

Diabetic retinopathy: loss of neuroretinal adaptation to the diabetic metabolic environment

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Diabetic retinopathy (DR) impairs vision of patients with type 1 and type 2 diabetes, associated with vascular dysfunction and occlusion, retinal edema, hemorrhage, and inappropriate growth of new blood vessels. The recent success of biologic treatments targeting vascular endothelial growth factor (VEGF) demonstrates that treating the vascular aspects in the later stages of the disease can preserve vision in many patients. It would also be highly desirable to prevent the onset of the disease or arrest its progression at a stage preceding the appearance of overt microvascular pathologies. The progression of DR is not necessarily linear but may follow a series of steps that evolve over the course of multiple years. Abundant data suggest that diabetes affects the entire neurovascular unit of the retina, with an early loss of neurovascular coupling, gradual neurodegeneration, gliosis, and neuroinflammation occurring before observable vascular pathologies. In this article, we consider the pathology of DR from the point of view that diabetes causes measurable dysfunctions in the complex integral network of cell types that produce and maintain human vision.

Keywords: diabetic retinopathy; neurodegeneration; neurovascular unit; maladaptation; metabolism

Clinical features of diabetic retinopathy

Diabetic retinopathy (DR) afflicts approximately 93 million people worldwide, and 28 million of these have vision-threatening DR.^{1–3} These numbers are expected to increase as the prevalence of type 2 diabetes continues to climb.⁴ The diagnosis and treatment of DR are primarily focused on vascular abnormalities that appear at later stages of the disease. DR is staged into several levels of severity, including mild, moderate, and severe non-proliferative DR (NPDR), followed by proliferative DR (PDR), depending on the extent of vascular lesions. As DR severity increases, vascular abnormalities, including plasma leakage, dilation, microaneurysms, and hemorrhages, occur at increasing frequency, with the growth of abnormal capillaries (angiogenesis) defining PDR. This vascular focus is largely due to the fact that retinal vasculature abnormalities are unambiguously identified by visual inspection, and advanced vascular abnormalities correlate with a disruption of vision. Diabetic

macular edema (DME) results when fluid accumulation increases retinal thickness and causes light-distorting fluid-filled cysts within retinal tissue, as well as serous detachments separating the neural retina from the underlying pigmented epithelium. Retinal edema is examined noninvasively by optical coherence tomography (OCT) imaging of the retina. OCT can precisely measure retinal layer thickness while detecting intraretinal cysts and serous retinal detachments. Fluorescein angiography clearly defines microaneurysms as hyperfluorescent dots, often associated with additional diffuse fluorescence within the retinal tissue, indicating dye leaking from the vasculature. If unchecked, this focal vascular leakage leads to precipitation of vision-obstructing opaque deposits of plasma lipoproteins (hard exudates). Vitreous cavity hemorrhages, caused by penetration of abnormal and bleeding capillaries into the vitreous gel, occur with the progression to PDR. These vessels fail to form a tight blood–retinal barrier (BRB) and thus leak and contribute to edema. Untreated PDR leads to fibrovascular

tissue formation, and the resulting epiretinal membranes require surgical removal by vitrectomy, as they adhere to the vitreous and cause traction retinal detachment.

The ability of vascular endothelial growth factor (VEGF) to promote both vascular permeability and angiogenesis made it a likely contributor to the vascular dysfunctions observed in severe DR. Fluid balance within retinal tissue is controlled by the balance of transport across the inner vascular BRB and fluid resorption across the retinal pigmented epithelium.⁵ Like the blood–brain barrier, the BRB is composed of tight junctions between endothelial cells that stringently control the flux of molecules between plasma and neural tissue. Breakdown of this normally tight barrier is thought to be a major factor in the pathogenesis of DME,⁶ although compromise of normal water removal mechanisms via aquaporin proteins could also contribute to the intraretinal accumulation of fluid.^{7–9} VEGF causes disassembly of endothelial cell junctions and acts as a potent endothelial cell mitogen, so inappropriate accumulation of VEGF in the diabetic retinas was hypothesized to promote both edema and angiogenesis. In 1994, Aiello *et al.*¹⁰ found markedly increased VEGF protein levels in the vitreous fluid of patients with DR. Their initial report and numerous subsequent studies demonstrated that the concentration of VEGF in vitreous fluid of DME and PDR patients can be increased to 10 times that of normal levels.^{10–20} Furthermore, VEGF levels are significantly higher in vitreous fluid from patients with active PDR compared to the vitreous fluid from those with inactive or quiescent PDR.^{17,18,21,22} These discoveries led to the concept that blocking VEGF action in the retina would improve DME and stall the progression of PDR. This hypothesis was clinically tested by an off-label application of humanized antibody against VEGF165 (bevacizumab, Genentech) that had been developed for cancer therapy, and several small trials have suggested efficacy toward edema and improved vision in approximately 25% of patients.²³ In these studies, patients received a limited number of bevacizumab injections (from one to nine), often on an as-needed basis. Ranibizumab (Genentech) is an Fc antibody fragment designed specifically for intraocular use, and it is now FDA approved for DME treatment on the basis of randomized trials.^{24,25} A VEGF receptor fusion protein (aflibercept; Regeneron) also improved vi-

sual acuity greater than 15 letters in up to 46% of patients after 1 year of treatment every 4 weeks.²⁶ Also, ranibizumab reduced the risk of NPDR severity progression in eyes treated for DME by approximately 67% over a 2-year period of monthly injections.²⁷ This last finding, if confirmed, suggests that VEGF overexpression may be directly related to the progression of established DR in a significant portion of patients.

Although success has not been total, these anti-VEGF treatments represent a major advance in DR therapy. Intravitreal injections are invasive but well tolerated by patients, and the risks associated with repeated intravitreal injections are surprisingly low. For example, endophthalmitis rates are less than 1/2000, and to date, no major long-term ocular or systemic risks of anti-VEGF treatments have been identified.²⁸ The success of anti-VEGF treatments and indications that additional factors may be involved in the control of retinal vascular permeability and angiogenesis in DME and PDR have encouraged research on alternative therapeutic targets (reviewed in Ref. 29). Additional proangiogenic factors are increased in vitreous fluid from PDR patients, including angiopoietin-2 (Ang-2),^{17,18,30} cysteine-rich 61 (CYR61),^{31,32} erythropoietin (EPO),^{16,18,33} osteopontin (OPN),^{34,35} platelet-derived growth factor (PDGF),^{36–39} and stromal cell–derived factor (SDF-1, CXCL12).^{40,41} Concentrations of several antiangiogenic factors are also decreased in the vitreous fluid of PDR patients and/or increased following laser photocoagulation therapy. These include angiostatin (AS),⁴² endostatin (ES),⁴³ pigment epithelium–derived growth factor (PEDF),^{16,30,34} and tissue kallikrein (TK).⁴⁴ In addition, after treatment of PDR with laser surgery or intravitreal bevacizumab, a transition from angiogenesis to fibrosis can occur when vitreous levels of VEGF decrease and levels of connective tissue growth factor (CTGF, CCN2) increase.⁴⁵

The hope is that targeting these alternative permeability-inducing or proangiogenic factors will help patients for whom anti-VEGF treatments are not effective. However, the greater goal is to address the early pathophysiologic changes that lead to vision loss so that patients with diabetes can maintain good vision without the need for invasive or destructive procedures.⁴⁶ This objective requires finding means to halt the progression of DR pathology before the accumulation of appreciable vascular

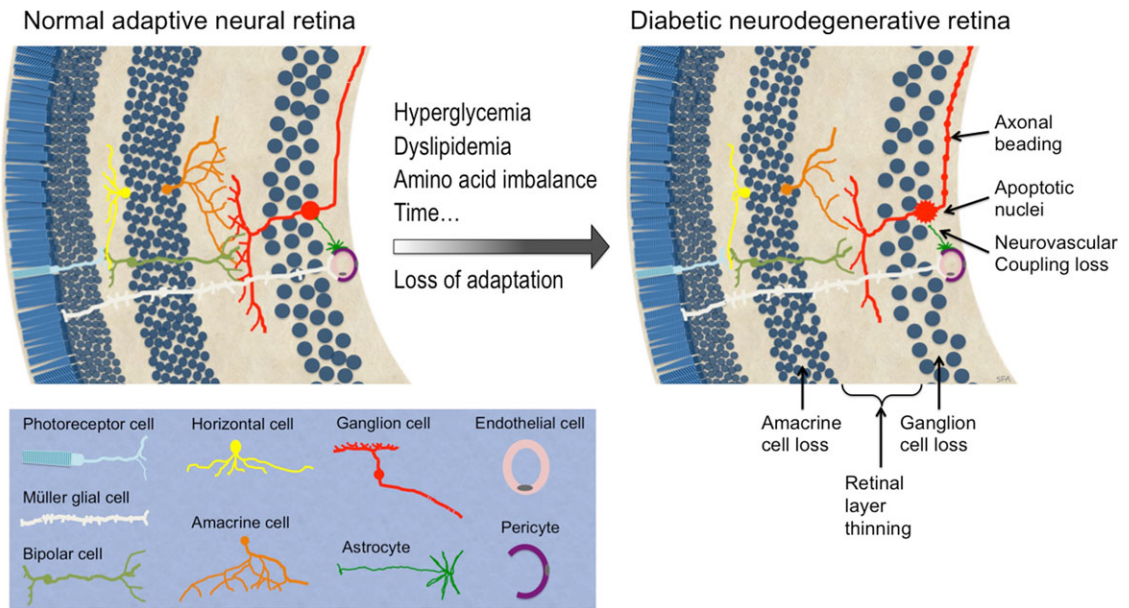


Figure 1. DR changes to the neural retina. Over time, neurons in the inner retinal layers lose adaptation to systemic metabolic alterations caused by diabetes and succumb to cell stress, as evidenced by reduced axonal and dendritic process branching, axonal beading, apoptotic cell death, accumulative cell loss, and retinal layer thinning.

defects, thus avoiding the detrimental effects of edema, microaneurysms, hemorrhages, and inappropriate angiogenesis.

Insufficient vascular coupling of retinal metabolic demand suggests that retinal neurovascular unit dysfunction is an early sign of DR

The challenge of maintaining good vision in an expanding population of patients with diabetes requires a new understanding of the pathophysiology of DR. It is not entirely clear why some organs (e.g., retina, peripheral nerves, and kidney) are relatively susceptible to diabetic complications. DR and diabetic nephropathy have been considered microvascular complications of diabetes, thus suggesting that the commonality is their dependence on microvascular functions. However, this apparent link does not explain the susceptibility of peripheral nerves or the recently appreciated cerebral complications of diabetes.^{47,48} In addition, while retinal and kidney microvessels share some parallels, they are fundamentally different; for example, kidney vessels do not possess a tight blood–tissue barrier. Thus, an important question to consider is what may link retina, brain, and peripheral nerves to make these

tissues relatively susceptible to diabetic complications. Lesions within the neurosensory retina are now understood to play an important role in DR. There are clear indications that retinal function is disturbed shortly after the onset of diabetes, and that neurodegeneration is an ongoing component of DR pathology.

We propose that the retinal response to diabetes involves a successful adaptation to altered systemic conditions, followed by eventual loss of neurovascular unit function in response to progressive metabolic disruption, resulting in subtle preclinical findings, followed by eventual advanced vision-threatening retinopathy (Fig. 1 and Table 1). Retinal function depends on the synergy of multiple neuronal subtypes, including photoreceptors, horizontal and bipolar cells, and amacrine and ganglion cells, along with their supporting glia (astrocytes and Müller cells), as well as inner vascular (endothelial cells and pericytes) and outer blood–retinal (choroidal vessels and pigmented epithelium) barriers that mediate the supply of nutrients and control the transfer of water and ions into and out of the tissue.⁴⁹ This complex integral network of cell types and structures allow humans to see across a wide range of light intensity (10 orders of magnitude),

Table 1. Evolution of diabetic retinopathy and failures of adaptive mechanisms

Preclinical retinopathy			NPDR		PDR	
	Symptoms	References	Symptoms	References	Symptoms	References
Clinical signs	None		None, blurred vision, or glare		None, floaters, or decreased vision	153–160
	Normal-appearing retina		Retinal vasodilation, microaneurysms, hemorrhages, cotton-wool spots, venous beading		Partial vitreous gel separation from retina; retinal and/or iris neovascularization; epiretinal membranes	
Functional events	Decreased ERG oscillatory amplitudes and increased latency	76–78	Normal or decreased visual acuity		Visual acuity usually decreased	
	Decreased visual field sensitivity	161	Visual field defects	153,161,162	Progressive visual field defects	
	Decreased flicker responses	63–65,67	Increased blood flow	163	Reduced dark adaptation	
	Decreased blue–yellow color sensation	164,165	Fluorescein angiography: vascular leakage and occlusion	166	Fluorescein leakage from neovascularization	
Cellular alterations	Synaptic loss	167			Reduced vasoconstriction in response to oxygen	
	Neuronal apoptosis	80,95,100,168,169	Cytoplast bodies and nerve fiber layer swelling	82,105–107,170,171	Retinal and/or iris neovascularization	
	Nerve fiber loss and retinal thinning	172–174	Neuronal loss and degeneration; lipid- and fluid-containing cysts	107,175	Progressive neuronal and axonal degeneration	
	Glial cell dysfunction	81,176,177	Gliosis of Müller cells and reduced astrocytes	176		
	Reduced endothelial cell tight junctions	178	Early fibrotic reactions with increased TGF- β	179,180	Epiretinal membranes	
Metabolic alterations	Reduced insulin receptor/Akt activity	89,90	Vascular occlusion and intraretinal shunt vessels	181		
	Reduced retinal protein synthesis	183	Microglial cell proliferation	182		
	Altered retinal lipid composition	151,184,185	Increased cytokine and proangiogenic proteins		Immune cells in epiretinal membranes	
	Defective crystallin expression	113,186				
	Reduced retinal glutamate/glutamine metabolism	119,187,188				
	ER stress	93				
	Oxidative stress	91,92				
	Impaired K _(ATP) current and purinergic toxicity	69,72,73				
	Reduced synaptic protein expression	167				
	Altered retinal amino acid profiles	189				

and to discern nearly infinite degrees of colors. This functional network appears to adapt fairly well to the systemic metabolic alterations caused by diabetes, for patients can maintain vision and exhibit no apparent clinical pathology for 5–10 years after the onset of diabetes. However, the complexity and functional demands of the retina may make it susceptible to eventual loss of tissue homeostasis in the presence of diabetes.

An important aspect of neuronal tissue equilibrium is balancing local blood supply and metabolic demand. It has been known for many years that cerebral blood flow responds to brain metabolism, and in the 1980s and 1990s it was realized that vascular tone is mechanistically coupled to neuronal metabolism; it was then hypothesized that this neurovascular coupling may involve signaling through astrocytes.^{50–53} Such a link was demonstrated in 2003 by Zonta *et al.*⁵⁴ using brain slices. Subsequently, Iadecola⁵⁵ was the first to popularize the term *neurovascular unit* to describe the concept that neurons, astrocytes, smooth muscle cells (or pericytes), and endothelial cells form a functional unit that controls cerebral blood flow in response to metabolic demand. The term has proved useful in understanding the links between neural degeneration and vascular dysfunctions that occur from stroke, Parkinson disease, and other neurodegenerations.⁵⁶ Matea and Newman⁵⁷ applied the neurovascular unit concept to describe the functional and structural interactions between neurons, glial cells, and vascular cells in the inner retina. The outer retina photoreceptors and Müller cells receive nutrients and dispose of waste products via the choroidal blood supply through the pigmented epithelium. Thus, ironically, the oxygen-rich outer retina is devoid of vessels, whereas the oxygen-poor inner retina has a well-defined, though relatively sparse, vascular supply. In both the inner retina and brain, neurovascular coupling regulates blood flow to meet the oxygen and nutrient demands created by metabolic and electrical activities, while the blood–tissue barriers control the flux of water and ions, protect against the influx of plasma proteins, and regulate inflammation.

Thus, the neurovascular unit allows integration of metabolic needs and vascular tone by integrating multiple molecular signals in context to maintain normal visual function throughout a range of phys-

iological conditions. The functions of the neurovascular unit in the brain and retina are demonstrated by a normal adaptive response of retinal blood vessels to match metabolic demand and to minimize excessive or insufficient blood and nutrient delivery, termed autoregulation.⁵⁸ In humans, retinal vascular diameter and blood flow respond dynamically to changing physiologic conditions, including blood pressure, blood gas concentration, and visual stimulation. For example, retinal function is protected from wide variations in systemic arterial pressures; retinal blood flow remains constant over a range of perfusion pressures up to an increase of 36% over baseline.⁵⁹ Other features of autoregulation are revealed by vasoconstriction in response to breathing 100% oxygen (hyperoxia) and vasodilation resulting from exposure to hypercapnia (increased pCO₂).⁶⁰ Hyperoxia reduces the volume of blood flow needed to provide the retina with appropriate oxygen influx, whereas hypercapnia increases the requirement for blood flow. These physiologic responses occur within seconds to minutes and diminish rapidly when the stimulus is removed.

The cellular coupling that links the neurovascular unit includes light-induced vasodilation and vasoconstriction of retinal arterioles. Flickering light stimulation of the retina increases metabolic demand in the inner retina, which is accompanied by vasodilation of arterioles.^{60–62} Metea and Newman⁵⁷ found that these responses result from direct glial–vascular signaling without neuronal involvement, and are mediated by integrated responses to arachidonic acid intermediates, nitric oxide, and K⁺. Specifically, stimulating or inhibiting nitric oxide synthase determines if signals initiated by 5–6-epoxyeicosatrienoic acid (5–6-EET) and 20-hydroxy-5,8,11,14-eicosatetraenoic acid (20-HETE) lead to vasodilation or vasoconstriction in response to light. These same arachidonic acid derivatives also mediate light-induced vasomotor responses and are associated with increased glial cell [Ca²⁺]. Metea and Newman⁵⁷ concluded that glial-evoked vasomotor responses are due to direct glial-to-vessel signaling without neuronal intermediates. Also, light- and glial-evoked vasomotor responses are mediated by the same arachidonic acid metabolites, with only light-evoked vasodilation and vasoconstriction depending on neuron-to-glia signaling. Thus, retinal vascular responses depend on multiple local and systemic factors.

Deficient neurovascular coupling, as evidenced by altered vasoconstriction and flicker light responses, is an early sign of retinal disequilibrium caused by diabetes. Multiple studies have examined the effects of diabetes on the reactivity of retinal vessels. Before the appearance of clinically evident retinal vascular lesions or edema, patients with pre-diabetes or overt type 2 diabetes exhibit greater than 50% reductions of vasoconstriction in response to hyperoxia and vasodilation in reaction to flicker stimulus compared to healthy controls.^{63,64} Similar changes occur in type 1 diabetes.⁶⁵ The impairment does not appear to result from a defective response to nitric oxide release, as vasodilation stimulated by oral nitroglycerin is normal in the diabetic subjects.⁶⁶ Pemp *et al.* conclude that, “neither the reduced vasodilator response to flicker stimulation nor abnormal retinal autoregulation, as observed previously, is the consequence of a generally reduced vascular reactivity of retinal vessels in this disease.”⁶⁶ Additional work by the Schmetterer laboratory shows that patients with well-controlled type 1 diabetes (mean HBA_{1c} < 7.5%) for less than 10 years with clinically normal retinas and normal pattern electroretinographic responses exhibit increased basal diameters of retinal arterial and reduced flicker-induced vasodilation in both retinal arteries and veins compared to nondiabetic controls.⁶⁷ This result suggests the mechanisms that regulate flicker responses may be distinct from those that mediate electrical responses of the neurosensory retina. The increased vascular diameter is an early manifestation of disturbed autoregulation that begins shortly after the onset of diabetes.⁶⁴ These early events suggest the possibility that the retina changes its adaptive reflexes in response to mild hyperglycemia or another metabolic perturbation of diabetes. Dorner *et al.* found that short-term hyperglycemia reduces the vasodilation response to flicker by 55% in healthy subjects.⁶⁸ Thus, diabetes appears to alter the ability of the retina to autoregulate, but the specific mechanism remains uncertain.

The mechanism by which diabetes affects the neurovascular coupling response to flickering light is not clear. Vasoreactivity also depends on focal electrical responses along the course of retinal arteriole/capillary segments.⁶⁹ Diabetes accelerates the axial decay of voltage along microvessels by fivefold compared to normal.⁷⁰ This defect results from spermine-induced inhibition of voltage-gated cal-

cium channels in arterioles⁷¹ and of K_(ATP) channels in capillaries, thus eliminating the normal topographical heterogeneity of functional K_(ATP) channels required for normal vasoresponses.⁷² Impeded axial voltage transmission would be expected to hinder the control of blood flow by impeding upstream responses to downstream signals. Vessels from diabetic rat retinas are also susceptible to death due to purinergic toxicity via pore formation following activation of P2X(7) purinoceptors by extracellular NAD⁺.⁷³ Together, these changes limit the ability of retinal vessels to autoregulate and increase neuronal vulnerability to death from metabolic insults. They reveal important regional responses of retinal vessels to ionic and small molecule signals that regulate the neurovascular unit under physiologic conditions.

Defects detected by electroretinography suggest that deficiencies in neuroglial function are early features of DR

Neuroglial function of the retina is assessed by electroretinography (ERG), the ocular equivalent of electroencephalography. The ERG has been used to investigate the ocular effects of diabetes for at least five decades, with the first studies finding delays in the response rate (increased implicit time) in patients with severe nonproliferative and proliferative retinopathy.⁷⁴ The studies were interpreted to indicate that Müller cells are damaged in advanced DR. More recent studies revealed defects in the ERG responses of patients with newly diagnosed type 1 and type 2 diabetes who have no clinically evident retinopathy.^{75–77} Multifocal ERG revealed focal areas of electrical depression and increased latency (implicit time) in the peripheral retina that predicted the development of retinal vascular lesions in specific retinal zones.⁷⁸ Leclaire-Collet *et al.*⁷⁹ observed that alterations in the amplitude and implicit time pattern of ERGs in patients with diabetes correlate with deficits in flicker light-induced vasodilation. These changes were interpreted to indicate altered function of Müller, ganglion, amacrine, and bipolar cells, all of which are adversely affected by experimental diabetes.^{80–83} The ERG is a highly sensitive index of retinal function but is difficult to apply in routine clinical practice because of the length of testing (>1 h for dark-adapted testing) and need for corneal contact lenses.

Together, these findings of depressed vascular coupling and altered neuroglial electrical responses

that occur before the onset of clinically evident vascular lesions suggest that diabetes causes considerable alterations to retinal function before the onset of typical clinical signs of DR. These neurosensory retina alterations might be early adaptations to diabetes or may simply be negative consequences of adaptive mechanisms necessary to maintain function of the retina in the diabetic state. These findings do not necessarily indicate the blood vessels are normal during this period. Indeed, mice with deficient PDGF receptor β expression in brain pericytes exhibit blood–brain barrier breakdown at 1 month of age, while behavioral function and brain cellular structure remain intact until 6 months of age and neuroinflammation occurs at 16 months of age.⁸⁴ Therefore, primary blood–brain barrier defects do not cause immediate brain damage in animals that are otherwise healthy, implying that the central nervous system has intrinsic cytoprotective mechanisms, as suggested by Iadecola.⁸⁵ Degenerative brain diseases exhibit concomitant defects in neuroglial and vascular function (reviewed in Ref. 86), but the sequence of neurovascular unit disintegration in DR remains uncertain at this time. Thus, normal-appearing retinal blood vessels in diabetic patients may not reveal subtle defects that could indicate early retinopathy.

Maladaptive changes observed in animal models of diabetes and human specimens

The transition from physiologic adaptation to disease occurs slowly over the course of years, and indications of maladaptive changes may lie below the resolution of clinical evaluation; therefore, studies in animal models are particularly important. Functional changes, such as impaired ERG and flicker responses, similar to those in humans occur in rats and mice within several months of diabetes onset.^{87,88} Concurrent biochemical alterations characteristic of diabetes (e.g., reduced insulin receptor/Akt activity,^{89,90} oxidative stress,^{91,92} and endoplasmic reticulum stress⁹³) occur within 1–6 months after the onset of diabetes and in the context of histologically normal retinas. These alterations may have direct effects on retinal neurovascular unit function by causing cellular dysfunction, perturbations in neurotransmitter production, astrogliosis, and neuroinflammation. In diabetic rats, retinal cell death and astrogliosis were reversed both by reduction of hyperglycemia via treatment with the

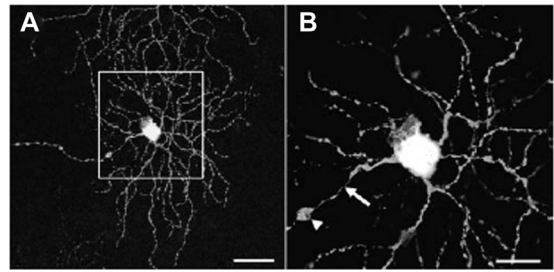


Figure 2. Axonal swellings of Thy1-YFP⁺ retinal ganglion cells in a mouse model of diabetic retinopathy. An example of axonal swellings on retinal ganglion cells of Thy1-YFP transgenic mice crossed with Ins2^{Akita} diabetic mice after 3 months of diabetes. (A) An entire dendritic arbor of a retinal ganglion cell in a diabetic retina; (B) an enlarged image of the boxed region in A, with axon swelling (arrowhead) at approximately 60 μm from the soma and preceded by a prominent thinning of the axon (arrow). Scale bars: 50 μm in A and 20 μm in B. Taken from Gastinger *et al.*,¹⁰⁹ with permission.

sodium-linked glucose transporter inhibitor phloridzin (without added insulin) and by injecting very small doses of insulin in the subconjunctival space to restore retinal insulin receptor signaling (without affecting systemic glycemia).⁹⁴ Both manipulations restored retinal Akt activity. Akt, also known as protein kinase B (PKB), is a key positive regulator of cell metabolism, growth, and survival.⁹⁵ Akt is highly responsive to insulin receptor binding leading to PI3K activation, which occurs in nervous tissues as well as classically insulin-responsive tissues (liver, muscle, and adipose).⁹⁶ Thus, the corrective effects of local insulin delivery were expected, while the effects of glycemic normalization were somewhat surprising. We interpret these results to indicate that both central features of type 1 diabetes, insulin deficiency and associated hyperglycemia, can contribute directly or indirectly to cellular dysregulation in the rodent retina. The findings do not exclude contributions from dyslipidemia or altered amino acid metabolism, which are also part of the diabetic milieu.

If the retina appears normal and has normal visual function as assessed by standard methods (visual acuity and fields), clinicians generally deem that retinopathy is not present. Nevertheless, studies in animal models reveal multiple cellular alterations, suggesting that diabetes causes subtle but progressive dysfunction and degeneration of the neural retina. Notably, multiple studies have shown that uncontrolled insulin-deficient diabetes increases the

basal rate of neuronal cell death by several fold, beginning within 1–3 months after the onset of hyperglycemia and in postmortem retinas of humans with less than 5 years of diabetes.^{80,97–102} Diabetic rodent models do not generally exhibit the advanced stages of DR pathologies observed in humans, such as DME and PDR, but they eventually exhibit breakdown of the BRB and vascular cell death.¹⁰³ The rapid onset of neuronal apoptosis was not initially expected because the earliest vascular cell death in diabetic rats requires at least 6 months to appear.¹⁰⁴ It should be noted that several groups have failed to detect increased neuronal apoptosis in diabetic rats or mice when they examined retinal cross sections.^{88,105–107} Indeed, Barber *et al.*⁸⁰ also found no quantitative increase in TUNEL-positive cells when using cross sections, even though occasional sections revealed TUNEL-positive cells. However, when retinal flat mounts are examined and sufficient animals are studied to provide appropriate statistical power, the sampling error of sections is circumvented and the infrequent but increased rate of cell death is apparent.⁸⁰ Fort *et al.*⁹⁴ also reported the ability of a nucleosome ELISA to consistently detect modest increases in retinal cell death and responses to treatments in diabetic rodents. Therefore, we submit that the accelerated retinal cell death in diabetes is a conserved event in all species; however, appropriate group numbers and methods must be used if its occurrence is to be accurately detected.

Moreover, diabetes affects both neurites (axons and dendrites) and cell bodies of retinal neurons, as evidenced by neuritic swellings^{108,109} (Figs. 2–4), TUNEL-positive nuclei, and caspase-3 activation⁸² within a few months of diabetes onset. These changes in well-defined animal models reveal ganglion cell body swelling and axonal fragmentation, and closely mimic features observed in postmortem human retinas from patients with diabetes.¹¹⁰ Both retrograde and anterograde axonal transport are impeded in the optic nerve of diabetic rats.^{111,112} Retinal ganglion cell axonopathy, including reactive gliosis of axonal astrocytes, occurs within weeks of diabetes, before loss of retinal ganglion cells.¹¹² This effect on retinal ganglion cells differs from that in glaucoma, in which axonal degeneration is thought to be the primary defect.^{113,114}

The presence of ongoing retinal cell death in animals and humans under moderate metabolic

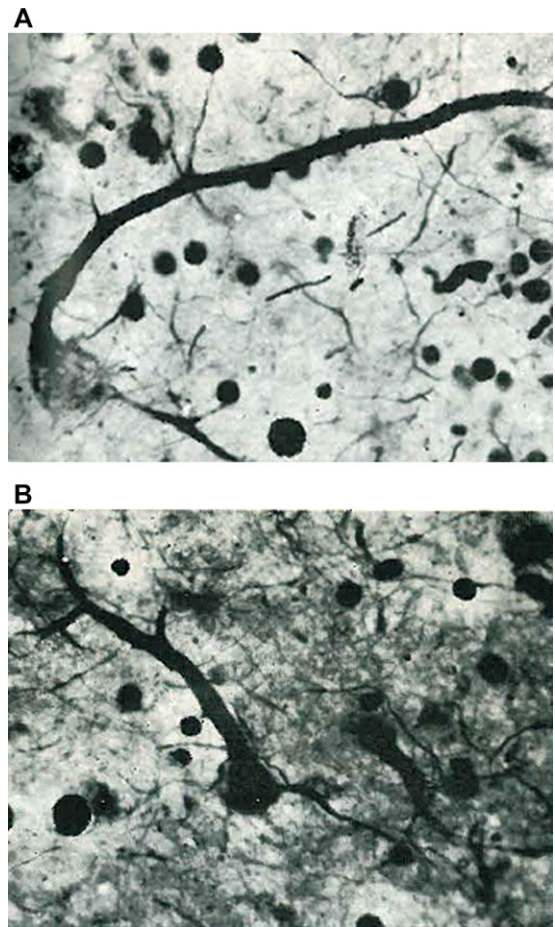


Figure 3. Early evidence of degenerating ganglion cells in human diabetic retinopathy. Ganglion cell bodies and neurites are fragmented and the dendrites are swollen. Hirtaga neuroglia stain of frozen sections. Reprinted from Wolter,¹¹⁰ with permission.

control⁸⁰ suggests several potential explanations. One possibility is that hyperglycemia or insulin resistance is sufficient to injure neurons. Indeed, mild DR occurs in approximately 8% of people with prediabetes,¹¹⁵ much as diabetic peripheral neuropathy occurs in up to 25% of people with prediabetes,¹¹⁶ and multiple cases of DR have been reported in patients with normal or minimally altered glucose tolerance (reviewed in Ref. 117). These established clinical observations suggest that hyperglycemia alone may not be a necessary or sufficient cause of DR.

Another explanation for early and ongoing cell death is that intrinsic adaptive mechanisms fail

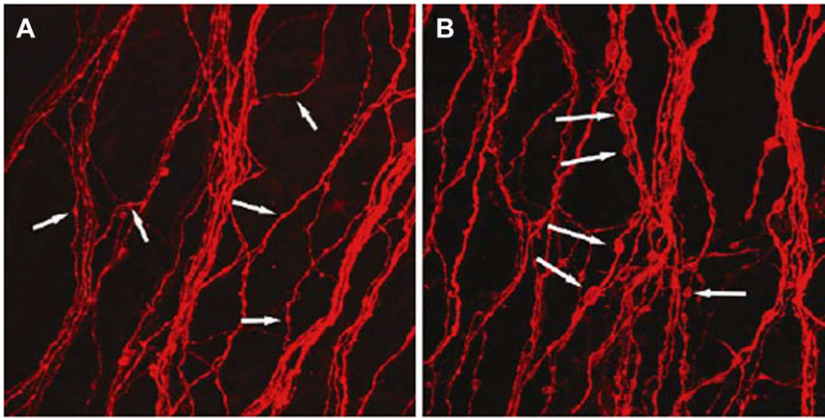


Figure 4. Enlarged axonal beading of parasol cells in human diabetic retinopathy. (A) Axons within a control retina exhibiting low-caliber beading consistent with transportation beads (arrows). (B) Axons within a diabetic retina showing abnormally large and irregular beads along the axons (arrows). Taken from Meyer-Rüsenberg *et al.*,¹⁵⁷ with permission.

to serve their function, as illustrated with two examples. First, α -crystallins belong to the small heat shock protein family of molecular chaperones and regulate apoptosis by inhibiting the proapoptotic Bcl-2 family member Bax. In diabetic rats, the expression of these chaperones increases while their solubility decreases, leading to disruption of their interaction with Bax.¹¹⁸ Second, autophagy, a lysosome-based process to recycle organelles and long-lived proteins, normally protects neurons from death, but we have found that the expression and cleavage of retinal autophagy proteins are altered in clinical and experimental diabetes (unpublished data). Iadecola and Anrather⁸⁵ have summarized a host of intracranial and systemic endogenous protections against stroke, including enhanced growth factor production, bone marrow-derived progenitor cells, and blood pressure control. At present, protective mechanisms in the retina are less well understood, but the potential for therapeutic benefits of endogenous neuroprotection could be substantial. Additional work is needed to determine how diabetes causes cellular stress, how stress response mechanisms are altered in retinal neurons, and when neurovascular coupling is lost beyond the ability to prevent vision loss.

Proposed mechanisms of DR related to impaired Akt/mTOR signaling

Clearly, the diabetes epidemic mandates new strategies to prevent retinal cell death and preserve vision,^{119,120} and understanding the mechanisms of

neural cell death in DR is essential because neuronal integrity is central to vision.¹²¹ The mechanisms of retinal neurodegeneration are complex and likely multifactorial. At a systemic level, insulin-deficient diabetes is fundamentally a consequence of deficient insulin receptor action in sensitive tissues, notably liver, skeletal muscle, and adipose tissue, with attendant unopposed excess action of glucagon, leading to a catabolic state with breakdown of energy stores, impaired substrate oxidation, and accumulation of lipids, glucose, and some amino acids within the blood. In contrast to the acutely insulin-sensitive tissues (liver, skeletal muscle, and adipose), the retina differs in that insulin receptor kinase activity does not fluctuate with feeding and fasting.¹²² In addition, even though insulin at high nanomolar concentrations can stimulate retinal insulin receptors, insulin-like growth factors 1 and 2, rather than insulin, are probably the primary endogenous ligands for retinal insulin receptors (unpublished data). Nonetheless, the retina uses largely conserved insulin receptor signaling pathways, notably PI 3-kinase (PI3K), Akt isoforms 1 and 3, and p70 S6 kinase, but not p42/44 MAP kinase, to mediate cell survival.^{89,94,123,124} Analysis of enzyme activities by immunoprecipitation and quantification of substrate phosphorylation reveals that the activity of the retinal insulin receptor–PI3K–Akt pathway is approximately double that of the gastrocnemius muscle in normal rats.¹²⁵ This relatively high enzyme activity also parallels a higher specific rate of protein synthesis in the retina compared to muscle

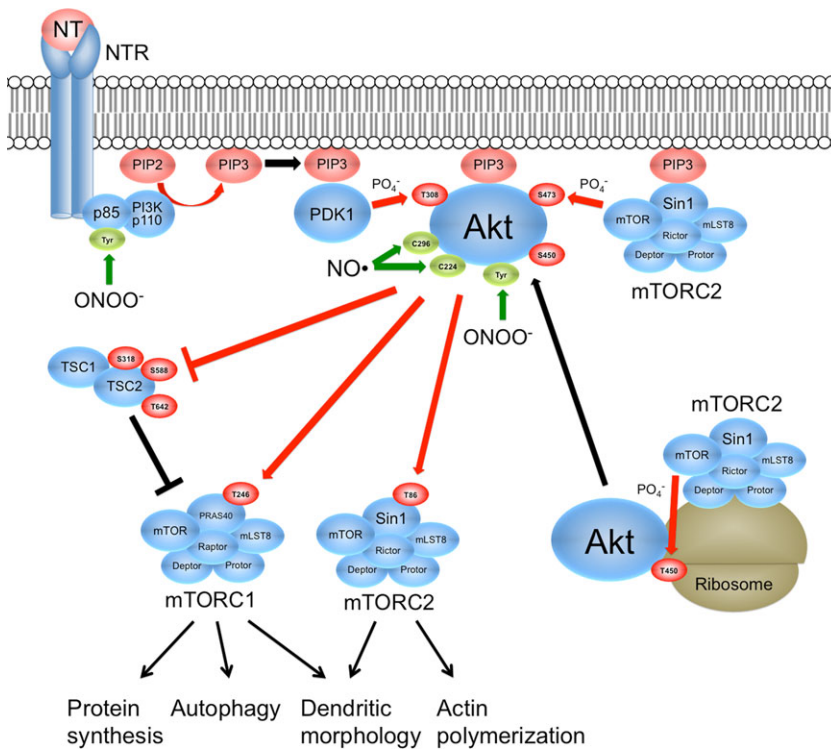


Figure 5. Summary of known Akt and mTOR regulation pathways. The mTOR kinase within rictor-containing mTORC2 complex located at ribosomes catalyzes the co-translational phosphorylation of Akt Thr450, thus stabilizing the newly formed Akt protein. Neurotrophins (NT) (e.g., NGE, IGFs, BDNF) bind their respective receptors (NTR), thus activating phosphoinositide 3-kinase (PI3K), resulting in formation of phosphoinositide(3,4,5)-triphosphate (PIP₃). PIP₃ recruits phosphoinositide-dependent kinase 1 (PDK1), Akt, and mTORC2 complex to the membrane by association with pleckstrin homology (PH) domains. PDK1 phosphorylates Thr308 of Akt and mTOR within mTORC2 phosphorylates Akt Ser473, thus fully activating Akt. TSC2 inhibits mTORC1 through GTPase activation of the small G protein Rheb (not shown). Akt-induced phosphorylation of TSC2 at several sites relieves inhibition of mTORC1. In addition, Akt increases the phosphorylation of PRAS40 in mTORC1 and Sin1 in mTORC2. mTORC1 primarily stimulates protein synthesis via its effects on mRNA translation and regulates autophagy, whereas mTORC2 controls dendritic morphology and actin polymerization (see text). Phosphorylations are shown in red and nitrations and nitrosylations are shown in green. Protein nitration occurs when peroxynitrite (ONOO⁻), formed from nitric oxide (NO) and superoxide (O₂⁻), reacts with tyrosine residues (Tyr) forming nitrotyrosine. Nitration has been implicated in inhibition of PI3K and Akt activities. Protein nitrosylation occurs when NO reacts with the thiol group of cysteine residues. Nitrosylation of Cys298 and Cys224 have been implicated in the inhibition of Akt activity.

(unpublished data). This may be due to the extremely high metabolic and synthetic rates of photoreceptors. Rajala *et al.* have detailed how photons activate the insulin receptor of photoreceptors to mediate cell survival via hexokinase^{126,127} and phototransduction by activating photoreceptor cyclic nucleotide gated channels.^{128,129} Accordingly, deletion of the insulin receptor or Akt2 removes neuroprotective inputs to photoreceptors, making them highly susceptible to light-induced stress.^{130,131}

The mechanism of diminished Akt activity observed in retinas of diabetic rats does not coincide

with loss of phosphorylation associated with growth factor signaling.⁸⁹ We recently completed an exhaustive mass spectrometric analysis of all Akt1 protein phosphorylation changes occurring in diabetic rat retinas in which Akt1-specific activity is significantly downregulated. Although approximately 4% of Akt1 protein from the diabetic retinas exhibited a novel dual phosphorylation of S124 and S129 in the hinge region, no changes in activating phosphorylations were identified (unpublished data). Thus, loss of activating phosphorylation does not seem to be the mechanism of decreased Akt1 activity in the diabetic retina. It is also probable that diabetes-induced

protein oxidative and nitrosive alterations impair Akt in the neural retina. Such modifications would explain diminished Akt activity without loss of activating phosphorylations. Increased protein nitration in the neural retina of diabetic humans and rats has been documented. For example, nitrotyrosine immunoreactivity was found throughout the inner neural retina, including the nerve fiber, ganglion cell, inner plexiform, and inner nuclear layers of retinas from diabetic humans and rats.^{132,133} Work by El-Remessy *et al.* demonstrated how nitration of the antiapoptotic TrkA receptor plays a key role in the loss of NGF signaling and neurodegeneration in the diabetic retina¹³⁴ and how nitration of PI3K p85 subunit inhibits VEGF survival signaling in retinal endothelial cells.¹³⁵ High-fat diet–induced nitration of insulin signaling proteins and insulin resistance were reversed by catalytic removal of peroxynitrite, the reaction product of superoxide and nitric oxide, which causes nitration of tyrosine residues.^{136,137} Although nitration of tyrosines in Akt is likely in DR, two cysteines, Cys296¹³⁸ and Cys224,¹³⁹ have also been identified as nitrosylation sites of Akt1 (Fig. 5). This Akt nitrosylation mechanism accounts for loss of Akt activity in aging muscle and brain ischemia, where activating phosphorylations of Akt were not decreased, but nitrosylation of Akt was increased.^{140,141} Akt nitrosylation is thought to contribute to insulin resistance in diabetic muscle.¹⁴² In high-fat diet–induced prediabetes and diabetes, muscle insulin resistance coincided with nitrosylation of the insulin receptor β , insulin receptor substrate 1 (IRS-1), and Akt.¹⁴³

We hypothesize that defects in mTOR signaling may also underlie the neurodegenerative aspects of DR, as it does brain degenerations,^{144,145} because it coordinates protein synthesis and autophagy in response to growth factors and amino acids.^{146,147} mTOR is associated with two relatively independent complexes, mTORC1 and mTORC2, which contain regulatory-associated protein of mTOR (raptor) and rapamycin-insensitive companion of mTOR (rictor) proteins, respectively (Fig. 5). Raptor-associated mTORC1 promotes protein synthesis in muscle and liver by phosphorylating initiating factor 4E-binding protein (4E-BP1) and S6 kinase (S6K1), and is inhibited by both torin and rapamycin.¹⁴⁸ In contrast, rictor-associated mTORC2 promotes cellular viability and cytoskeleton organization by phosphorylating Akt Ser473 and protein kinase C α ,

and is inhibited by torin but not rapamycin.¹⁴⁸ Conditional deletion of *Rictor* in postnatal mice and flies impairs synaptic efficacy via reduced actin polymerization^{149,150} and causes cerebellar Purkinje neurons to be severely stunted.¹⁵¹ Both raptor and rictor regulate dendrite arborization in hippocampal neurons in response to insulin and IGF-1.¹⁵² Therefore, it is reasonable to postulate that disruption of neuronal mTOR function may contribute to defects in axons and dendrites that are essential for vision. mTOR also suppresses autophagy.¹⁵³ Depending on the context, autophagy can protect neurons or promote neuronal cell death,^{154,155} but increasing evidence suggests that autophagy dysfunction can contribute to neurodegeneration by allowing the accumulation of mutant or defective proteins.¹⁴⁵ We recently showed that hyperglycemia decreases mTOR activity in diabetic rat retinas and R28 cells by lowering phosphatidic acid content, and replenishment of phosphatidic acid inhibits retinal cell death induced by IL-1 β .¹⁵⁶ These findings reinforce the importance of further understanding how diabetes impairs Akt and alters mTOR signaling in the retina.

Conclusion

Numerous studies over the past five decades conclusively show that neurosensory retinal defects evolve with or before the onset of the earliest vascular lesions that define DR pathology. This information may allow early detection of DR using sensitive techniques that measure retinal sensory neuropathy. However, several questions remain, such as (1) the nature of the insults that lead to retinal damage; (2) how and when the retina loses adaptation to ongoing diabetes resulting in vision loss; (3) how clinical tests can be optimized to detect early retinal neuropathic changes in clinical practice; and (4) how such a diagnosis can be employed to prevent appreciable vision loss in persons with diabetes.

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Conflicts of interest

The authors declare no conflicts of interest.

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