

Diabetes Reduces Basal Retinal Insulin Receptor Signaling

Reversal With Systemic and Local Insulin

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Diabetic retinopathy is characterized by early onset of neuronal cell death. We previously showed that insulin mediates a prosurvival pathway in retinal neurons and that normal retina expresses a highly active basal insulin receptor/Akt signaling pathway that is stable throughout feeding and fasting. Using the streptozotocin-induced diabetic rat model, we tested the hypothesis that diabetes diminishes basal retinal insulin receptor signaling concomitantly with increased diabetes-induced retinal apoptosis. The expression, phosphorylation status, and/or kinase activity of the insulin receptor and downstream signaling proteins were investigated in retinas of age-matched control, diabetic, and insulin-treated diabetic rats. Four weeks of diabetes reduced basal insulin receptor kinase, insulin receptor substrate (IRS)-1/2-associated phosphatidylinositol 3-kinase, and Akt kinase activity without altering insulin receptor or IRS-1/2 expression or tyrosine phosphorylation. After 12 weeks of diabetes, constitutive insulin receptor autophosphorylation and IRS-2 expression were reduced, without changes in p42/p44 mitogen-activated protein kinase or IRS-1. Sustained systemic insulin treatment of diabetic rats prevented loss of insulin receptor and Akt kinase activity, and acute intravitreal insulin administration restored insulin receptor kinase activity. Insulin treatment restored insulin receptor- β autophosphorylation in rat retinas maintained *ex vivo*, demonstrating functional receptors and suggesting loss of ligand as a cause for

reduced retinal insulin receptor/Akt pathway activity. These results demonstrate that diabetes progressively impairs the constitutive retinal insulin receptor signaling pathway through Akt and suggests that loss of this survival pathway may contribute to the initial stages of diabetic retinopathy. *Diabetes* 55:1148–1156, 2006

A growing body of evidence suggests that the neural retina undergoes significant deterioration early in the course of diabetes. In humans, this evidence includes altered electroretinograms, diminished color vision, and contrast sensitivity before the clinical diagnosis of diabetic retinopathy (1,2). Retinal neurons undergo apoptosis within 1 month after the onset of experimental diabetes in rats (3), and the *Ins2^{Akita}* diabetic mouse model demonstrates elevated retinal caspase 3 activity by 4 weeks (4). Intensive diabetes control has the greatest therapeutic effect before the development of early diabetic retinopathy (5), so it is of paramount importance to understand the initiating events in the pathogenesis of diabetic retinopathy in order to develop the means to prevent vision impairment.

Insulin signaling in retina has received little attention, but recent work indicates that insulin receptor signaling may be important for retinal physiology because the retina expresses amounts of insulin receptor protein equivalent to the liver and brain. Retinal insulin receptors autophosphorylate and activate downstream signaling kinases (6–10) and may contribute to retinal development (11–13). In normal rats, autophosphorylation of the retinal insulin receptor is equivalent to that of fasted liver insulin receptor; however, the kinase activity of retinal insulin receptors is equivalent to that of postprandial liver insulin receptors and twofold greater than that of liver insulin receptors from fasted rats (6). Retinal insulin receptor kinase activity is equivalent to that of the brain and does not fluctuate with the feeding/fasting cycle. Furthermore, retinal Akt kinase activity is several-fold higher in normal animals than in skeletal muscle or liver (6). Thus, the basal insulin receptor and Akt activity is distinct from liver, muscle, and fat basal insulin receptor activity and appears to be important for the normal function of retina.

In retinal neurons, insulin provides trophic support for neurons via phosphatidylinositol 3-kinase (PI3-K)/Akt and p70S6 kinase pathways (14,15), and this trophic function of insulin on retinal neurons is impaired by exposure to elevated glucose and glucosamine (16). Exogenous insulin

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GSK, glycogen synthase kinase; IGF, insulin-like growth factor; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; PI3-K, phosphatidylinositol 3-kinase; STZ, streptozotocin.

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stimulation of whole retina tissue activates the insulin receptor substrate (IRS)-2/Akt branch of the insulin receptor signaling network, with no change in extracellular signal-related kinase-1/2 phosphorylation (6,8). Insulin stimulates Akt phosphorylation within the same layers of retinal neurons where apoptosis is increased in diabetic rats and humans (3,6). These data suggest that the Akt pathway is utilized in retinal neurons as a continuous survival signal.

This study tested the hypothesis that diabetes alters the basal pro-survival insulin receptor signaling pathway in retina. The results show that insulin-deficient diabetes significantly reduced constitutive retinal insulin receptor kinase activity beginning after 4 weeks, with further loss of insulin receptor autophosphorylation, expression, and activity after 12 weeks. However, the insulin receptor remained responsive to exogenous insulin. Short-term diabetes also reduced retinal IRS-1/2-associated PI3-K activity, Akt kinase activity, glycogen synthase kinase (GSK)-3 β phosphorylation, and p70S6K kinase activity despite normal expression of the signaling proteins. Long-term diabetes reduced IRS-2 but not IRS-1 content and did not alter retinal p42/p44 mitogen-activated protein kinase (MAPK) activity. Together, these results suggest that constitutive insulin receptor/Akt/p70S6K pro-survival signaling in retina is impaired by diabetes and may contribute to neural degeneration in the development of diabetic retinopathy.

RESEARCH DESIGN AND METHODS

Induction of diabetes and insulin therapies. Age-matched male Sprague-Dawley rats (Charles River, MA) were used in all experiments. Rats were housed under a 12-h light/dark cycle with free access to a standard rat chow and water. All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Resolution on the Care and Use of Laboratory Animals. Diabetes was induced by intraperitoneal injection of streptozotocin (STZ) (65 mg/kg; Sigma, St. Louis, MO) dissolved in sodium citrate buffer, pH 4.5, and control rats received equivalent volumes of buffer alone as described previously (3). STZ-injected rats were considered diabetic when exhibiting blood glucose levels >13.9 mmol/l (250 mg/dl) within 5 days after diabetes induction (One-Touch meter; Lifescan, Milpitas, CA). Continuous insulin therapy was begun 5 days after induction of diabetes by implanting a subcutaneous insulin pellet to deliver ~2 units bovine insulin/day for the duration of the experiment (LinShin Canada, Toronto, Ontario, Canada) (3). A second implant was given after 4 weeks for longer time points. Acute, short-term insulin therapy consisted of two injections daily of 5 units Humulin Regular/5 units Humulin Ultralente for 3 days before the animals were killed (17). Intravitreal insulin (5 μ l of a 100-nmol/l solution of bovine crystalline insulin [Sigma]) was injected in one eye and vehicle (PBS with 0.1% BSA) in the other eye, under ketamine/xylazine anesthesia (4 mg/kg/0.4 mg/kg). Before death, rats were anesthetized with injection of 100 mg/kg sodium pentobarbital i.p. and killed 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 (see below).

Ex vivo retina cultures. Experiments were performed essentially as described (6). This technique preserves retinal energetic function as measured by ATP, ADP, and phosphocreatine levels. This method of incubating retinas in an insulin-containing medium is analogous to the widely used brain slice method and mimics the in vivo environment in which retina is exposed to insulin via the vitreous fluid and choroid (18). Briefly, individual whole retinas were incubated in 1 ml modified Eagle's medium (Sigma) with 5.5 mmol/l glucose, 5 mmol/l pyruvate, and 10 mmol/l HEPES at 37°C and 5% CO₂. After 15 min, retinas were treated with insulin (10 nmol/l bovine crystalline; Sigma) or 0.9% saline vehicle for 5 min. Retinas were then snap frozen under liquid nitrogen and stored at -80°C until analysis.

Immunoprecipitation and immunoblotting. Preparation of retinal lysates, immunoprecipitation, SDS-PAGE, immunoblotting, and reprobing of the nitrocellulose membranes were performed as described (6,14). Anti-insulin receptor- β , insulin-like growth factor (IGF)-1R β , and anti-p70S6 kinase antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-

tyrosine, IRS-1, IRS-2, Gab-1, pan p85 (also recognizes p50 α and p55 α /AS53 [19]), and phospho-Akt Thr (308) were from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-Akt Ser (473), pan-Akt, phospho-GSK-3 β Ser (9), anti-phospho-p70S6K (Thr389), phospho-p44/42 (Thr202/Tyr204), and Elk-1 fusion protein were from Cell Signaling Technology (Beverly, MA). Anti-actin was from Sigma. For immunoprecipitations, the protein A Sepharose bead slurry was from Amersham Pharmacia Biotech (Piscataway, NJ). Protein concentrations of tissue lysates were determined using a DC protein assay kit (Bio-Rad, Hercules, CA) compared against a BSA standard curve. Equivalent amounts of protein were loaded per lane for SDS-PAGE and each immunoprecipitation reaction.

Kinase activity assays. The kinetic activities of the insulin receptor, IGF-1 receptor (IGF-1R), Akt, and p70S6k were performed as described previously (6,15,20). The immunoprecipitating antibodies against the insulin receptor- β , IGF-1R β , and Akt-1 were from Santa Cruz Biotechnology, and anti-Akt-3 was from Upstate Biotechnology. γ -³²P-ATP for all kinase assays was from Amersham.

The PI3-K assays were performed as described (21) with slight modifications. Retinas were homogenized in ice-cold buffer A consisting of 50 mmol/l Tris-HCl (pH 7.5), 0.1% Triton X-100 (wt/vol), 1 mmol/l EDTA, 1 mmol/l EGTA, 50 mmol/l sodium fluoride, 10 mmol/l β -glycerophosphate, 5 mmol/l sodium pyrophosphate, 1 mmol/l sodium orthovanadate, a protease inhibitor cocktail (Sigma), 0.1% β -mercaptoethanol (vol/vol), and 1 mmol/l microcystin. The tissue homogenates were centrifuged at 14,000 rpm for 10 min at 4°C, and supernatants (75 μ g protein) were subjected to dual immunoprecipitation by incubation overnight at 4°C with 2 μ g each of anti-IRS-1 and anti-IRS-2 (Santa Cruz Biotechnology), which were pre-conjugated (2 h at 4°C) to Gammabind G Sepharose (Amersham). The immune complexes were washed once with buffer A containing 0.5 mol/l NaCl, once with buffer B (50 mmol/l Tris-HCl, pH 7.5, 0.03% Brij-35 [vol/vol], 0.1 mmol/l EGTA, and 0.1% β -mercaptoethanol [vol/vol]), and once with TNE buffer consisting of 20 mmol/l Tris-HCl, pH 7.5, 100 mmol/l NaCl, 0.5 mmol/l EGTA, and 0.1 mmol/l sodium orthovanadate. The immune complexes were then incubated at 35°C for 10 min in 50 μ l TNE buffer, pH 7.4, in the presence of γ -³²P-ATP (10 μ Ci/assay) and the substrate phosphatidylinositol (20 μ g/assay). The reaction was stopped by adding 20 μ l of 6 N HCl and 160 μ l of CHCl₃/CH₃OH (1:1). The organic phase was then spotted on a thin-layer chromatography plate and subjected to ascending chromatography using the solvent CHCl₃/CH₃OH/H₂O/NH₄OH (60:47:11.3:2). Phosphatidylinositol 3-phosphate, thus resolved, was quantified by phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA).

p44/42 Kinase activity was assayed by immunoprecipitating the protein, incubating the immunoprecipitated protein with γ -³²P ATP and Elk-1 fusion protein substrate, and immunoblotting with anti-phosphor-Elk-1 (S383) antibody.

Immunohistochemistry and insulin radioimmunoassay. Immunohistochemistry was performed as described previously on frozen sections of rat retina (22). The primary antibodies against p70S6K were from Santa Cruz. Plasma insulin was quantified with a sensitive LINCO (St. Charles, MO) rat radioimmunoassay per manufacturer's instructions.

Statistics. For all experiments, the data were normalized to the controls before analysis. ANOVA models with heterogeneous variances, adjusted for the replication of the experiment, were fit to the data to assess differences between STZ-induced diabetic rats and control rats. The means \pm SEM, *P* values, and statistical tests are reported. Analyses were performed using InStat 2.0 (San Diego, CA) and the SAS statistical software package (SAS Institute, Cary, NC).

RESULTS

Rat characteristics. The induction of STZ-induced diabetes and insulin treatment of all rats was consistent with our previous reports (3,22). Control rats gained 113.7, 250.7, and 227.6% of their original body weights and were normoglycemic at means of 4.2, 4.5, and 4.4 mmol/l glucose over the 4-, 8-, and 12-week time courses, respectively. Diabetic rats gained less weight (65.7, 118.6, and 112.9% increase after 4, 8, and 12 weeks, *P* < 0.0005, *P* < 0.001, and *P* < 0.0005, respectively) and were hyperglycemic (means 20.6, 16.1, and 19.8 mmol/l after 4, 8, and 12 weeks, respectively, all *P* < 0.0005) compared with age-matched control rats. Diabetic rats that were treated with insulin pellets did not have statistically different weight or blood glucose levels compared with their age-matched controls. Diabetic rats that were treated acutely with

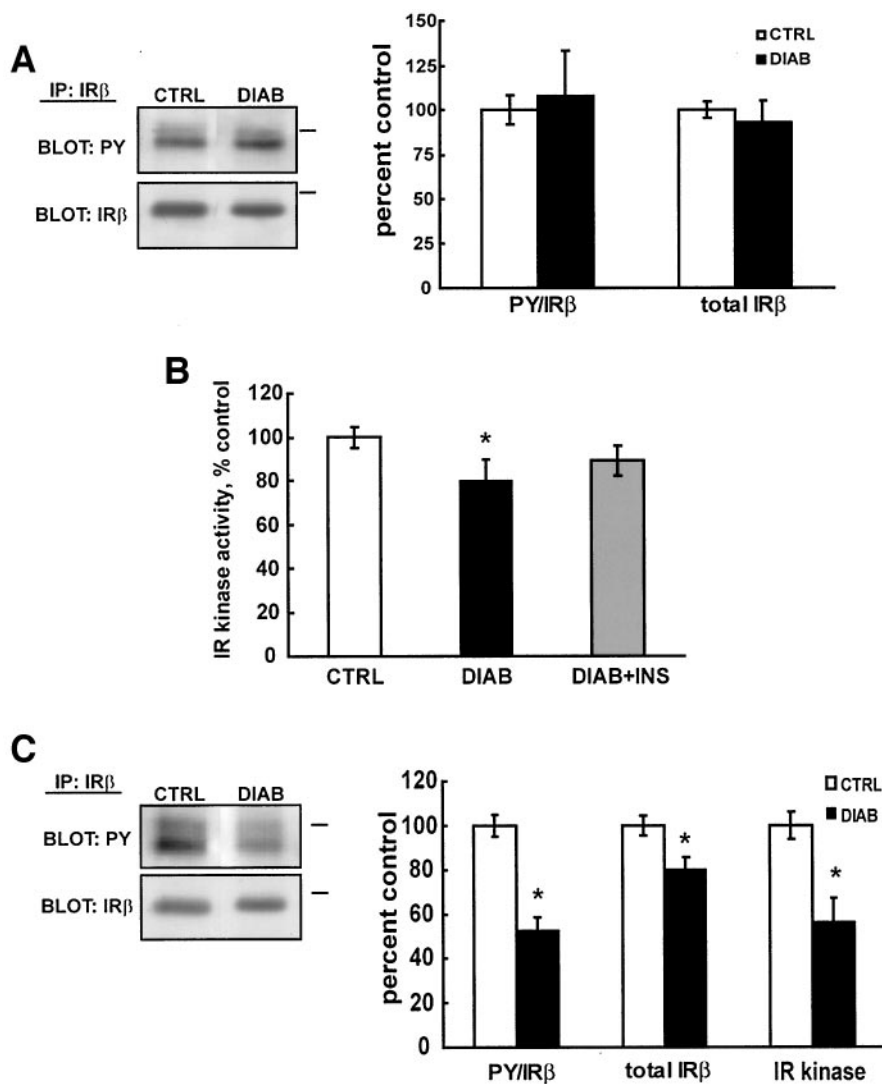


FIG. 1. Reduced retinal insulin receptor phosphorylation and kinase activity in diabetes. The insulin receptor (IR) from retina was immunoprecipitated (IP) and analyzed for tyrosine phosphorylation (PY) or kinase activity. **A:** Analysis of immunoblots for tyrosine phosphorylation of the insulin receptor- β (IR β) and total insulin receptor- β reveal equivalent phosphorylation and expression in retina tissue of control (CTRL) and 4-week-diabetic (DIAB) rats (representative immunoblots shown, $n = 8$ control and 5 diabetic rats). **B:** Four weeks of diabetes ($n = 26$) reduces insulin receptor kinase activity compared with controls ($n = 27$) ($P < 0.001$). Retinal insulin receptor kinase activity was normal in diabetic rats treated with insulin pellets (INS) ($n = 14$) when compared with controls ($P = 0.12$). Retinal insulin receptor kinase activity was not significantly reduced in 4-week-diabetic rats when compared with diabetic rats treated with insulin pellets ($P = 0.23$); however, treatment with insulin pellets trended toward improved insulin receptor kinase activity. **C:** Twelve weeks of diabetes reduces retinal insulin receptor- β autophosphorylation and expression and further reduces insulin receptor kinase activity (* $P < 0.01$, ** $P < 0.0001$; $n = 11$ for control and 12 for diabetes for phosphotyrosine/insulin receptor- β and total insulin receptor- β ; $n = 4$ per group for insulin receptor kinase). The dashes (-) in (A) and (C) indicate mobility of the band for IGF-IR β .

insulin for 3 days before death (see RESEARCH DESIGN AND METHODS) did not gain weight (64.0% increase; $P < 0.005$) and had highly variable blood glucose levels that were still elevated compared with control rats (mean 13.3 mmol/l; $P < 0.05$) at the time of death.

Basal retinal insulin receptor kinase activity and phosphorylation are progressively reduced by diabetes. The effect of STZ-induced diabetes on retinal insulin receptor autophosphorylation and kinase activity toward an exogenous substrate was examined in rats after 4, 8, and 12 weeks of diabetes. Autophosphorylation was analyzed by insulin receptor immunoprecipitation, protein separation on SDS-PAGE gels, and phosphotyrosine immunoblotting. Insulin receptor kinase activity was measured by the addition of poly Glu-Tyr to insulin receptor immunoprecipitates in the presence of 32 P-ATP. After 4 weeks of diabetes, retinal insulin receptor autophosphorylation remained unchanged (Fig. 1A). However, retinal insulin receptor kinase activity was reduced 26% ($P < 0.05$), and diabetic rats treated with subcutaneous insulin pellets had normal retinal insulin receptor kinase activity (Fig. 1B). Plasma insulin levels were 4.0 ± 0.9 , 0.3 ± 0.05 , and 3.9 ± 0.5 ng/ml for control, untreated diabetic, and insulin-treated animals, respectively. A similar 25% reduction of insulin receptor kinase activity ($P < 0.05$) without an observable alteration in insulin receptor phosphoty-

rosine content was observed after 8 weeks of diabetes. However, after 12 weeks of diabetes, we observed significant reductions of both the basal retinal insulin receptor autophosphorylation (47%, $P < 0.0001$) and kinase activity (44%, $P < 0.05$; Fig. 1C). Insulin receptor- β subunit content in retina, as determined by pull-down assays at this time point, was also decreased 20% ($P < 0.01$; Fig. 1C), and immunoblotting of retinal lysates showed no change in insulin receptor- β content at 4 weeks and a 62% reduction after 12 weeks (Fig. 1C). By contrast, after 4 weeks of diabetes, there was no change in retinal IGF-1R kinase activity or insulin receptor/IGF-1R hybrid expression as determined by immunoblotting (not shown). Therefore, insulin-deficient diabetes incites a specific, progressive loss of the basal retinal insulin receptor kinase activity and expression.

Insulin-stimulated insulin receptor autophosphorylation is retained in retinas of diabetic rats. A second approach to evaluate retinal insulin receptor signaling utilized ex vivo retina cultures to examine the specific effects of hormone stimulation on the retina. In agreement with our previous report (6), 10 nmol/l insulin significantly increased retinal insulin receptor- β autophosphorylation. In retinas of diabetic rats, however, 10 nmol/l insulin increased insulin receptor- β autophosphorylation 40% more ($P < 0.05$) than in retinas of control rats (Fig. 2). This

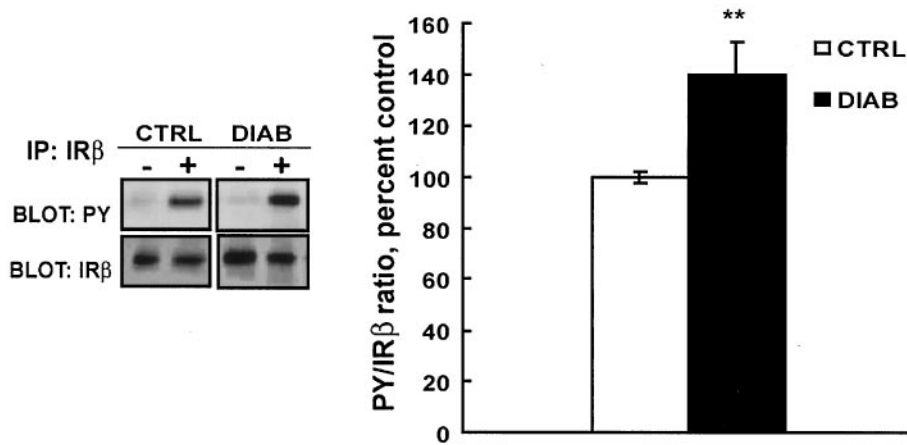


FIG. 2. Increased insulin receptor (IR) auto-phosphorylation of insulin-stimulated ex vivo retina. Retinas of control ($n = 4$) (CTRL) and 4-week-diabetic ($n = 5$) (DIAB) rats were incubated ex vivo with either vehicle (-) or 10 nmol/l insulin (+) for 5 min. Insulin-stimulated insulin receptor- β auto-phosphorylation was increased 40% ($*P < 0.01$ by t test) in retinas of diabetic rats compared with controls. Results are representative of two separate experiments. IP, immunoprecipitation; PY, phosphotyrosine.

result demonstrates that the retinal insulin receptor retains its ability to respond to insulin in the diabetic state and suggests that loss of ligand contributes to the reduced basal insulin receptor activity in diabetes. A similar response to insulin was observed in the retinal vasculature of diabetic mice (23), and in the ex vivo model, retinal neural cells also respond (6).

IRS-2 but not IRS-1 content in retina is progressively reduced by diabetes. We (6) and Rajala et al. (24) have observed that insulin stimulates retinal IRS-2 but not IRS-1 tyrosine phosphorylation. IRS-2 knockout mice exhibit increased apoptosis of retinal neurons, including those in the inner retina (25). IRS-2 content was determined by immunoblotting. As shown in Fig. 3A and C, IRS-2 content was unchanged after 4 weeks of diabetes ($n = 8$ control and 7 diabetic rats) but was reduced 60% ($n = 4$ control and 4 diabetic rats; $P < 0.05$) after 12 weeks of diabetes

(Fig. 3B and C). Straight immunoblotting of proteins from retinal lysates from rats with 12 weeks of diabetes also revealed a 55% reduction in IRS-2 with no change in IRS-1 content (not shown). These data suggest that diabetes causes progressive loss of the insulin receptor signal transduction machinery at a relatively early time in the course of diabetes.

IRS-1/2-associated PI3-K activity is reduced in retina of diabetic rats. Insulin-stimulated retinal neuronal survival is mediated via PI3-K (14), therefore retinal IRS-1/2-associated PI3-K activity was analyzed by co-immunoprecipitation. This approach was utilized to assess PI3-K activity that associates with known IRSs in vivo (6,24) and in retinal cell culture (16). After 4 weeks of diabetes, IRS-1/2-associated PI3-K activity was reduced 48% by diabetes in retina ($P < 0.0005$; Fig. 4A). However, we did not observe any alteration in p85

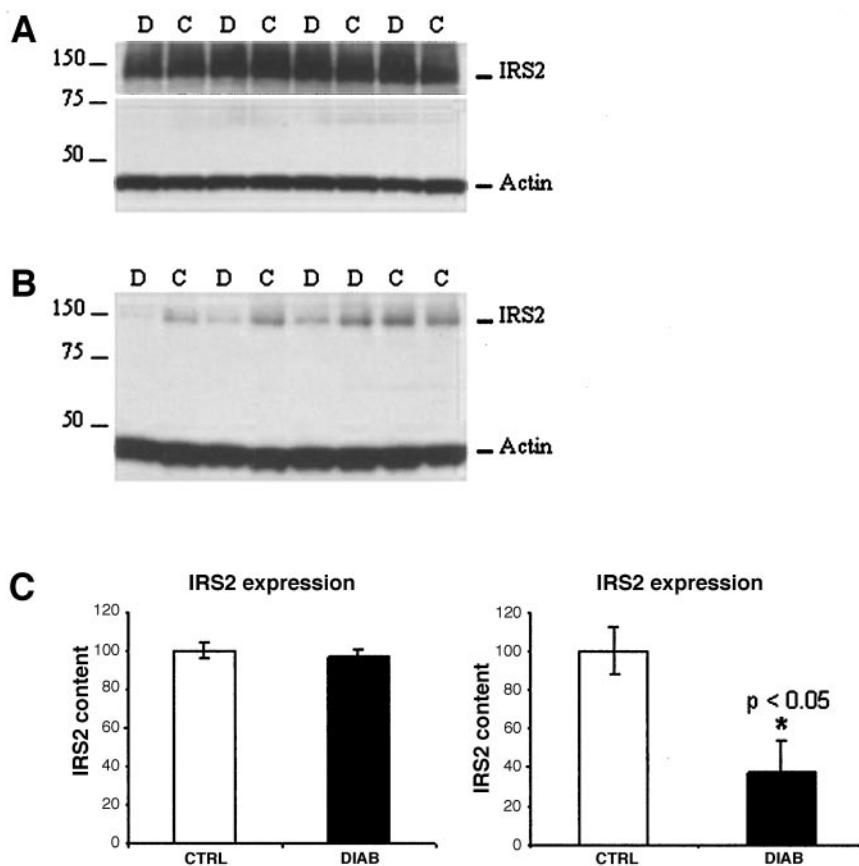


FIG. 3. Decreased retinal IRS-2 expression in diabetes. IRS-2 was immunoprecipitated from retinal lysates and the membranes immunoblotted for phosphotyrosine and total content. Four weeks of diabetes had no effect on total IRS-2 content or tyrosine phosphorylation ($n = 8$ control and 7 diabetic rats; not shown), but 12 weeks of diabetes reduced IRS-2 content by 60% ($n = 4$ /group; $P < 0.05$). Straight immunoblots of retinal lysates revealed no change in IRS-2 content at 4 weeks ($n = 4$ /group), but after 12 weeks, IRS-2 was reduced by 55% ($n = 4$ control and 5 diabetic rats; $P < 0.05$).

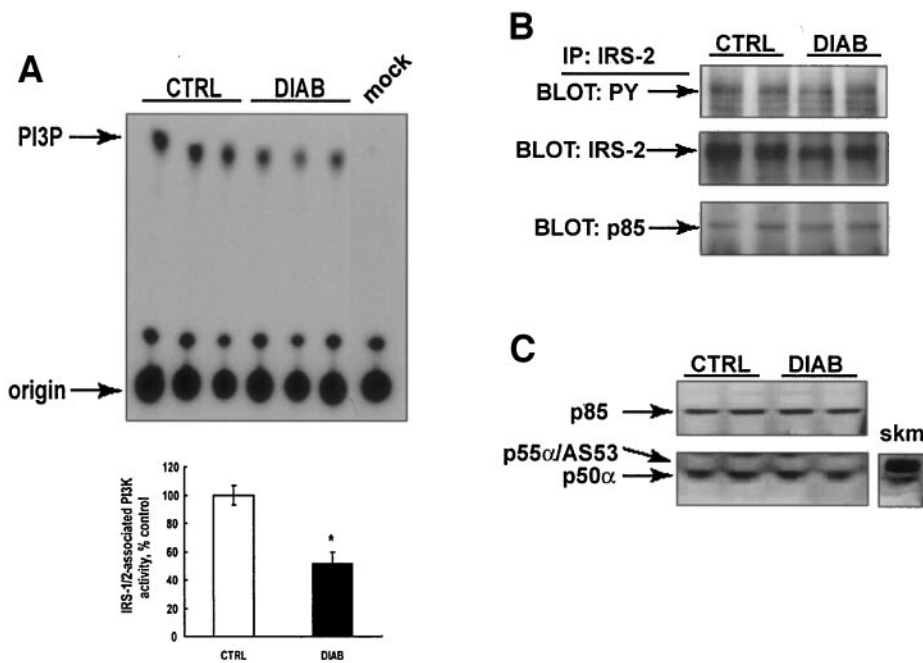


FIG. 4. Reduced IRS-1/2-PI3-K activity in retina of diabetic rats. **A:** Retinal lysates were dual-immunoprecipitated for IRS-1/2, and PI3-K activity was measured. A representative TLC plate image is shown demonstrating reduced phosphatidylinositol 3-phosphate (PI3P) formation in lysates of 4-week-diabetic rats ($n = 14$ control [CTRL] and 15 diabetic [DIAB] rats), with corresponding graphic representation. **B:** P85 association with IRS-2 and total phosphotyrosine (PY)-containing proteins were not changed between control and diabetic rats ($n = 8$ control and 7 diabetic rats). **C:** The splice-variant of p85, termed AS53 or p55 α , was not detected in retinal lysates by immunoblotting. The expression of p85 and p50 α was unaltered between control and diabetic rats ($n = 4$ per group). Lysates of skeletal muscle from normal rats (skm) served as a positive control for AS53 expression by immunoblot analysis.

association with either IRS-2 or all phosphotyrosine-containing proteins at this time point (Fig. 4B). The alternative splice form of p85 (AS53 or p55 α) was undetectable in retina of control and diabetic rats (Fig. 4C). Moreover, IRS-1, IRS-2, and Gab-1 contents remained stable in retina after 4 weeks of diabetes (not shown). Thus, IRS-1/2-associated PI3-K activity was reduced in retina, similar to the reduction in retinal insulin receptor, without a detectable change in the amount of p85 association, expression, or tyrosine phosphorylation of IRS-2 at this time point. These data suggest that diabetes impairs the enzymatic activity of pro-survival enzymes shortly after the onset of pancreatic β -cell destruction.

Akt-1 and -3 kinase activities are reduced by diabetes in retina tissue. We previously observed that retina expresses mRNAs for all three Akt isoforms, and Akt-1 and -3 are the predominant isoforms. Basal Akt-1 activity is several-fold higher in retina than in muscle or liver of normal animals, and insulin stimulates only Akt-1 activity (6). Therefore, the activities of Akt-1 and -3 were measured in control and diabetic retina tissue. First, in agreement with previously published data (26), we observed no change in Akt content or phosphorylation of the consensus serine 308 or threonine 473 sites in retinal lysates (Fig. 5A). However, when kinase activities of these proteins were measured in lysates after 4 weeks, diabetes reduced Akt-1 activity by 54% (Fig. 5B); after 12 weeks, Akt-1 activity was reduced $39.6 \pm 6.9\%$ ($P = 0.048$) in eight diabetic rats compared with six controls. The amount of Akt-1 pulled down in the immunoprecipitation was equal for control and diabetic retinas (Fig. 5A, bottom panel). Akt-3 kinase activity was decreased by 24% after 4 weeks and by 79% after 8 weeks of diabetes (Fig. 5C). There was no detectable change in the amount of Akt-3 immunoprecipitated; however, with the polyclonal Akt-3 precipitating antibody, it was difficult to resolve Akt-3 from IgG (not shown). Insulin treatment partially restored Akt-1 and -3 kinase activity (Fig. 5C). Akt-2 activity did not change at 4 or 8 weeks (not shown). Moreover, p44/42 MAPK activity did not show consistent changes after 4 or 8 weeks of

diabetes. Therefore, diabetes induces isoform-specific changes in Akt activity in retina without changing Akt content or the phosphorylation status of two well-described residues associated with insulin-stimulated activity.

GSK-3 β phosphorylation is reduced in retina of diabetic rats. Serine 9 phosphorylation on GSK-3 β is a substrate for Akt that leads to inhibition of GSK-3 activity. Increased GSK-3 β activity associated with decreased serine 9 phosphorylation is associated with neurodegeneration (27). Using phospho-specific antibodies of retinal lysates, a 25% ($P < 0.001$) decrease in GSK-3 β phosphorylation in diabetic rat retina was detected, and this effect was only partially ameliorated with insulin therapy (Fig. 6). These data strengthen the observation that Akt kinase activity is reduced in diabetic rat retinas and provide a likely link to retinal neurodegeneration in diabetes.

p70S6 Kinase signaling is reduced in diabetic rat retinas. Previously, it was demonstrated that p70S6K, a regulator of translational control of protein synthesis, mediates insulin-stimulated retinal cell survival in serum-deprived retinal neurons (15). Therefore, p70S6K activity was assayed in control and diabetic retina. Four weeks of diabetes significantly decreased p70S6K activity to 62% of control, and short-term insulin administration partially restored the activity (Fig. 7A) in diabetic rat retina. Of particular note, retina had the highest basal p70S6K activity among retina, liver, and muscle based on activity normalized to milligrams of total protein. Immunohistochemistry and immunoblotting for total p70S6K content indicate no change between control and diabetic retinas (not shown). Immunohistochemistry for p70S6K showed specific immunoreactivity in the inner and outer plexiform layers and photoreceptor outer segments (Fig. 7B). This high basal p70S6K activity is consistent with the high basal insulin receptor kinase and Akt kinase activity reported previously (6). Thus, impairment of p70S6K signaling by diabetes likely occurs in the neural cells of the retina, including the synaptic layers.

Intravitreal insulin restores retinal insulin receptor kinase activity. Systemic insulin initiated shortly after the onset of diabetes prevented the loss of retinal insulin

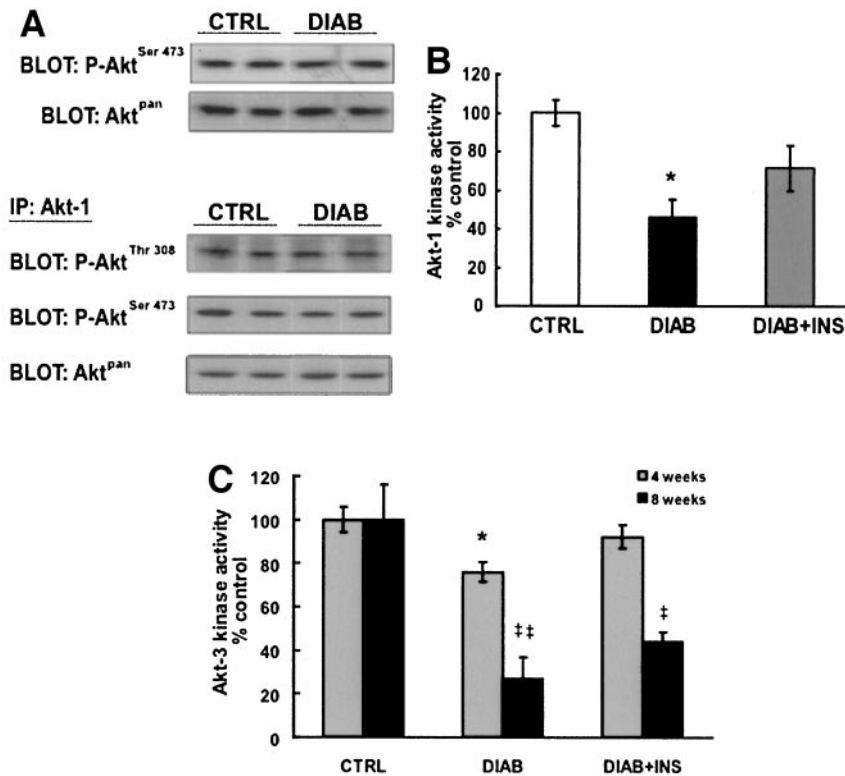


FIG. 5. Reduced Akt kinase activity by diabetes in rat retina. **A:** Retinal lysates from control (CTRL) and diabetic (DIAB) rats were analyzed by immunoblot analysis. In retina, Akt phosphorylation of threonine (Thr) 308 and serine (Ser) 473 and total expression were unaltered in the diabetic state. **B:** After 4 weeks of diabetes, Akt-1 kinase activity in retina was reduced by 54% (* $P < 0.01$ by ANOVA and Tukey-Kramer multiple comparisons post hoc test, $n = 7$ /group). **C:** Akt-3 kinase activity is reduced by 24% at 4 weeks (* $P < 0.05$ vs. control) and normalized with insulin therapy ($n = 17$ control, 19 diabetic, and 20 insulin-treated [INS] diabetic mice). Akt-3 kinase activity is reduced by 76% ($\ddagger P < 0.01$) after 8 weeks of diabetes and 57% ($\dagger P < 0.05$) in 8-week insulin-treated diabetic rats ($n = 3$ control, 3 diabetic, and 4 insulin-treated diabetic mice).

receptor kinase activity (Fig. 1), and data in Fig. 2 show that the responsiveness of the insulin receptor in diabetic rat retinas remains intact. Therefore, a direct role for insulin in restoring the basal insulin receptor kinase activity lost during diabetes was tested by intravitreal injection. Insulin (~ 10 nmol/l final concentration) was injected into one eye and vehicle (PBS with 0.1% BSA) into the other eye of anesthetized control and diabetic rats. After 30 min retinas were removed, insulin receptor- β was immunoprecipitated, and kinase activity to poly Glu:Tyr was analyzed. Data shown in Fig. 8 demonstrate that intravitreal insulin did not augment the basal retinal insulin receptor kinase activity in control animals but restored the depressed insulin receptor activity in diabetic animals. These data demonstrate that lost insulin receptor kinase activity can be restored by direct insulin delivery.

DISCUSSION

In this study, we examined the effects of insulin-deficient diabetes on the basal insulin receptor/Akt signaling pathway in retina. We showed previously that the retina is an insulin-sensitive tissue, with higher basally active insulin receptor and Akt signaling activity than in liver and muscle (6). We now report that in retinal tissue from insulin-deficient diabetic rats, the elevated basal kinetic activity of the insulin receptor, PI3-K, Akt-1 and -3, and p70S6K was progressively downregulated and IRS-2 content was reduced, concomitant with neuronal cell death and increased vascular permeability (3,22). GSK-3 β phosphorylation was also decreased, consistent with increased activity and neuronal degeneration. Systemic insulin therapy from the onset of diabetes prevented loss of retinal insulin receptor kinase activity, and importantly for therapeutic implications, intraocular insulin restored lost insulin receptor kinase activity. Moreover, these changes were specific for the insulin receptor, since there was no change in retinal

IGF-1R kinase or p44/42 MAPK activity. To the best of our knowledge, this is the first study to demonstrate specific serial defects in insulin receptor signaling kinase activities in the retina and restoration with exogenous hormone.

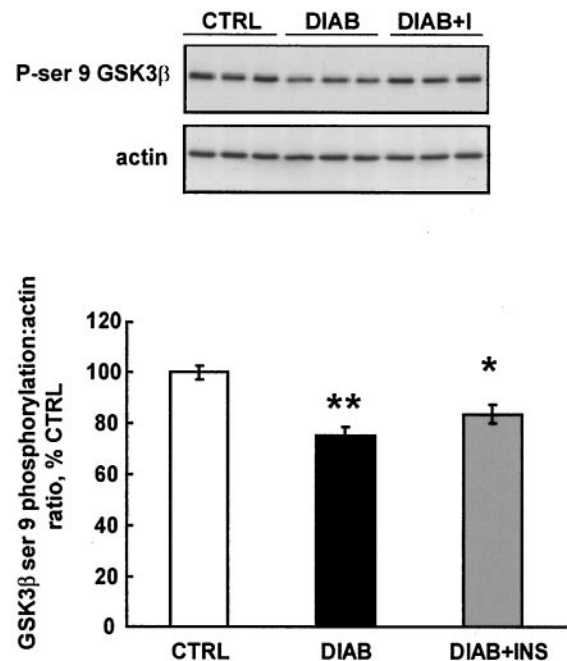


FIG. 6. GSK3 β serine (Ser) 9 phosphorylation is reduced in diabetic rat retina. Retinal lysates of control (CTRL) and 4-week-diabetic (DIAB) rats were analyzed for GSK-3 β phosphorylation and normalized to actin. Diabetes reduced GSK-3 β serine 9 phosphorylation (** $P < 0.001$ by ANOVA and Tukey-Kramer multiple comparisons post hoc test), and insulin pellet-treated (INS) diabetic rats also had reduced GSK3 β phosphorylation (* $P < 0.05$ by ANOVA, $n = 16$ control, 13 diabetic, and 17 insulin-treated diabetic rats).

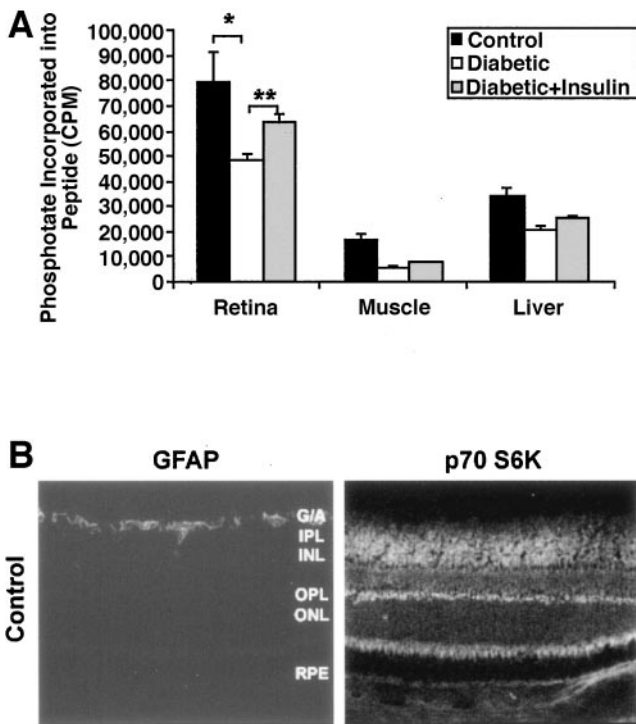


FIG. 7. Diabetes reduces retinal p70S6k activity. Four weeks after STZ injection, 13 diabetic rats were injected subcutaneously with human insulin (5 units Humulin R and 5 units Humulin U) twice daily; another 11 rats were injected with vehicle, and 10 were normal controls. **A:** Retina, liver, and muscle were removed and p70S6K kinase activity analyzed. * $P < 0.05$; ** $P < 0.01$. **B:** Normal rat eyes were enucleated, frozen in liquid N_2 with optimal cutting temperature, cryosectioned, fixed with 2% paraformaldehyde, and immunostained with p70S6K antibody. *Left:* No primary antibody negative control. *Right:* With primary antibody demonstrating specific p70S6K immunoreactivity primarily in the ganglion cell, inner and outer plexiform, and photoreceptor outer segments. Original image was taken with a $\times 20$ objective.

These observations lead to the novel concept that insulin receptor/Akt signaling in retina, like that in muscle and liver, is susceptible to the deleterious effects of β -cell destruction and insulin deficiency. The insulin receptor, IRS proteins, PI3-K, Akt, GSK-3 β , and p70S6K, which mediate intermediary metabolism in peripheral tissues, also appear to mediate cell survival in the central nervous system, and defects in these signaling components are associated with neurodegeneration. Thus, it is reasonable to

postulate that impaired insulin receptor signaling in diabetes could also contribute to retinal neurodegeneration.

A key finding in this report is that expression and phosphorylation of signaling proteins were unchanged after 4 weeks of diabetes, while kinase activity was reduced. A reduction in insulin receptor autophosphorylation and IRS-2 expression only existed after prolonged (12 weeks) of untreated diabetes. Some studies have demonstrated that insulin receptor- β phosphotyrosine content and autophosphorylation remain stable in skeletal muscle (28,29), while others have shown reduced autophosphorylation and kinase activity of the insulin receptor (30). Kinase assays arguably provide a more sensitive measure of insulin receptor function, and modifications to the insulin receptor, such as serine/threonine phosphorylation, may reduce insulin receptor activity without changing tyrosine phosphorylation (31). These differences may also account for the elevated basal activity and loss of insulin receptor activity without observable changes in phosphotyrosine content.

In this study, we also observed that Akt activity decreased in the diabetic retina without a change in serine 473 or threonine 308 phosphorylation. In agreement with Gerhardinger et al. (26), we found no difference in total retinal Akt expression and Akt serine 473 phosphorylation between control and diabetic rats but observed a significant decrease in Akt-1 and -3 activity. This incongruity in enzyme phosphorylation and kinetic activity has been reported for Akt-1 in muscle biopsies of obese individuals (32) and for total Akt expression and Akt serine 473 phosphorylation in vagus nerve of STZ-induced diabetic rats (33). Indeed, Akt kinase activity can be independent of serine 473 phosphorylation (34). Likewise, Cai and Helke (33) found reduced PI3-K activity in vagus nerve with no change in p85 expression, and we did not observe a change in retinal p85 expression or alternative splicing. The mechanisms that inhibit Akt activity in retina are incompletely understood, but possibilities include phosphorylation of other sites on Akt (such as tyrosine 474 for activation [35] or threonine 34 for inactivation [36]), and inhibitory interactions with protein kinase C ζ (37). Thus, the posttranslational mechanisms by which diabetes inhibits signal transduction are not simply the converse of stimulatory mechanisms. Since many peptide hormones signal through the PI3-K/Akt pathway, diminished insulin receptor signaling may not be the only route leading to reduced Akt activation in retina.

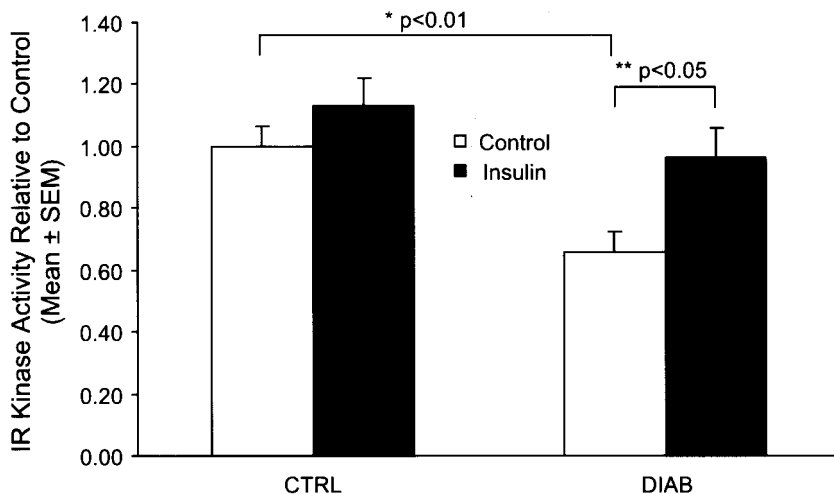


FIG. 8. Intraocular insulin restores retinal insulin receptor (IR) kinase activity. Control ($n = 7$) (CTRL) and 4-week diabetic ($n = 6$) (DIAB) rats were microinjected with either vehicle (PBS) or bovine insulin (final concentration ~ 10 nmol/l), and retinas were harvested 30 min later. PBS microinjection and no-injection eyes served as controls. Insulin receptor- β was immunoprecipitated from retinal lysates, and in vitro kinase assays were performed in the presence of ^{32}P -ATP and exogenous poly Glu:Tyr (4:1) substrate. Local insulin administration restored the diabetes-induced decrease in insulin receptor kinase activity.

This report focused on the loss of basal insulin receptor signaling in retina in diabetes, whereas most previous reports evaluated insulin signaling in response to superphysiological insulin injections (23,38,39). We found that even with very little circulating insulin, the basal retinal insulin receptor kinase activity in vivo is reduced only ~26% after 4 and 8 weeks of diabetes, suggesting that the receptor may still receive stimulation by hybridization with the IGF-IR, other ligands such as IGF-1 and -2, or light (40). This loss of insulin receptor kinase activity is equivalent to that found in the spontaneously insulin-deficient *Ins2^{Akita}* mouse (4); therefore, these changes clearly reflect the diabetic state and not STZ toxicity. Moreover, the degree of reduction of kinase activity in retina is equivalent to that found in skeletal muscle in STZ-induced diabetes (41). However, the retina possesses a high basal activity that is reduced in diabetes and is distinct from the muscle tissue response that depends on the feeding cycle and β -cell production of insulin.

The current study does not distinguish which cell type(s) in retina have disrupted insulin receptor signaling. All types of retinal cells express insulin receptors, with particularly high expression on Müller cell end-feet and neuronal dendrites (42). The net reduction of insulin receptor signaling kinase activities found in this study may reflect changes in all cell types. Thus, vascular and neuronal survival could be compromised by reducing insulin receptor activity directly on those cells and/or indirectly by altering glial or microglial cell function, on which they depend. Alternatively, specific cell subtypes may be particularly susceptible to diabetes. Studies to investigate these relative contributions are in progress but beyond the scope of this report.

The signaling defects we report in retina involving the insulin receptor/PI3-K/Akt/p70S6K pathway suggest that retinal metabolism and cellular survival are highly conserved across evolution. Indeed, disruption of insulin receptor signaling has a profound impact on retinal cell growth and development in *Drosophila*, chickens, and rodents (13,43,44). Brain insulin receptor knockout mice have increased elevated GSK-3 β activity associated with neurodegenerative disorders (45), similar to the findings in this report. Retinal neurons (14) and vascular endothelial cells (46) depend on insulin-mediated PI3-K activity for survival, and both types of cells are known to die by apoptosis in both human and rat models of diabetes (3,47). Therefore, it is reasonable to predict that long-term disturbances in retinal insulin receptor signaling may accelerate cell death and impair insulin-dependent anabolic activities, such as protein synthesis (48).

In these studies, both systemic and intravitreal insulin administration restored deficient insulin receptor signaling. This finding is similar to that of Brusee et al. (49), who found that direct insulin application reduces diabetic peripheral neuropathy. This result could indicate that exogenous intraocular insulin compensates for deficiency in these ligands or overcomes the metabolic stress the retina experiences in the diabetic state. In any case, the findings strongly implicate an important functional role for retinal insulin receptor signaling in preventing the neurodegenerative component of diabetic retinopathy. In light of insulin's stimulation of retinal IRS-2 but not IRS-1 tyrosine phosphorylation (6,24) and loss of IRS-2 but not IRS-1 in diabetic rat retinas, it is noteworthy that IRS-2 knockout mice exhibit reduced retinal Akt phosphorylation, increased active caspase-3 and transferase-mediated

dUTP nick-end labeling immunoreactivity, and compromised postnatal inner and outer retinal neuronal cell survival, independent of diabetes (25). Together with this work, the accumulated evidence strongly suggests that diminished growth factor signaling contributes directly to retinal degeneration. These data do not exclude a potential contribution of excess glucose or lipids to local impairment of retinal insulin receptor signaling. In fact, we have shown previously that excess glucose impairs insulin-stimulated Akt phosphorylation and neuronal survival (16). Thus, it appears that the retinal insulin receptor signaling pathway provides "neurotrophic" support and that diabetic retinopathy may be considered as a neurotrophin-deficient and/or -resistant state. Further studies are underway to characterize the specific means by which diabetes disrupts retinal insulin receptor signaling and the specific cells that are preferentially affected. These results also have potential importance for understanding the effects of intensive insulin therapy in humans.

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