetes, Fig. 14A, pg. 116). Because exogenous insulin treatment of retina results in IRS-2 phosphorylation more so than IRS-1 (30), IRS-2 PY content and p85 association was also examined. After 4 weeks of diabetes, basal IRS-2 PY content and p85 association with IRS-2 were unaltered in retina (Fig. 14B, pg. 116). Furthermore, in immunoprecipitates of all PY-containing proteins in retinal lysates, 4 weeks of diabetes had no effect on p85 association (Fig. 14C, pg. 116). It was also asked if differential expression of the p85α splice variants occurs in retina of diabetic rats as it does in *ob/ob* mouse liver and muscle (239). Using an antibody that recognizes both p50α and p55α/AS53 (176), immunoblot analysis was performed on retinal lysates from normal and diabetic rats. Total content of p85 and p50α remained equivalent, respectively, between control and diabetic rats in retina. Furthermore, there was no
detection of the p55α/AS53 splice-variant in retina in either control or diabetic rats
(Fig 14D, pg. 116). These results suggest that downregulation of the IR signal is not
due to reduced signaling protein expression or ability of the intermediates to
associate. They also point to differences in the insulin signaling systems in retina
versus other peripheral tissues. Signaling from IGF-IR, IR/IGF-IR hybrids, or other
growth factors, such as BDNF which also utilizes IRS proteins (209), may also
compensate for reduced IR signaling to maintain basal IRS-2 tyrosine
phosphorylation.

4.3.4 IRS-1 and IRS-2-associated PI3K activity is reduced after 4 weeks of diabetes

in retina

Immediately downstream of IRS-1 and IRS-2 is PI3K, which is activated by
the binding of SH2 domains on its regulatory protein, p85, to PY-X-X-M motifs
(240). Although retinal content of IRS-1 and -2 remained stable with diabetes (Fig.
14A, pg. 116), we investigated PI3K activity associated with both IRS-1 and -2 by
dual immunoprecipitation. This approach was utilized to assess PI3K activity that
can associate with known IR substrates in retina in vivo and in cultured retinal
neurons (30, 180). As seen in Figure 15A (pg. 117), IRS-1/2-associated PI3P
production was reduced in retinas from diabetic rats compared to controls as assessed
by in vitro kinase assay with phosphoinositol as a substrate. Quantification of the
PI3P spots revealed a 48.5% decrease (p < 0.001) in IRS-1/2-associated PI3K activity
in retinas of diabetic rats (Fig. 15B, pg. 117). Similar to our analysis of IR activity and content, the content of the upstream scaffolding molecules IRS-1 and -2 in retina is not altered by diabetes, but PI3K activity associated with IRS-1/2 is diminished by diabetes within the same time frame as increased retinal apoptosis (130).

4.3.5 Akt-1 and -3 kinase activity and GSK3β ser 9 phosphorylation in retina is reduced after 4 and 8 weeks of diabetes

In the canonical pro-survival signaling pathway stimulated by insulin, Akt is activated following PI3K by binding to phosphoinositides and subsequent phosphorylation on consensus thr 308 and ser 473 sites (13). In fasted rats, basal Akt-1 activity is 40% greater in retina than in liver and 4-fold greater than in muscle, and exogenous insulin further stimulates a selective increase in retinal Akt-1 activity (30). Therefore, basal Akt-1 activity in retina of control and diabetic rats was investigated. In agreement with previous studies in retina (224), we observed no change in Akt ser 473 phosphorylation or total content between control and diabetic rats using pan-Akt and pan-phospho-Aktser473 antibodies (Fig. 16A, pg. 118), but constitutive Akt-1 kinase activity in retinas of diabetic rats was 51.7% (p < 0.005) of controls (Fig. 16B, pg. 118). Overall, Akt-1 expression and phosphorylation on residues thr 308 and ser 473 were equivalent between control and diabetic rats (Fig. 16C, pg. 118). Also examined was Akt-3 kinase activity in retina between control and diabetic rats. Akt-3 basal kinase activity was slightly lower after 4 weeks of
diabetes, and was significantly diminished after 8 weeks ($p < 0.005$, Fig. 16D, pg. 118). There was no significant change in Akt-2 kinase activity after 4 or 8 weeks.

Ser 9 on GSK3β is a substrate for Akt (16). Using phospho-specific antibodies, a 20% ($p < 0.005$) decrease in GSK3β phosphorylation was detected (Fig. 17, pg. 119), supporting the observation that Akt kinase activity is reduced in retinas of diabetic rats. Insulin treatment of the diabetic rats recovered the diminished basal phosphorylation of GSK3β on ser 9. This result implies that the insulin deficiency in uncontrolled Type 1 diabetes reduces constitutive Akt kinase activity in retina by an unidentified mechanism, but independent from thr 308 and ser 473 phosphorylation. This reduction in Akt-1 and -3 kinase activities is likely to decrease retinal cell survival (130), or alter nutrient metabolism.

4.3.6 Retinas from diabetic rats are insulin responsive in normal glucose conditions

In previous work (180), hyperglycemic media reduced insulin stimulated Akt phosphorylation in retinal neurons. Therefore, it was asked if retinas from diabetic rats, cultured under normoglycemic conditions, would have normal IR autophosphorylation in response to insulin. This question was examined using ex vivo retina preparations in normal (5 mM) glucose from control and STZ diabetic rats treated with physiological insulin doses (10 nM) for 5 min, the time point of maximal IRβ tyrosine phosphorylation (30). Interestingly, retinas from diabetic rats displayed a greater response to insulin in terms of IRβ autophosphorylation (Fig. 18, pg. 120),
as has been reported for skeletal muscle of insulinopenic diabetic rats in response to a superphysiological insulin injection (241). In retina, insulin-induced stimulation of IRβ autophosphorylation was increased 39.6% in rats that were diabetic for 4 weeks. As before (Fig. 13A, pg 115), IRβ expression was equivalent between control and diabetic rats. These results suggest that the retinal IR function is dysregulated by uncontrolled diabetes without altering IR expression.
4.4 Discussion

This study examined alterations in the constitutive IR signal as a potential mechanism that would contribute to retinal cell death (130) and the early pathogenesis of DR. The results presented in this report (in agreement with other observations by Wu and Gardner, manuscript submitted) are the first to demonstrate insulin signaling defects of the retina in a diabetic animal model. It was previously shown that the retina is an insulin-sensitive tissue with higher basal and constitutively active IR→Akt-1 signaling capacity compared to liver and muscle, and exogenous insulin further stimulates this pathway (30). In retina from insulin deficient diabetic rats in vivo, the elevated basal kinetic activity of the IR, PI3K, and Akt-1 were downregulated, and GSK3β upregulated, at the same time points that neuronal cell death and increased vascular permeability occur (130). These observations lead to the novel concept that the retina, to a similar degree as muscle and liver, is susceptible to the deleterious effects of diabetes and the metabolic dysregulations associated with insulin resistance. A key, but unanticipated finding in this report is that the expression level and post-translational modifications, such as phosphorylation, of insulin signaling mediators were maintained, but their kinase activity were impaired. Although alternative splicing of p85α is differentially regulated in liver and muscle of ob/ob mice, this did not occur in retina of diabetic rats. Together, these results demonstrate a progressive disruption of the higher steady-state pro-survival IR/PI3K/Akt signal by diabetes in retina.
In contrast to reports in which insulin signaling in STZ rats was evaluated in response to superphysiological insulin injections (232, 241), this study was designed to analyze the constitutive signaling properties in the retina within the time frame of increased apoptosis (130). However, even with nearly total absence of insulin, retinal IR autophosphorylation in diabetic rats is similar to control rats, and the retinal IR kinase activity is reduced only ~26%, suggesting the receptor is still stimulated by IR/IGF-IR hybrids, other ligands such as IGF-I or IGF-II, and/or other undefined mechanisms. This loss of IR kinase activity is equivalent to that found in the spontaneously insulin-deficient Ins2Akita mouse (Barber and Gardner, manuscript in preparation), so these changes reflect the diabetic state and not STZ toxicity. Our findings also suggest that the constitutively elevated IR→Akt signal in retina is diminished in vivo due in part to insulin deficiency since insulin treatment restored lost retinal IR kinase activity. However, over time with uncontrolled diabetes, the progressive loss of PI3K, Akt, mTOR, and p70S6K activity (Wu and Gardner, manuscript submitted) exceed the loss of IR kinase activity, strongly suggesting that other inhibitory effects may be operative to further reduce the activity of the signaling kinases in retina. Thus, the constitutive IR→Akt signal in retina undergoes “signalosome resistance” in diabetes.

The observation that retinas from diabetic rats remain responsive to insulin when placed in a normoglycemic ex vivo environment, further demonstrate retinal IR signaling dysfunction due to diabetes. In muscle and liver, fasting and diabetes upregulate insulin binding and IR phosphorylation in response to a systemic insulin
bolus (232, 241). In retina, insulin-stimulated IR phosphorylation was upregulated in
ex vivo retinas of diabetic rats with no change in IR expression. Fasting also does not
significantly alter insulin binding in brain (242). Therefore, the constitutive IR signal
in retina is reduced by STZ diabetes in vivo, but IR autophosphorylation is also
dyregulated in retinas of diabetic rats treated with insulin ex vivo.

The signaling defects we report in retina involve the PI3K/Akt pathway,
suggesting that retinal metabolism and cellular survival may be compromised.
Elevated GSK3β activity, which is associated with neurodegenerative disorders by
modulating apoptosis and protein synthesis (243), may also contribute to retinal cell
death, and is an area of future investigation. Evidence also suggests that diabetes
affects distal Akt signaling proteins such as mTOR and p70S6K1 (Wu and Gardner,
manuscript submitted). Because fasting has no effect on the IR kinase activity or IR
expression in the central nervous system (30, 40, 242), this implies that both reduced
circulating insulin and insulin resistance influence the intracellular insulin signaling
cascade in retina. The consequences of impaired retinal IR signaling are probably
multiple. Retinal neurons (111) and vascular endothelial cells (78) depend on insulin-
mediated PI3K activity for survival, and both types of cells die by apoptosis in human
and rat models of diabetes (130, 215, 244). Therefore, it is reasonable to predict that
long term disturbances in retinal IR signaling will accelerate cell death and impair
insulin-dependent anabolic activities, such as protein synthesis (245). In the broadest
sense, the IR signaling pathway senses cellular nutritional status and coordinates
anabolic activity with energy demand (246), so downregulation of this system may
have deleterious long-term consequences. A recent report by Schubert et al (247) showed the neuronal IR knockout mice have reduced Akt and GSK3β phosphorylation in brain. The IR was required for prevention of KCl-induced apoptosis of isolated cells, but the intact, non-stressed brain did not have increased neuronal apoptosis. By contrast, retina appears more susceptible to loss of IR activity, just as it develops much more profound complications associated with diabetes.

Insulin causes its intracellular effects by increased tyrosine phosphorylation of the IR and IRS proteins, binding of PI3K to IRS, and ser/thr phosphorylation of Akt. An unexpected, but important finding of this study is that enzymatic activities of these kinases are inhibited in vivo without a loss of total content or a decrease in phosphorylation of known residues that contribute to enzyme activity. Studies of diabetic animal tissues employing anti-PY antibodies have produced some conflicting data; in some reports, IRβ PY content and autophosphorylation were stable [(248, 249) and Fig. 13A, pg. 115] while others have shown reduced autophosphorylation and kinase activity of the IR towards an exogenous substrate [(229, 249) and Fig. 13B, pg. 115]. While kinase assays may provide a more sensitive measure of IR function, other modifications of the IR, such as serine/threonine phosphorylation (250), may also contribute to decreased IR function in different tissues in diabetic models. In agreement with previous findings by Gerhardinger et al (224), no change in total Akt expression and Akt ser 473 phosphorylation was found in retina between control and diabetic rats. This discrepancy in enzyme phosphorylation and kinetic
activity has also been reported for Akt-1 in muscle biopsies of obese individuals (251), and for total Akt expression and Akt ser 473 phosphorylation in vagus nerve of STZ diabetic rats (252). Likewise, Cai and Helke (252) found reduced PI3K activity in vagus nerve with no change in p85 expression. The mechanism for this effect remains under investigation, but several possibilities exist including other phosphorylation sites on Akt [such as tyr 474 for activation (253) or thr 34 for inactivation (254)] and inhibitory interactions with PKCζ [(254, 255) and T. Fox and M. Kester, unpublished observations] because total Akt kinase activity may be independent of ser 473 phosphorylation (256). Since many peptide hormone receptors signal through the PI3K/Akt pathway, diminished IR signaling may not be the only contributor to reduced Akt activation in retina. For instance, alterations in NGF and NT-3 retrograde transport (257) and BDNF mRNA and protein content (258) could contribute to altered activation of the PI3K/Akt pathway.

In diabetes, hyperglycemia and hyperlipidemia exacerbate the insulin resistant state. Although the mechanisms are not completely understood, evidence suggests that hyperglycemic induction of the hexosamine biosynthetic pathway (HBP) is involved, and transgenic overexpression of glutamine:fructose-6-phosphate amidotransferase (GFAT, the rate-limiting enzyme of the HBP) induces whole body insulin resistance and decreased glucose disposal (259). It has been demonstrated that excessive glucose and glucosamine abrogate insulin’s neuroprotective effects on cultured retinal neurons, and insulin restores retinal cell survival in an Akt-dependent manner when the neurons are cultured in high glucose and treated with a
pharmacological inhibitor of GFAT (111, 180). Improper pro-IR maturation and reduced insulin-stimulated IRβ autophosphorylation may play an important role in reducing post IR signaling events in retina. This improper IR maturation in retinal neurons may be similar to findings in muscle in which HBP activation downregulates the insulin signal by increasing O-linked N-acetylglucosamine modifications on IRS proteins (260). Hyperlipidemia also impairs muscle insulin signaling in vivo (261-263), and is associated with severity of retinopathy (264). Activation of protein kinase C, serine/threonine phosphorylation of insulin signaling proteins, ceramide generation (T. Fox and M. Kester, unpublished observations), and inhibition of glycogen synthesis are mechanisms whereby free fatty acids induce insulin resistance (263, 265-267). These mechanisms could potentially downregulate insulin pro-survival and metabolic signaling in retina. Increased circulating amino acids in the cachexic diabetic rat may also downregulate insulin signaling as they have been shown to reduce insulin-stimulated, but not basal, proximal signaling events such as IRS-1 tyrosine phosphorylation, IRS-1:p85 association, and PI3K activity (268). In particular, glutamine may exacerbate this process by enhancing GFAT activity and increasing HBP flux. Together, the excessive metabolic substrates are thought to contribute to insulin resistance and diabetic complications by increasing free radical production (269). Additional work is now in progress to determine the mechanism by which the metabolic dysregulation of diabetes downregulates retinal insulin signaling.

Characterization of retinal insulin signaling and the early molecular events in the pathogenesis of DR provides insight into possible novel therapies for DR. The IR
and IGF-IR both play a significant role in controlling retinal blood vessel growth in animal models of retinopathy of prematurity (270), yet the interplay among the endothelium, Müller glia, and different neurons in the healthy and diseased retina are not clearly defined (271, 272). A recent report on humans with Type 1 diabetes has shown that retinal neural abnormalities arise earlier, and in the same locations where vascular lesions develop one year later (221). Insulin reduces retinal apoptosis in diabetic rats (130), retinal neurons in culture (111), and further activates Akt \textit{in vivo} (30). Excess glucose alone, however, does not induce retinal neuronal cell death, but interferes with insulin-mediated survival (180). Therefore, perturbation of the IR signaling pathway may manifest in reduced pro-survival signaling, so DR may be considered a neurotrophin deficient and/or resistant state. In conjunction with pathologic and leaky blood vessel growth, neuronal function and cell survival of the retina may be restored by proper growth factor signaling. These findings suggest the possibility that impaired IR signaling could be a common mechanism underlying the initiation of diabetic complications, and provide a possible explanation for the beneficial effects of long-term intensive insulin treatment (139).
4.5 Figures, Tables, and Legends

<table>
<thead>
<tr>
<th></th>
<th>Starting weight (g)</th>
<th>Ending weight (g)</th>
<th>Percent increase in weight</th>
<th>Blood-glucose (mmol/L)</th>
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<tr>
<td><strong>4 weeks</strong></td>
<td></td>
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<tr>
<td>Control rats</td>
<td>183.5 ± 1.6</td>
<td>391.6 ± 5.8</td>
<td>113.7 ± 3.3</td>
<td>4.2 ± 0.1</td>
</tr>
<tr>
<td>Diabetic rats</td>
<td>182.2 ± 2.4</td>
<td>301.5 ± 8.2*</td>
<td>65.7 ± 4.4*</td>
<td>20.6 ± 0.5*</td>
</tr>
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|                  |                     |                   |                           |                        |
| **8 weeks**      |                     |                   |                           |                        |
| Control rats     | 145.4 ± 3.8         | 509.4 ± 16.4      | 250.7 ± 8.4               | 4.5 ± 0.2              |
| Diabetic rats    | 144.3 ± 3.3         | 314.9 ± 16.0***   | 118.6 ± 10.8***           | 16.1 ± 0.6***          |
| Diabetic, +insulin pellets | 141.8 ± 3.1 | 468.0 ± 7.1* | 231.6 ± 9.3 | 5.2 ± 0.6 |

Table 3. Beginning and ending mean (± SEM) weights (g) and blood-glucose levels (mmol/L) in age-matched control, STZ-diabetic, and diabetic rats treated with insulin pellets. All diabetic rats weighed significantly less than their age-matched counterparts, and therefore had a reduced percentage weight increase. Likewise, all diabetic rats had significantly elevated blood-glucose levels. Insulin pellets were implanted 5 d after diabetes induction with a subcutaneous insulin pellet delivering ~2 U insulin per day for the duration of the experiment. * p < 0.0005 by Student’s t-test compared to control rats in 4 week studies. For 8 week studies, *** p < 0.001 from control, and * p < 0.05 from control.
Figure 13. Diabetes reduces retinal IR kinase activity. Retinas from STZ-diabetic and age-matched control rats were analyzed for basal IRβ tyrosine phosphorylation and kinase activity as described in Methods. (A) Representative immunoblots for PY and IRβ are shown for IRβ immunoprecipitates from retina tissue of control (CTRL) and STZ-diabetic (DIAB) rats. The dash (---) indicates mobility of the IGF-IRβ band (not shown), which is detected in IRβ immunoprecipitates by PY immunoblotting, but not on reprobed membranes for the IRβ. Quantification of the immunoblots are expressed as PY/IRβ ratios (n = 8 CTRL and n = 5 DIAB). There was no statistical difference in IRβ PY content or total IRβ expression between control and diabetic rats in retina. The experiment was repeated again after 4 weeks of diabetes with similar results. (B) To measure IR kinase activity, IRβ immunoprecipitates were incubated with ATP and an exogenous substrate (poly 4Glu:1Tyr), and the kinase reaction was stopped by spotting the reaction supernatant onto p81 filters and scintillation counting. Although there was no detectable change in retinal IRβ tyrosine phosphorylation after 4 weeks of diabetes, retinal IR kinetic activity was reduced 26.0% in diabetic rats. [** p < 0.01 by ANOVA, 95% CI (63.4, 84.6), n = 14 for CTRL and DIAB]. Retinal IR kinase activity was reduced similarly by 24.8% after 8 weeks of diabetes, with no difference in diabetic rats treated long-term with insulin pellets [DIAB * p < 0.05 by ANOVA and log₁₀ transformation, 95% CI (53.5, 96.9)].
Figure 14. IRS-1, -2, and Gab-1 content and p85 association and expression profile is stable in retinas of diabetic rats. (A) Immunoblot analysis was performed on retinal lysates of control (CTRL, left two lanes) and 8-week diabetic rats (DIAB, right two lanes) for IRS-1, IRS-2, and Gab-1 content. A representative immunoblot is shown, and quantification revealed no statistical change in expression of these proteins (n = 7 for CTRL and n = 6 for DIAB). (B) IRS-2, which conducts the insulin signal greater than IRS-1 in retina, was analyzed for PY content and p85 association by immunoprecipitation (IP) followed by immunoblot analysis. Total PY content was unchanged in retinal lysates between CTRL (left two lanes) and diabetic rats (right two lanes, 4 weeks), and the amount of p85, the regulatory subunit of PI3K, that co-precipitates with IRS-2 is not different between CTRL and diabetic rats (n = 8 for CTRL and n = 7 for DIAB, representative blots). (C) Retinal lysates were immunoprecipitated for all PY-containing proteins for total p85 association in control (n = 4, left two lanes) and 4-week diabetic rats (n = 4, right two lanes). Immunoblot analysis revealed no change in p85 association with PY-containing proteins (representative blot shown). (D) Content of p85 and the splice-variants (p50α and p55α/AS53) were analyzed by immunoblotting retinal lysates from control and 4-week diabetic rats. Representative immunoblots for p85 (top panel) and p50α (bottom panel) reveal that content did not change between control (n = 8, left two lanes) and diabetic (n = 8, right two lanes). The p55α/AS53 splice variant was undetectable in retina; skeletal muscle (skm) lysates from a control rat were used as a positive control for p55α/AS53 expression.
Figure 15. IRS-1/2-associated PI3K activity is reduced in retinas of diabetic rats. Retinal lysates of control (CTRL) and STZ-diabetic (DIAB) rats were dual immunoprecipitated for IRS-1 and IRS-2, and PI3K activity was measured on the captured immune complex. (A) A representative phosphoimage of a TLC plate is shown demonstrating reduced PI3P production from retinas of 4-weeks diabetic rats compared to their age-matched controls. The mock immunoprecipitate served to blank the assay. (B) Quantification of PI3P production revealed a 48.5% decrease in IRS-1/2-associated PI3K activity in retinas of diabetic rats after 4 weeks [* p < 0.001 by ANOVA, 95% CI (28.9, 75.8), n = 14 for CTRL and n = 15 for DIAB].
Figure 16. Retinas of diabetic rats have reduced Akt-1 kinase activity after 4 weeks. (A) Equivalent protein was separated on SDS-PAGE for assessment of Akt\textsuperscript{Ser\textsuperscript{473}} phosphorylation and total expression. A representative immunoblot is shown demonstrating no statistical difference between control (CTRL, left two lanes) and 4-week diabetic rats (DIAB, right two lanes). (B) Retinal lysates were immunoprecipitated for Akt-1 and subject to kinase assays. Akt-1 kinase activity was significantly reduced 48.3% in retinas of diabetic rats compared to CTRL [* p < 0.005 by ANOVA, 95% CI (22.0, 81.9), n = 15 for CTRL and n = 14 for DIAB]. (C) The immune complexes from part B were separated on SDS-PAGE and immunoblotted using pan-Akt antibodies that recognize the consensus thr 308, ser 473, and total Akt (representative blots shown). Akt-1 phosphorylation and expression remained equivalent in retina between CTRL and diabetic rats. (D) Akt-3 kinase activity was significantly diminished after 8 weeks of STZ diabetes [p = 0.128 by ANOVA, 95% CI (38.2, 109.1), n = 4 at 4 weeks; * p < 0.005 by ANOVA, 95% CI (-15.6, 69.5), n = 3 at 8 weeks].
Figure 17. GSK3β ser 9 phosphorylation is reduced in retinas of diabetic rats. Immunoblot analysis of GSK3β ser 9 phosphorylation demonstrated a 19.8% reduction of this site in diabetic rat retinas, which was partially recovered in long-term insulin treated diabetic rats [** p < 0.01 by ANOVA, 95% CI (69.7, 92.7), n = 8 for CTRL, n = 5 for DIAB, and n = 8 for DIAB + INS]. The experiment was repeated once more with similar results.
Figure 18. The retinal IR from diabetic rats responds to insulin. Retinas from age-matched control (CTRL, open bars) and diabetic (DIAB, black bars) were isolated for *ex vivo* analysis of IR autophosphorylation, and were analyzed as described in Methods. The retinal lysates were subject to immunoprecipitation (IP) for the IRβ, followed by immunoblotting for PY, and reprobing for normalization to total IRβ. Representative immunoblots (left) are shown of *ex vivo* retinas treated with vehicle (-) and 10 nM insulin (+). Quantification of the immunoblots and expression of the PY/IRβ ratio (right) demonstrated a 39.6% increase in the IRβ PY content following insulin stimulation of retinal explants of diabetic rats. (* p < 0.05 by ANOVA and Student-Newman-Keuls post-test, DIAB insulin-stimulated value compared to CTRL insulin-stimulated value within each time point; 4 weeks, n = 4 for CTRL and n = 5 for DIAB).
CHAPTER V

GROWTH FACTOR CONTRIBUTION TO THE CONSTITUTIVE INSULIN RECEPTOR AUTOPHOSPHORYLATION IN RETINA
5.1 Abstract

The insulin receptor (IR) in retina displays constitutive autophosphorylation and kinetic activity that, in contrast to liver IR signaling, does not change in fasted rats. The purpose of this study is to examine how insulin, insulin-like growth factor (IGF) -I, and IGF-II contribute to the constitutive IR autophosphorylation in retinas of normal, fasted rats. The first approach acutely reduced circulating insulin by somatostatin (SST) infusion, and the second approach utilized intravitreal injection of antibodies against insulin, IGF-I, and IGF-II. SST infusion for 180 min significantly reduced circulating insulin and reduced both IR and insulin receptor substrate (IRS) -2 PY content in liver, but had no effect on retinal IR autophosphorylation. Of the three antibodies injected vitreally, only αIGF-II antibodies reduced the constitutive retinal IR and IGF-IR PY content. These results suggest that IGF-II contributes to the basal IR, as well as IGF-IR, signal in retina, and that novel mechanisms exist in retina to regulate growth factor signaling.
5.2 Introduction

Circulating insulin fluctuates with ingestion of a meal and a concomitant elevation in blood glucose levels, and it is widely accepted that insulin sensitive tissues, such as skeletal muscle and adipose, rapidly clear glucose from the blood, while gluconeogenesis in the liver is reduced by insulin. Within 1.5 h following a meal, insulin secreted from the pancreas directly affects the liver by increasing IR and IRS-1 tyrosine phosphorylation, and increases p85 binding to IRS-1 (38). Therefore, circulating insulin correlates positively with increases in insulin signal transduction in liver.

IRs are also expressed widely throughout the central nervous system, including the retina (31), yet their functions and mechanisms of regulation are not fully understood. The IR in the brain and retina display constitutive kinase activity that is not altered by feeding, which is in stark contrast to IRs expressed in muscle and liver (30, 38, 40). When treated with insulin ex vivo, retinal IRs respond to exogenous insulin and preferentially activate Akt-1 via increased IRS-2 tyrosine phosphorylation, and this response is found in the retinal nuclear layers (30). However, a mechanism which regulates constitutive IR autophosphorylation and activity in retina or brain has not been described.

Two splice variants of the IR (IR-A, or –exon 11, and IR-B, or +exon 11) are expressed in mammalian cells. Exon 11 constitutes twelve amino acids at the carboxy-terminal of the extracellular domain of the IR α subunit. The retina
predominantly expresses IR-A in contrast to the liver which predominantly expresses IR-B (49, 50). Interestingly, the affinities for insulin and IGFs differ between IR-A and IR-B even when heterodimerized with the IGF-IR, in that the IR-A binds IGF-II with higher affinity (52, 201). We have also shown that pathophysiological levels of IGF-II can activate retinal IRs (30). Therefore, it is hypothesized that growth factors, in addition to insulin, may contribute to the constitutive IR phosphorylation that is observed in retina, and data contained in this chapter suggests that IGF-II contributes to IR and IGF-IR autophosphorylation.
5.3 Results

5.3.1 Somatostatin infusion reduced basal circulating insulin in 24 h fasted rats

Following 180 min of SST or vehicle infusion, plasma samples were analyzed for insulin content to determine the efficacy of the protocol on reducing basal circulating insulin. In agreement with previous publications (165), the SST infusion protocol significantly reduced the basal levels of insulin compared to vehicle infused rats (Fig. 19, pg. 132). Vehicle infused rats had similar circulating insulin as in previous work examining IR autophosphorylation in fasted rats [Table 2, pg. 87 and (30)]. Blood-glucose levels were elevated and statistically significant in rats receiving the SST infusion, but the values were still well within normal physiological range for a fasted rat (3.20 ± 0.17 mmol glucose for vehicle infused rats and 3.94 ± 0.15 mmol glucose for SST infused rats; p < 0.05 by Student’s t-test).

5.3.2 SST reduced liver IR signaling, but not constitutive retinal IR signaling.

It has previously been demonstrated that fasting (30) and re-feeding (38) dramatically alters the IR autophosphorylation and kinase activity in liver, but there is no effect of fasting on retinal IR autophosphorylation or activity (30). Therefore, it was examined if lowering basal circulating insulin further (Fig. 19, pg. 132) in a fasted rat would alter the constitutive IR signal in retina. Immunoprecipitation and
immunoblot analysis of liver lysates demonstrated that the basal autophosphorylation of the IRβ and the downstream substrate, IRS-2, were significantly lowered by SST (Fig. 20, pg. 133). This is in contrast to retinal IRβ and IRS-2 in the same rats in which lowering basal circulating insulin for 180 min with SST had no effect on retinal IRβ autophosphorylation or IRS-2 PY content. Furthermore, as previously published (30), retinal IRβ kinase activity was greater than liver IRβ activity in the fasted rats (data not shown). These results suggest that the regulation of the constitutive IR signal in retina is vastly different from other peripheral tissues which respond rapidly to insulin for immediate nutrient metabolism.

5.3.3 IGF-2 contributes to the constitutive autophosphorylation of the IR and IGF-IR in retina

The IR-A isoform has slightly different ligand binding properties than the IR-B isoform and is considered a receptor for IGF-II, even when hybridized with IGF-I receptors (52, 201). Because the retina exclusively expresses the IR-A isoform (50), the hypothesis that neutralizing antibodies against insulin, IGF-I, or IGF-II, injected intravitreally, would reduce the constitutive autophosphorylation of the IR, and possibly the IGF-IR, was tested. Insulin was also injected intravitreally (5 µL of 100 nM bovine insulin, final concentration in the vitreous ~10 nM) as a positive control for IR autophosphorylation ~4-fold. As seen in Figure 21 (pg. 134), insulin increased basal IR autophosphorylation. Interestingly, in eyes receiving αinsulin or αIGF-I
antibodies, no appreciable decrease in IR or IGF-IR autophosphorylation was observed. However, αIGF-II antibodies consistently reduced both IR and IGF-IR autophosphorylation (Fig. 21, pg. 134). These results suggest that IGF-II derived from ocular tissues, possibly the neural retina (197), may play a significant role in modulating IR, IGF-IR, and IR/IGF-IR signaling in the retina.
5.4 Discussion

The retina expresses a functional IR in vivo that signals to conserved downstream pathways as in other insulin-sensitive tissues (30), and that uncontrolled diabetes reduces the constitutive IR→Akt pro-survival signaling pathway. The rate of insulin transport across the blood-retinal barrier (BRB) is slower (30, 70) than across the fenestrated endothelium of other peripheral insulin-sensitive tissues. Therefore, other mechanisms are likely operative in retina which contribute to the constitutive autophosphorylation of the retinal IR, and in this chapter, the hypothesis that intraocular ligands contribute to this was tested. The major finding of these studies is that neutralization of IGF-II in the vitreous reduced retinal IR autophosphorylation in retina tissue.

Reducing circulating insulin by fasting does not reduce retinal IR autophosphorylation as does diabetes (30). Therefore, the first approach utilized somatostatin (SST) to further suppress circulating insulin in fasted rats. Reducing circulating insulin ~75% in this manner consistently reduced liver IR autophosphorylation and IRS-2 tyrosine phosphorylation, but did not alter retinal IR autophosphorylation or IRS-2 tyrosine phosphorylation. This suggests the retinal IR, in contrast to liver, is not as acutely sensitive to a reduction of plasma insulin concentrations. Therefore, pancreatically-derived insulin may not regulate retinal IR signaling in the short-term. However, based on previous work (166), a 3 h reduction in circulating insulin by somatostatin reduces brain glucose uptake, but IR signaling
changes were not documented. In retina, we show that reducing circulating insulin with SST for 3 h does not effect retinal IR and IRS-2 tyrosine phosphorylation, but a longer reduction of circulating insulin may be required to observe effects on retinal insulin signaling. SST receptors have also been described in mouse retina (273), which, by altering cAMP levels, may also alter other downstream signaling components in retina.

The second approach examined if an ocular-derived ligand for the IR-A form contributes to retinal IR autophosphorylation. Interestingly, αinsulin and αIGF-I antibodies did not reduce retinal IR or IGF-IR autophosphorylation, but αIGF-II antibodies did. This suggests that IGF-II contributes to the constitutive autophosphorylation of the IR-A, and IGF-IR, in retina, and future studies will examine the contribution of IGF-II to the basal kinetic activity of both the IR and IGF-IR. This study is based on ligand concentrations (73, 167, 168) that were determined in the vitreous, so levels of each ligand within the extracellular space in the retina may exceed those values measured from the vitreous, which would explain why no effect was observed with αinsulin or αIGF-I antibodies. Furthermore, because IGF-II is also a ligand for IR-A even when hybridized to IGF-IR (201), this does not rule out that a significant portion of retinal IR signaling may be dependent on the formation of IR/IGF-IR hybrids, which have been described in retina tissue (Chapter 4). Therefore, by knocking down available IGF-II in the retina, both holoreceptors (IR-A/IR-A and IGF-IR/IGF-IR) and hybrids would be affected. These
data suggest novel ligand-dependent activation of retinal IRs which depends on IGF-II.

To date, only one ligand-independent mechanism has been described to alter retinal IR activation: light. Dark adapted rats exposed to light have significantly elevated IR autophosphorylation and associated PI3K activity in rod outer segments (124). Rajala et al (124) hypothesize that this is a mechanism to prevent light-induced retinal damage, and it may be expected that IRs expressed elsewhere in the retina do not respond in the same manner. However, one may speculate that a local increase in IR ligand concentrations in rod outer segments with light exposure may be contributing to IR activation. Determining which cells in retina secrete IGF-II will be determined in future studies.

IGF-II expression has been described in adult rat neural tissues (197), and studies involving gene deletion of the IR, IGF-IR, and IGF-II suggest that in mouse development, IGF-II plays a major developmental role by signaling through the IR, while insulin is responsible for post-natal growth (196, 274). Genetic analysis has revealed that the perinatal lethal phenotype of IGF-II−/− mice are rescued when the IGF-IIR/M6P gene is also deleted (275). The overall contribution of IGF-II to the maintenance of adult tissues is unknown, but it is most likely mediated through the IGF-IR as IGF-IIR/M6P deletion results in overgrowth of tissues, as clearance of IGF-II is reduced causing further stimulation of the IGF-IR (275). In the brain of diabetic rats, IGF-II mRNA expression is reduced (276), while in the vitreous, higher levels of IGF-II have been reported in people with retinopathy and rubeosis, but not
significantly elevated in patients with retinopathy alone (167, 277). This may be a result of increased vascular permeability in the retina or ciliary body epithelium. However, if IGF-II production is reduced also within the retina as in brain, this may be a possible mechanism in which IR signaling activity is downregulated by diabetes.
5.5 Figures and Figure Legends

Figure 19. Circulating insulin is reduced in rats receiving SST infusion. Fasted rats (18 h) received either vehicle (VEH) or SST infusion as described in Materials and Methods. The SST treatment significantly reduced circulating insulin below what is found in a fasted rat (* p < 0.05 by Student’s t-test, n = 10 per group)
Figure 20. SST infusion reduced liver, but not retina, basal IR and IRS-2 phosphorylation. Liver and retina tissue were examined for tyrosine phosphorylation of IRβ and IRS-2 following vehicle (n = 10) or SST (n = 10) infusion by immunoprecipitation and immunoblotting, and representative immunoblots are shown. SST infusion reduced both IRβ autophosphorylation and IRS-2 PY content, but in the same rats, retinal IRβ autophosphorylation and IRS-2 PY content were unaffected.
Figure 21. Antibodies against IGF-II reduce basal IRβ and IGF-IRβ autophosphorylation. Rats received either insulin or anti-insulin, -IGF-I, or -IGF-II antibodies vitreally, and IRβ and IGF-IRβ autophosphorylation was analyzed 1 h later. As a positive control, vitreal insulin increased IRβ phosphorylation (n = 4). In rats receiving antibodies, only αIGF-II reduced both IRβ and IGF-IRβ autophosphorylation (n = 4 per group). The percent change (± SEM) in IRβ or IGF-IRβ autophosphorylation with either insulin or each antibody (+) treatment is shown below each representative immunoblot relative to vehicle (-) treatment (set to 100%).
CHAPTER VI

OTHER DISTINCT RETINAL INSULIN RECEPTOR SIGNALING

CHARACTERISTICS
6.1 R28 Retinal Neurons as a Model of \textit{in vivo} Growth Factor Signaling

Prior to these experiments that examine insulin action in an intact retina either \textit{in vivo} or \textit{ex vivo}, Dr. Gardner’s laboratory had acquired transformed retinal neurons as a model system. The description of their derivation from post-natal day 6 rat pups (175) and the method of culture can be found elsewhere [Methods and Materials and (111)].

6.1.1 Insulin and IGF-1 activate the IR, IGF-IR, and IR/IGF-IR hybrids

To assess the pro-survival effects of insulin and IGF-1 on R28 cells, the hypothesis that physiological doses of insulin (10 nM) and IGF-1 (1.3 nM) activated specifically their cognate receptors was tested. Serum-starved R28 cells were treated with insulin or IGF-I for either 2 min or 24 h. The IR$\beta$ and IGF-IR$\beta$ were immunoprecipitated for analysis of autophosphorylation by anti-PY immunoblotting. Interestingly, neither the IR nor IGF-IR display constitutive autophosphorylation in this cell culture model (Fig. 22, pg. 149), which differs from whole retina tissue (as discussed in Chapters 3-5). Treatment of R28 neurons with insulin for 2 min resulted in a robust increase in IR$\beta$ autophosphorylation; likewise, the IGF-IR$\beta$ was autophosphorylated with IGF-I treatment for 2 min. Interestingly, R28 cells express IR/IGF-IR hybrid receptors, similar to retina tissue \textit{in vivo}, which are detected on the anti-PY immunoblots, and when the membranes are reprobed for the IR$\beta$ or IGF-IR$\beta$
from IGF-IRβ or IRβ immunoprecipitates, respectively (not shown). For instance, in R28 cells treated with IGF-I, a PY doublet is consistently observed in IRβ immunoprecipitates suggesting phosphorylation of the β subunits of the IR/IGF-IR hybrids. The signal from the IRβ/IGF-IRβ doublet on the PY immunoblots of IGF-IRβ immunoprecipitations from insulin-treated cells is not as intense as IRβ immunoprecipitations from IGF-I-treated cells suggesting that insulin is not activating the IR/IGF-IR hybrids in this system. After 24 h of insulin or IGF-I treatment, receptor autophosphorylation was down regulated as the intensity of the receptor autophosphorylation bands is diminished compared to 2 min. Down regulation of IR and IGF-IR expression in response to prolonged growth factor treatment, as in hyperinsulinemic states, will be investigated in future experiments. These data suggest that insulin and IGF-I act primarily through their cognate holo- and hybrid receptors, but at the doses used, insulin and IGF-I did not activate IGF-IR or IR holoreceptors, respectively. These cells are a widely used *in vitro* model to study normal and diseased retina physiology (111, 180, 237).

6.1.2 Insulin specificity for pro-survival signaling: comparison with BDNF, NGF, and EGF

The goal of the following experiment was to test the efficacy of HNMPA-(AM)$_3$, a putative inhibitor of the IR kinase, in cultured retinal neurons for further use *in vivo*. HNMPA-(AM)$_3$ had been used previously to determine IR isoform-specific
signaling and gene regulation in β-cells (278), and had also been used to determine the contribution of the IR to Ca\(^{2+}\) influx in photoreceptors (121). Because of the similarities between the IR and IGF-IR, it was determined that HNMPA-(AM)\(_3\) also inhibited the IGF-IR and prevented IGF-I-induced Akt phosphorylation in R28 retinal neurons (not shown). Nevertheless, a compound specifically inhibiting the IR/IGF-IR signaling system would be a useful tool to examine knock-down of the constitutive signaling pathway in vivo and study the physiological and molecular outcomes. To reaffirm specificity of HNMPA-(AM)\(_3\) to the IR/IGF-IR proteins, R28 retinal neurons were treated with insulin, BDNF, NGF, and EGF with and without HNMPA-(AM)\(_3\) and analyzed for Akt, p70, and ERK 1/2 pathway activation (Fig. 23, pg. 150). First, it was learned that HNMPA-(AM)\(_3\) is a poor choice to inhibit the IR/IGF-IR pathways because it alone reduced phosphorylation of the putative activation site, thr 389, on p70 S6K, possibly increased other phosphorylation events on p70 S6K inducing a gel-shift and resolution into multiple bands consistent with hyperphosphorylation, and stimulated phosphorylation of ERK 1/2 on residues consistent with activation of those kinases. Therefore, the use of this compound was abandoned for in vivo use. However, an interesting observation is noted when examining the retinal neuronal cell cultures treated without the inhibitor. All growth factors increased p70 S6K phosphorylation on thr 389, suggesting they may contribute to the regulation of translation initiation; however, only insulin increased Akt ser 473 phosphorylation. ERK 1/2 phosphorylation was not stimulated by any growth factor. This observation suggests that insulin may have a unique function in
neuronal cells in regulating the Akt kinase and promoting neuronal cell survival.

Further documentation will be required examining the time-course of Akt, p70, and ERK phosphorylation in response to each growth factor and if the specificity is also observed in vivo.
6.2 PP132: A Potential Novel Substrate for the IR in Retina

Receptor tyrosine kinases (RTKs), which include the IR, transduce the hormonal signal across the cell membrane by hydrolysis of ATP and transferring a phosphate to specific tyrosine residues on other proteins. For the IR, numerous substrates have been identified which are tyrosine phosphorylated (6, 108, 110). Using antibodies which recognize phosphorylated tyrosine residues, tyrosine phosphorylation events in the retina were examined in response to insulin using the \textit{ex vivo} retina model (see Materials and Methods). This method provides an unbiased approach to evaluation of IR signaling.

Retinal lysates from retinas treated \textit{ex vivo} with 10 nM insulin were immunoprecipitated with \(\alpha\)PY antibodies followed by immunoblot analysis for PY. As seen in Figure 24 (pg. 151), results were compared with lysates from insulin-treated liver tissue, and immunoprecipitation of the IR\(\beta\) from retina served as a positive control for the insulin treatment. As expected, IR\(\beta\) tyrosine phosphorylation increased in retina after two min, and the same molecular weight band was also detected in immunoprecipitations for total PY. In liver, the IR\(\beta\) band is detected with a slower migration as previously reported (30). Bands that correspond to IRS-1 and IRS-2, which are expressed in retina, appear to be detected in low quantities by this method (> 150 kDa), suggesting that they may not be expressed in as high a quantity as other PY-containing proteins in retina, or they may not be the major substrate for the IR kinase in retina. The most intense band visualized had a migration of \~132
kDa. Furthermore, the intensity of this band increased concomitantly with insulin
treatment and IRβ tyrosine phosphorylation, with maximal phosphorylation detected
after five min and decreasing to basal levels after 30 min. This suggests that this
protein (phosphoprotein 132, pp132) is a potential substrate for the IR tyrosine
kinase, and therefore, may also serve a similar function as IRS proteins. However,
the number of phosphorylated tyrosine residues on pp132 can not be determined in
this manner. To further characterize this protein, 2-dimensional PAGE and MALDI-
TOF mass spectroscopy will be utilized to identify if pp132 has already been
characterized.
6.3 Examination of Retinal IR Autophosphorylation Using Phospho-specific IR Antibodies

The observation that the retinal IR (as well as brain) possesses constitutive activity and autophosphorylation suggests that the regulation of the IR in neural tissues may differ from other well-studied systems and tissues such as CHO-IR cells, adipose, and muscle tissue. The main limitation of studies using αPY antibodies is the assumption that the antibody recognizes all phosphorylated tyrosine residues with equal affinity. If this assumption is not correct, and some phospho-tyrosine residues are more critical than others for IR activity, then this information can not be determined using “pan”-αPY antibodies. This is indeed the case as “pan”-αPY antibodies are raised against similar, but different antigens (279). Therefore, basal autophosphorylation of IRs was examined using an α(IR/IGF-IR)PY1158 antibody. Y1158 in the IR (and Y1131 in the analogous IGF-IR) is a tyrosine residue in the kinase domain of the receptor, but when mutated, along with single mutations of Y1162 and Y1163 of the IR kinase domain, the IR retains approximately half of its original insulin stimulated activity (280, 281). Using this antibody, Y1158 phosphorylation of the IR was examined in liver, retina, and brain of normal freely fed and fasted rats by immunoprecipitation and immunoblot analysis to test the hypothesis that Y1158 phosphorylation would be increased in liver of fed rats and Y1158 phosphorylation in retina and brain would not change and be similar to or higher than liver as in studies using αPY antibodies. As expected, this was the case
for liver tissue (Fig. 25, pg. 152). However, unanticipatedly, Y1158 phosphorylation was less in both retina and brain compared to liver, and did not change between fed and fasted rats. This suggests that in neural tissue, such as retina and brain, IR activity may be maintained with a different phosphorylation pattern or other protein modifications, and the “pan”-αPY antibodies may not be recognizing the tyrosine residues required for IR activity in retina or brain. By comparing IR kinase activity in immunoprecipitations of the IRβ, followed by a second round of immunoprecipitations with “pan”-αPY compared to αPY1158 (or against PY1162 or PY1163), one may gain insight into the contribution of the tyrosine residues of the IR kinase domain that regulate activity in retina. Additional studies using MALDI-TOF analysis will shed light on a possible unique phosphorylation pattern of the IR in retina.
6.4 Ins2\textsuperscript{Akita} Diabetic Mice Display Reduced IR Kinase Activity

The Ins2\textsuperscript{Akita} mouse strain has been described and is an accepted model of Type I diabetes (169, 170). With a mutation in the Insulin-2 gene, the insulin protein is misfolded, retained in the ER, and not properly secreted by the β cells. The induced ER stress then contributes to β-cell failure and fasting hyperglycemia at ~7 weeks of age (170). Work by others in Dr. Gardner’s laboratory have characterized vascular abnormalities and increased active caspase-3 immunoreactivity in retinas of Ins2\textsuperscript{Akita} mice compared to their wild-type litter mates (Barber et al, manuscript in preparation). Therefore, the hypothesis that the constitutive IR kinase activity is reduced in retinas of the Ins2\textsuperscript{Akita} mice was tested. Retinas from wild-type (n = 9) and diabetic (n = 8) Ins2\textsuperscript{Akita} mice were analyzed for IR kinase activity as described in Methods and Materials. Results show a ~28% reduction in IR kinase activity in retinas of diabetic Ins2\textsuperscript{Akita} mice compared to wild-type control litter mates (Fig. 26, pg. 153). This result is similar to that found in retinas of STZ-diabetic rats (see Fig. 13, pg. 115), supporting the hypothesis that diabetes reduces the constitutive retinal IR kinase. Because this is a genetic model of diabetes, this refutes the possibility that STZ-toxicity plays a role in reducing retinal IR signaling. Further examination of the retinas of diabetic Ins2\textsuperscript{Akita} mice will be required to determine if the IR→Akt signaling pathway is reduced and contributes to increased neural apoptosis in retina.
6.5 Transgenic Ablation of Retinal IRs Alters Downstream Constitutive Signaling

It is evident from the previous studies that the regulation of the retinal IR and its downstream signaling intermediates is regulated differently than in other peripheral insulin-sensitive tissues. The retina does not respond to pancreatically derived insulin as dynamically compared to muscle which rapidly clears and metabolizes glucose from the blood. Moreover, uncontrolled insulinopenic diabetes, as in the STZ model, reduces the IR→Akt signalsome activity. Therefore, a genetic mouse model in which the expression of the IR in retina is reduced, without diabetes, was employed to examine proximal IR signaling in retina (courtesy of Dr. Domeneco Accili, Columbia University, see Chapter 2). The pitfall in using the TTR-IR mouse is that a common background strain used in mouse transgenic studies is the albino FVB, which carries a mutation in the pde6 gene, resulting in a retinal degeneration (rd) phenotype and a loss of photoreceptors (172). This limitation was overcome by the breeding strategy described in Methods and Materials (Chapter 2, Fig. 4) to produce an excellent model in which to study IR function in retina.

Retinas from one set of littermates (IR+/+, n = 2; IR+/-, n = 3; IR-/-, n = 2; all mice expressed the TTR-IR transgene) were analyzed at eight months of age to determine if IR expression was reduced in retina. Skeletal muscle was also examined as a positive control for the lack of TTR, and therefore, IR transgene expression. As expected, there was an ~50% reduction of retinal IR expression in mice on an IR+/-
background, and very little IR detected in mice on an IR-/- background (Fig. 27, pg. 154). This is mostly likely contamination from RPE cells which are known to express TTR mRNA (282).

In both skeletal muscle and retina, the expression of the IGF-IR and Akt were maintained, as well as Akt$^{ser473}$ phosphorylation. Additional sets of littermates will be analyzed to determine if IGF-IR and Akt expression and kinase activity changes to compensate for a loss or IR expression in retina. Overall, this model will increase the understanding of IR function in retina and potentially, a retinal cell-specific manner. Future experiments will also analyze the ERG to determine a functional outcome of a lack of IR expression in retina.
6.6 Periocular insulin administration activates the retinal IR in vivo

In vivo and ex vivo, insulin activates the pro-survival kinase Akt in retinal neurons. Activation of this kinase may have therapeutic implications in preventing neuronal cell death in DR (130) and other diseases of the eye such as glaucoma, another condition in which retinal neurons undergo increased rates of apoptosis (217). Since molecules as large as 70-kDa dextran and immunoglobulins have been reported to diffuse into the retina after a subconjunctival injection by transscleral diffusion (283, 284), the hypothesis that insulin injected subconjunctively activates Akt in retina was tested. Furthermore, for this to have therapeutic implications in individuals with diabetes, a dose response was performed initially to determine a dose which would not effect blood-glucose levels.

As seen in Figure 28A (pg. 155), low doses of insulin (< 0.0325 U/100g body weight) injected under the subconjunctiva in rats do not induce hypoglycemia. Next, it was determined if this route and dose can stimulate the pro-survival kinase Akt in retina tissue. A cohort of young rats (< 300g) and aged rats (> 500g) were used. Representative immunoblots in Figure 28B (pg. 155) demonstrate that 0.0325 U/100g induces a statistically significant (p < 0.05) increase of Akt phosphorylation in retina in both groups of rats after 30 min. The rate of insulin clearance by the retina is unknown, but based on other insulin delivery methods, repeated injections would be required to treat a patient as the effect of insulin is short (t1/2 ~3 h for Humulin® R, Lilly, injected subcutaneously). Therefore, future work will focus on long-term
delivery methods, such as time-release gels, that can be applied to the eye to deliver a sustained trans-scleral dose.
6.7 Figures and Figure Legends

Figure 22. Insulin and IGF-I activate their cognate and IR/IGF-IR hybrid receptors in R28 neurons. Serum-starved R28 cells were incubated with insulin (10 nM) or IGF-I (1.3 nM) for the indicated times and analyzed for IRβ and IGF-IRβ autophosphorylation. The IR and IGF-IR were activated by their respective ligands without cross-reactivity, except for hybrid IR/IGF-IR activation. Immunoblots are representative of three independent experiments.
Figure 23. Insulin, unlike other growth factors, preferentially phosphorylates the pro-survival Akt in retinal neurons. Serum-starved R28 neurons were treated for 5 min either insulin, BDNF, NGF, or EGF, with (+) and without (-) the addition of 200 µM of a putative IR/IGF-1R kinase inhibitor, HNMPA-(AM)₃. Cells were then harvested and equivalent protein was analyzed for Akt ser 473, p70 thr 389, and ERK 1/2 thr 202/tyr204 phosphorylation. HNMPA-(AM)₃ alone (compare the first two lanes from the left) activated ERK 1/2 and reduced p70 thr 389 phosphorylation. In lanes treated with growth factors alone (-), insulin increased Akt and p70 phosphorylation, while BDNF, NGF, and EGF only increased p70 phosphorylation. No growth factor activated the ERK pathway.
Figure 24. An unidentified protein, pp132, may mediate insulin action in retina. Ex vivo retina were analyzed for total phosphotyrosine content by immunoprecipitation (IP) and immunoblotting for PY. Following 10 nM insulin treatment for the indicated times (0 to 30 min), pp132 phosphorylation increases in concert with IRβ phosphorylation. Insulin-stimulated liver tissue (L+) served as a control for comparison purposes, and demonstrates that pp132 is expressed in lower abundance, if at all, in liver.
Figure 25. Y1158 phosphorylation of retinal and brain IRs is diminished compared to liver. In this preliminary experiment (n = 2 per condition), the contribution of Y1158 phosphorylation to overall constitutive IR phosphorylation was examined. The IRβ was immunoprecipitated from insulin-stimulated liver (L+) as a positive control and from liver, retina, and brain from fasted (18 h, -) rats or freely fed (+) rats. Y1158 phosphorylation increased in liver of fed rats, but in retina and brain tissue, IR1158 was not highly phosphorylated.
Figure 26. Constitutive IR kinase activity is reduced in retinas of Ins2<sup>Akita</sup> mice. Retinas from control and age-matched diabetic Ins2<sup>Akita</sup> mice were analyzed for IR kinase activity as described in Materials and Methods. Similar to the STZ model of diabetes, the constitutive retinal IR kinase activity is reduced ~27% (*p < 0.05 by Student’s t-test).
Figure 27. Altered downstream protein expression in retinas with reduced IR expression. Equivalent protein from retina (right) lysates were examined for IR, IGF-IR, phospho-Akt, and total Akt expression. Muscle tissue (left) served as a control for TTR-driven gene expression. All samples were from TTR-hIR and wild-type pde6 mice with different endogenous mIR expression. M, male; F, female; +/-, +/-, and -/- refer to endogenous mIR genotypes. Ponceau S stain (top panels) confirmed equal loading. IRβ expression in retina mirrored IRβ expression in muscle, but IGF-IR expression increased slightly in mouse retina with reduced IR expression. Likewise, phospho- and total Akt expression also increased modestly in retinas of mice with reduced IR expression.
Figure 28. Periocular insulin activates the pro-survival kinase Akt. (A) Insulin injected under the subconjunctiva has the potential to reach the circulation. A dose-response shows that 0.0325 U/100g of insulin had minimal effects on blood-glucose values (BGV, mg/dL) after 30 min (y-axis, net decrease in BGV; x-axis, insulin dose). (B) Representative immunoblots and quantification of Akt phosphorylation in retinas of rats receiving subconjunctival insulin. Rats of varying weights (n = 5 < 300g, n = 5 > 500g) were injected under the subconjunctiva with 0.0325 U/100g insulin (+) or equivalent volumes of diluent (-) in the contralateral eye. After 30 min, retinas were analyzed for Akt phosphorylation. BGV did not decrease, and retinal Akt ser 473 phosphorylation was significantly elevated in eyes receiving insulin (*p < 0.05).
CHAPTER VII

DISCUSSION AND FUTURE DIRECTIONS
Diabetic retinopathy (DR) is generally characterized as a vascular disease, but recent evidence from this laboratory [as reviewed in (138, 271)] and others (137, 222) now suggest that DR is a neurovascular disease in which subtle neurological abnormalities arise before overt vascular damage is observed. This concept is illustrated in Figure 29 (pg. 165) in which abnormalities arise, not necessarily in a linear order, in the neural retina following the onset of diabetes; these alterations are not readily detected by visual inspection of the retina. Changes within the neurons such as increased glial reactivity, microglial activation, decreased ERG response, reduced color and contrast sensitivity, and elevated apoptosis are undiagnosed prior to altered retinal perfusion, elevated VEGF expression, occludin phosphorylation and redistribution in the endothelium, and the “vicious cycle” of pathological vascular leakage and neovascularization. Only the latter can be detected in a routine examination, and, unfortunately, the only treatment option at this stage of the disease is laser surgery to cauterize leaking blood vessels. By gaining an understanding of the early molecular abnormalities that arise in the retina with diabetes, such as dysregulation of the constitutive IR→Akt signaling pathway, novel therapies may be developed which reverse the changes with the goal of slowing the progression, or preventing, overt retinopathy.

The central aim of this thesis was to gain a better understanding of early insulin signaling events in the retina. Insulin is a potent pro-survival hormone for neurons in culture (27, 111), but insulin signaling in the retina in vivo and the effects of diabetes were unexplored at the onset of this work. In fact, it has been assumed
that the retina was not insulin sensitive and virtually all retinopathy research has focused on glucose-related changes. The three major findings of this work are 1) the IR and the downstream kinase Akt are constitutively active in retina in vivo and exogenous insulin further stimulates this pathway; 2) diabetes downregulates the activity of this pathway; and 3) novel mechanisms regulate constitutive IR signaling in retina — specifically IGF-II is a probable ligand for the retinal IR-A in vivo (Fig. 30, pg. 166). Therefore, the downregulation of the constitutive pro-survival IR→Akt signalsome may be an early indicator of neural dysfunction in DR (Fig. 29, pg. 165).

Prior to these studies, the retina, as well as the CNS as a whole, was regarded as insulin-insensitive, probably because of a lack of insulin-stimulated glucose uptake and glycogen synthesis in neurons (29). While true, this characterization is misleading as the same study found other insulin-dependent effects on neurons; therefore, the outcome of IR stimulation is simply not as clearly defined in neurons as the glucose uptake pathway in other cell types. This work (Fig. 7, pg. 85) and others (40) have found that the IR in retina and brain is constitutively active and unresponsive to the fluctuations of circulating insulin. This may be interpreted as “insulin-insensitivity,” but considering the length of time required for insulin transport across cultured retinal endothelium cells [~1 h, (70)] and in vivo with superphysiological doses (>30 min, Fig. 6, pg. 83), the function of IR signaling in the retina is probably considerably different than the well-characterized anabolic functions of IR signaling in other peripheral tissues. Even reducing circulating insulin further in a fasted rat with SST did not acutely reduce IR autophosphorylation
(Fig. 20, pg. 133). The ability of the BRB to selectively transport insulin, compared to endothelium of other insulin-sensitive tissues, suggests other mechanisms, such as alternative ligands, phosphatase activity towards the retinal IR, or light, regulate IR signaling in retina.

The ex vivo retina preparation provides an ideal system to study downstream effects of IR stimulation. To summarize, it was learned that physiological insulin levels stimulate the IRβ→IRS-2→Akt-1 pathway. Increases in IRS-1 tyrosine phosphorylation were minimal. This is in agreement with others (205, 206, 208, 285) who have demonstrated that IRS-2 is a major substrate for the IR/IGF-IR system in neurons. IRS-1 is expressed in retina [Fig. 12, pg. 93, (50, 105)], so it should not be excluded as having a role in retinal insulin signaling physiology; likewise, the role of pp132 in retinal insulin signaling needs to be elucidated (Fig. 24, pg. 151). Because retinal neurons lack the insulin-sensitive GLUT4 protein (183), it is not surprising that mRNA for the insulin-sensitive Akt-2 appeared in low abundance and was not activated with insulin treatment (Fig. 9 and 10, pgs. 90-91). Akt-1, which has the broadest tissue distribution, did have increased activity with insulin treatment, but Akt-3, expressed mainly in neural and other immune-privileged tissues, was unresponsive (Fig 10, pg. 91). Because Akt-1 kinase activity mirrored pan-Akt ser 473 phosphorylation, this suggests only Akt-1 is insulin-sensitive in retina. Although Akt-3 mRNA was detected in retina, the protein may be in low abundance or mechanisms may exist which regulate a constitutively active and insulin-insensitive Akt-3. Collectively, these data suggest that the retinal IR is operative in vivo,
constitutively activated, and regulates Akt-1 activity. The retina, as a whole, does not store significant amounts of glycogen as it is mainly partitioned to the Müller cell (286, 287). Therefore, future studies will focus on the connection between GSK3β, a substrate regulated by Akt, in Müller cell glucose metabolism and the contribution of GSK3β to protein synthesis and neurodegeneration in the neural retina.

Numerous studies have investigated dysfunctional IR signaling in diabetic animal models, yet to date, no study has examined the effects of diabetes on the complication-prone retina. The second aim of this study addressed how the IR→Akt signalsome is altered in retinas of diabetic rats. The major finding was that the enzymatic activity of the signaling proteins — IR (Fig. 13, pg. 115), PI3K (Fig. 15, pg. 117), and Akt-1 (Fig. 16, pg. 118) — were decreased as early as four weeks following induction of diabetes, which correlates with increased apoptosis in the retina (130), although the specific cell types undergoing apoptosis are unknown. Additionally, the degree of IR signaling downregulation may also be cell specific within the retina. Also, after four weeks of STZ diabetes, expression and phosphorylation of the signaling proteins were unaltered, suggesting that other mechanisms or protein modifications regulate signaling activity in retina. Although these data (Chapter 4 and Fig. 26, pg. 153) show an association between reduced retinal IR signaling capacity and increased cell death in retinas of diabetic animals (130), other models, such as genetic knock down or in vivo interfering RNA studies, may demonstrate causality for reduced IR→Akt-1/3 pro-survival signaling and increased retinal apoptosis. Overall, these data support the hypothesis that the
dysfunctional IR→Akt signalsome in retina may be an early event in the pathogenesis contributing to DR (Fig. 29, pg. 165).

The goal of the DCCT was to test the hypothesis that intensive insulin therapy, with the goal of lowering blood glucose as close to normal as possible in IDDM patients, would reduce the risk of diabetic complications. That hypothesis was supported by the data, but the results have often been misinterpreted that the excess glucose in circulation is the primary cause of diabetic complications. The excess glucose indeed may contribute to the downregulated IR signaling in retina since circulating insulin does not have rapid access to the retina. Because plasma insulin is depleted in the long-term STZ rat, and the retinal IR retains ~70% basal activity in diabetic rats compared to controls, future studies using the compound phloridzin, which blocks glucose re-uptake in the kidney by inhibition of the sodium-linked glucose transporter, can address the hypothesis that excess glucose contributes to dysregulated IR signaling in retinas of diabetic rats. Furthermore, antibodies have been raised against N-acetylglucosamine, a glycosylation moiety that has been implicated in altering protein function, and O-linked N-acetylglucosamine content in proteins has been reported to increase with increased flux through the HBP pathway (288). Increased HBP flux has been reported to occur in retinal neurons cultured with high glucose and glucosamine (180), but whether increased HBP flux and N-acetylglucosamine modification of retinal IR signaling proteins occurs in vivo remains to be determined.
Because insulin in circulation does not rapidly gain access to the retina compared to other tissues, other mechanisms may regulate the constitutive autophosphorylation of the retinal IR. For instance, preproinsulin mRNA has been detected in retina (92). Therefore, locally synthesized growth factors, behind the BRB, may contribute to constitutive IR-A autophosphorylation in an autocrine or paracrine fashion. In \textit{ex vivo} retina, 100 nM IGF-II maximally phosphorylates the IR-A (Fig. 8, pg. 88). \textit{In vivo}, injecting neutralizing antibodies against insulin, IGF-I, and IGF-II reveal that IGF-II contributes to the basal IR-A autophosphorylation (Fig. 21, pg. 134). IGF-II has been reported in the vitreous of humans (167) and IGF-II mRNA is expressed in adult rat brain (197) and reduced with diabetes (276); therefore, IGF-II expression by the neural retina, with and without the effects of diabetes, and transport properties to the neural retina and vitreous must be examined (Fig. 30, pg. 166).

Together, the data contained in this thesis support a retinal insulin signaling pathway (Fig. 30, pg. 166) which is constitutively active, and further stimulation results in IRS-2 tyrosine phosphorylation and Akt-1 activation. An unidentified phosphotyrosine-containing protein may also mediate IR signaling in retina. Furthermore, IGF-II contributes, in part, to the constitutive IR autophosphorylation in retina. In the STZ rat model of diabetes, the constitutive activity of this pathway in retina is altered at the level of the IR, PI3K, and Akt-1/3. This may contribute to increased apoptosis in the retina of diabetics (130). As shown in Figure 30, the effect
of diabetes on IGF-II expression and contribution to retinal IR-A and IR-A/IGF-IR signaling remains to be explored.

The notion of a constitutively active transmembrane receptor also implies that intracellular conditions play a role in regulating IR autophosphorylation. Phosphatase activity towards the IR in retina may differ from other tissues. Following an intravenous insulin injection (Fig. 6, pg. 83) or insulin treatment of ex vivo retinas (Fig. 8, pg. 88), the maximal increase in retinal IR tyrosine phosphorylation was ~4-fold greater than basal. This suggests that ~25% of retinal IRs are constitutively active. Gene deletion of phosphatases that regulate IR signaling in muscle upregulate IR signaling (289), but the role of phosphatase activity towards the retinal IR remains to be investigated. Another key difference in IR regulation in retina compared to other cell types may be the mechanisms involved in IR recycling following ligand stimulation. In general, a liganded IR is internalized to endosomes in which the signal is still operative until dissociation of the ligand and dephosphorylation of the IR by phosphatases (290). Any number of the internalization processes may be prolonged, and phosphatase activity towards the IR may be diminished in retinal cells to maintain a sustained IR signal.

The results presented thus far have interesting implications for treating retinopathy, or other eye diseases, in patients. As shown in Chapter 6.6, periocular insulin can reach the retina and activate the pro-survival kinase Akt. Currently, experiments are ongoing in the laboratory of Dr. Gardner to determine optimal delivery methods to the retina, and whether other growth factors may also inhibit
apoptosis in DR in vivo. IGF-II may also be a likely therapeutic candidate because the IR-A isoform expressed by retinal neurons have a greater affinity for IGF-II than the IR-B isoforms expressed on the vasculature. Therefore, IGF-II treatment may select for retinal neuron pro-survival signaling as opposed to IRs on retinal endothelial cells, which have been attributed to transient worsening of retinopathy (144, 270). Ultimately, by reducing or completely blocking retinal IR signaling dysfunction, the development of blinding DR may be prevented.
7.1 Figures and Legends

Figure 29. A model of the progression of DR. After the onset of diabetes, subtle changes occur in the retina prior to pathological neovascularization and overt diabetic retinopathy. Dysregulation of the IR signaling cascade in retina is a previously undescribed event in diabetic models. Preventing one or more of the early changes may slow the progression of the disease.
Figure 30. A model of constitutive IR signaling in retina and alterations with diabetes. It is accepted that insulin is the primary ligand for the IR and has lower affinity for hybrid receptors, but in retina, IGF-II likely plays a role in activating the IR-A and possibly IR-A/IGF-IR hybrids. Stimulation of the pathway results in IRS-2 phosphorylation and Akt-1 activation. The role of pp132 is currently undefined in retina. Diabetes, likely due to excess glucose, inhibits the constitutive activity of this pathway, and its effect on IGF-II expression remains to be defined.
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Chad E. N. Reiter and Thomas W. Gardner. Constitutive Insulin Receptor Signaling in Retina: Effects of Diabetes and Mechanisms of Regulation. Presented at the 1st Annual Cell Signalling Symposium, Dundee, Scotland, UK.

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