Characterization of insulin signaling in rat retina in vivo and ex vivo

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Departments of 1Cellular and Molecular Physiology, 2Pharmacology, 3Neuroscience and Anatomy, and 4The Penn State Retina Research Group, Ulerich Ophthalmology Research Center and Juvenile Diabetes Research Foundation Diabetic Retinopathy Center at Pennsylvania State University, The Pennsylvania State University College of Medicine, Milton S. Hershey Medical Center, Hershey, Pennsylvania 17033

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Reiter, Chad E. N., Lakshman Sandirasegarane, Ellen B. Wolpert, Marianne Klinger, Ian A. Simpson, Alistair J. Barber, David A. Antonetti, Mark Kester, and Thomas W. Gardner. Characterization of insulin signaling in rat retina in vivo and ex vivo. Am J Physiol Endocrinol Metab 285: E763–E774, 2003. First published June 10, 2003; 10.1152/ajpendo.00507.2002.—Insulin receptor (IR) signaling cascades have been studied in many tissues, but retinal insulin action 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 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 it remained constant between freely fed and fasted rats. Furthermore, IR signaling increased in the retina after portal vein administration of supraphysiological doses of insulin. Ex vivo retinas responded to 10 nM insulin with IR β-subunit (IRβ) and IR substrate-2 (IRS-2) tyrosine phosphorylation and AktSer473 phosphorylation. The retina expresses mRNA for all 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 prosurvival pathway. These findings may have implications in understanding normal and dysfunctions of insulin receptor; Akt; diabetic retinopathy

THE INSULIN RECEPTOR (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 (4, 44, 59). 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 (23, 52, 65, 66). It is expressed in all retinal layers and is homologous to neuronal IRs in brain that feature less β-subunit glycosylation and increased mobility on SDS-PAGE compared with 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 (58). Binding studies have also demonstrated the presence of the IR in the retinal microvasculature (27).

In brain, IR, IR substrate-1 (IRS-1), and phosphotyrosine (PY) immunoreactivities colocalize, suggesting a role for insulin action in neural tissue metabolism, growth, and/or survival (8, 41, 62). Other studies have revealed that the retina expresses a number of proteins associated with insulin signaling. Folli et al. (18) reported IRS-1 and phosphoinositide 3-kinase (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. (21) extended those observations in rat retina by demonstrating IRS-1 expression in the rod outer segments, and Diaz et al. (16) demonstrated that Akt and, to a lesser extent, extracellular regulated kinase (ERK1/2) phosphorylation increases after supraphysiological insulin treatment for 24 h in whole embryonic chick retina. The Ras and ERK1/2 pathway may play an important role in neuroretal development, as its activity diminishes in the fasted state (58).

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ishes upon differentiation. Oncogenic Ras transformation of chick neuroretinal cells induces proliferation via the Raf/MEK/ERK pathway (47). Collectively, these reports suggest that the insulin-signaling pathway is important in retinal physiology.

Activation of the Ser/Thr kinase Akt by PI3K is necessary for the prosurvival mechanism of insulin action (37). Akt inhibits apoptosis by phosphorylating a number of apoptosis regulators, including caspase-9 (13), Bad (15), glycogen synthase kinase (GSK)-3β (25), IκB kinase (IKK) (53), and apoptosis signal-regulating kinase-1 (30) in the cytosol. Akt translocates to the nucleus to phosphorylate Forkhead family transcription factors (9, 46). 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 (17), and activated Akt inhibits apoptosis by preventing p53 transcriptional activity in hippocampal neurons (71). Barber et al. (7) demonstrated Akt-dependent cell survival in cultured retinal neurons in response to insulin and IGF-I. Treatment of the retinal neurons with insulin and IGF-I 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 we sought to examine retinal insulin-signaling characteristics. We document retinal insulin responsiveness leading 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 a low-dose (50 μg) insulin injection. 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<sup>Ser473</sup> phosphorylation in the retinal nuclear layers in ex vivo cultures. Akt-1, Akt-2, and Akt-3 mRNAs are expressed in retina, and only Akt-1 kinase activity increased with insulin. These results suggest that insulin signaling plays a significant role in retinal physiology and is mediated through specific Akt isoforms.

**MATERIALS AND METHODS**

**Animals.** Male Sprague-Dawley rats (Charles River, MA), 200–350 g, were used and age-matched in all studies. They were housed at the Penn State University College of Medicine animal care facility, where they had free access to a standard rat chow and water and were maintained on a 12:12-h light-dark cycle in wire-bottom cages. Where noted, rats were fasted 18 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. Experiments were conducted under standard laboratory illumination to control for light-induced IR activation (51).

**Intraportal insulin injections and retinal explant experiments.** For in vivo insulin injection experiments, rats were fasted 18 h before being anesthetized with a 1:10 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) were injected via the portal vein; the abdominal cavity was opened, and nothing was injected into sham control rats. At the indicated times, gastrocnemius skeletal muscle and retina were removed and snap-frozen under liquid nitrogen and stored at −80°C until analysis. For ex vivo experiments, rats were anesthetized with pentobarbital sodium at 100 mg/kg and decapitated upon loss of motor reflexes; 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 min at 37°C, 5% CO<sub>2</sub>, and gentle shaking, essentially as described by us and others for the study of retinal metabolism and effects of growth factors (14, 33, 34). Insulin (10 nM), IGF-I, IGF-II (IGF-binding protein 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 min at room temperature for immunohistochemical analysis (see Immunohistochemistry). Plasma insulin and blood glucose were measured using a rat insulin ELISA kit (Alpco, Windham, NH) and a Lifescan One-Touch blood glucose meter (Milpitas, CA), respectively.

**ATP assay.** Intact retinas were homogenized in 500 μl of ice-cold 6% perchloric acid either immediately after extraction or after 15 and 45 min of incubation in the explant culture media (just discussed). The assay was performed exactly as previously described (33, 34, 42, 68) and measured using FluorMeasure software (C&L Instruments, Hummelstown, PA). Amounts of ATP were normalized to total protein in the sample and expressed as nanomoles of ATP per milligram protein.

**Immunoprecipitation and immunoblotting.** Tissue lysates were immunoprecipitated and immunoblotted essentially as previously described (6). All antibodies were purchased and used according to the manufacturer's suggestions. Anti-IRβ and -IGF-IRβ were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-IGF-I, -IR-1, and -IRS-2 were from Upstate Biotechnology; anti-phospho-Ser<sup>473</sup> and anti-total Akt and -phospho and -total p44/p42 were from Cell Signaling Technology (Beverly, MA). The protein A Sepharose bead slurry was from Amersham Pharmacia Biotech (Piscataway, NJ). Secondary antibodies were anti-rabbit horseradish peroxidase and anti-mouse biotin (Amersham); secondary incubations were with streptavidin-alkaline phosphatase (GBCO Life Technologies, Rockville, MD) (7). Detection with enhanced chemiluminescence kits (Cell Signaling) and enhanced chemiluminescence kits (Amersham) was performed according to the manufacturer's protocols. Immunoblot quantification for the assessment of signaling changes was performed using ImageQuant (Molecular Dynamics, Sunnyvale, CA), NIH Image 1.61, or GeneTools SynGene (K&R Technology, Frederick, MD) software. Where noted, membranes were stripped at 50°C for 1 h in a buffer containing 63 mM Tris (pH 6.8), 2% SDS, and 0.035% 2-mercaptoethanol and were confirmed by

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reblotting with secondary antibodies and detection before immunoblotting procedures were resumed.

**IR kinase assays.** Tissue lysates (500 μg) were immunoprecipitated using anti-IRβ antibodies (overnight at 4°C), as described above. The immune complex was washed twice in kinase buffer (73), which did not diminish the amount of IR bound as assessed by immunoblot analysis (not shown). 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 GluTyr agarose gel (Sigma), and 25 μCi/ml [γ-32P]ATP (Amersham) for 45 min with constant mixing. The reaction was stopped by brief centrifugation and spotting 25 μl of supernatant on Whatman p81 phosphorylase paper. Filter papers were washed three times for 5 min in 0.75% phosphoric acid and once for 5 min in acetone before counting proceeded on a Beckman LS 6000SC Scintillation Counter (Beckman Instruments, Fullerton, CA).

**Akt isoform-specific kinase assays.** Akt isoform-specific kinase assays were performed as previously described (54). The immunoprecipitates (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), -3 (Upstate Biotechnology) primary antibody, which is initially preconjugated (1 h 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), crotside peptide substrate (GRPRTSS-PAEG), 30 μM, Upstate Biotechnology), and [γ-32P]ATP (10 μCi/assay). The amount of 32P incorporated into crotside was determined using p81 phosphorylase papers (as above). The observed dpm values were corrected for nonspecific binding by subtracting the background values (enzyme blank) obtained with mock immunoprecipitates.

**Akt-1, -2, and -3 in situ hybridization.** The synthesis of 35S-labeled riboprobes and RNA in situ hybridization was performed essentially as described (31, 63) on optimal cutting tissue (OCT)-embedded cryostat sections, 16 μm thick, of whole eyes from normal rats. Vectors for the sense and antisense riboprobes of Akt-1 (bp 1696–2229), and -3 (bp 1476–1724) were kindly provided by Dr. Morris Birnbaum, University of Pennsylvania. Hybridization stringency was optimized by applying probes (105 cpm/ml) in formamide hybridization buffer to sections, oversharpening, 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–HCl, and 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 days, dipped in Kodak NTB3 photographic emulsion, stored with desiccant at 4°C for 10–14 days, and developed and stained with hematoxylin for evaluation. All sections were processed together to facilitate signal comparisons among the groups.

**Immunohistochemistry.** Ex vivo retinas (see above) were embedded in OCT after fixation and snap-frozen in dry ice-cooled 2-methylbutane. Sections (10 μm) from vehicle and 10 nM insulin-treated retinas were mounted on the same slide and washed twice for 10 min at room temperature in PBS-0.1% Triton X-100 (PBST). The slides were blocked with 10% donkey serum diluted in PBST for 1 h at room temperature before primary antibody incubations at 4°C overnight. Antiphospho-AktSer473 (IHC-specific, Cell Signaling) was diluted 1:50, and the secondary antibody was diluted 1:1,500, conjugated to rhodamine red-X (Jackson Immunoresearch, West Grove, PA), and incubated for 1 h at room temperature. Confocal images were captured with a Nikon E800 microscope equipped with a differential interference contrast (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.

**Statistics.** Quantified results are reported as means ± SE. Data were analyzed by Student’s t-test or by one-way ANOVA followed by a multiple comparison posttest, as described in Figs. 1–8, using InStat 2.00 software. Statistical significance was considered for P < 0.05.

**RESULTS**

**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 with 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. 1), suggesting an active physiological role for the IR in retina. The IRβ in retina and brain migrates slightly faster on SDS-PAGE compared with liver IRβ, suggesting less extensive glycosylation of retinal IRβ than has been reported for IRα (7, 65).

**Intravenous insulin administration increases retinal IRβ basal tyrosine phosphorylation.** To determine whether acute elevation in circulating insulin activates retinal IR in vivo as in other tissues, IR phosphorylation was measured after an insulin injection. A bolus (500 or 50 μg) of insulin was administered intraperitoneally to normal fasted rats (weight ~250 g). This dose and route of administration have been used to stimulate peripheral IR autophosphorylation (19, 61). At 15, 30, 45, and 60 min postinjection, 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. 2 A) from the same rats at each time point demonstrated a robust response in muscle and relatively delayed retinal IRβ phosphorylation. 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-to-IRβ ratios demonstrated an ~30-fold increase in

![Fig. 1. Immunoblot analysis of insulin receptor β-subunit (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 4 experiments. Note the slightly faster migrating IRβ in retina tissue compared with liver; as has been reported in brain, this is probably due to less glycosylation in neurons.](http://ajpendo.physiology.org/ajpendo media/189701/ajpendo189701f1.jpg)
PY content in muscle IRβ 15 min postinjection, which peaked at 30 min (Fig. 2B). Retinal IRβ phosphorylation, unlike muscle, did not increase significantly until 30 min postinjection, and it remained three- to four-fold elevated above that in vehicle-injected controls for 60 min (Fig. 2C). This difference in the fold increase in retina tissue may be due to the higher basal IRβ phosphorylation in the retinas of vehicle-treated rats (Fig. 2A) and to the blood-retinal barrier (BRB), which limits diffusion. Retinal lysates were also analyzed for Akt<sup>Ser473</sup> phosphorylation at 45 min postinjection, when IRβ phosphorylation was maximal. Insulin induced a 48% increase (*P < 0.05) in Akt<sup>Ser473</sup> phosphorylation over vehicle-injected controls; there was no statistical difference between sham and vehicle-injected controls (Fig. 2D).
The 50-μg dose of insulin elicited no detectable change in the tonic retinal IRβ phosphorylation state after 15 and 45 min (Fig. 2E). This is in contrast to the 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. Supraphysiological insulin further activates the IR-signaling cascade above basal states in retina in a dose-dependent manner, but with a different temporal course than in muscle, illustrating important differences between retinal insulin signaling compared with other peripheral tissues.

IRβ PY content and kinase activity remain constant in retina between fasted and freely fed rats. Because retinal IRβ phosphorylation increased in response to acute supraphysiological insulin administration, we examined whether the tyrosine phosphorylation and kinetic activity of the IRβ in retina diminished in fasted animals as they do in liver and muscle. Simon et al. (58) previously demonstrated that brain IR content in retina remains stable despite physiological changes in nutritional status and circulating insulin (Table 1).

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 given 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) 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. 3C). 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.

The IRβ is autophosphorylated by insulin 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 (33, 34) and others (14) to investigate retinal metabolism and effects of growth factors on the retina. After dissection, retinas were treated with either 10 nM insulin or vehicle and analyzed as we have

**Fig. 3. Retinal IRβ phosphorylation remains constant in freely fed and fasted rats.** A: tissue lysates were immunoprecipitated and blotted for IRβ tyrosine phosphorylation, which was found to be unaltered in retina. However, increases in muscle and liver compared with fasted rats (lanes 1–2, retina; lanes 3–4, muscle; lanes 5–6, liver; lanes 1, 3, and 5, fasted rats; lanes 2, 4, and 6, fed rats, n = 6/group).

B: quantification of blots in A expressed as PV/IRβ ratios. There was no statistical difference in IRβ phosphorylation in retinal tissue between fed and fasted rats. In the freely fed state, muscle and liver IRβ phosphorylation was significantly greater than that of retina IRβ (**P < 0.01 and ***P < 0.001 by ANOVA and Student-Newman-Keuls posttest over retina PY/IRβ ratio from fasted rats).

C: kinase assays were performed on liver, retina, and brain lysates from 18-h-fasted (n = 4) and freely fed rats (n = 4). As reported in brain, retinal IR kinase activity remains stable (P = 0.80), whereas in fasted rats, liver IR kinetic activity is significantly reduced compared with both retina and brain (**P < 0.05 by ANOVA and Student-Newman-Keuls posttest).
described. Increased IRβ phosphorylation was detected with 1 nM insulin (not shown), and a concentration of 10 nM insulin is sufficient to induce IRβ phosphorylation of cultured retinal neurons (7). The retinal IRβ displayed lower basal tyrosine phosphorylation (Fig. 4, A, C, and D), and within 2 min of 10 nM insulin stimulation, the IRβ exhibited nearly fourfold greater PY immunoreactivity, which remained elevated threefold but was diminishing after 30 (Fig. 4, A and B) and 60 min (not shown) of insulin stimulation. Expression of the IR in this model is stable (Fig. 4B). IGF-IRβ PY content remained stable with 10 nM insulin treatment, demonstrating no insulin-induced activation of the IGF-IR in this model (Fig. 4C). Because IGF-II is also a ligand for the IR (20, 35), and IGF-II mRNA has been detected in adult rat brain (36), we performed a dose response with IGF-II on retinal IRβ activation in retinal explants (Fig. 4D). IGF-II (10 nM) had no effect, whereas 100 nM and 1 μM IGF-II maximally phosphorylated the IR. Total ATP levels were measured as described (33, 34, 68), 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.

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 antisense strands were hybridized to 16-μm cryostat sections, and representative dark-field images are shown in Fig. 5. Akt-1 mRNA localized to every cell layer of the retina, consistent with its broad distribution among different tissues (28). 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 (43). Therefore, all Akt isoforms may potentially play a role in maintaining retinal survival and/or metabolism.

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 (7, 17, 24). With ex vivo use of the retina culture system described above, the characteristics of retinal Akt activation were investigated in response to insulin. Akt was maximally phosphorylated twofold above basal conditions \( (P < 0.05) \) on Ser\(^{473} \) 5 min after the addition of insulin (Fig. 6, A and B). Akt phosphorylation then declined to basal levels 30 min after the addition of insulin, possibly due to active phosphatases, and there was no statistically significant difference between vehicle- and insulin-treated retinas after 30 min. However, these data were dependent on antibodies that recognize phospho- and total Akt-1, -2, and -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 min to determine which Akt isoforms were activated (54). This time point was chosen because Ser\(^{473} \) phosphorylation is maximal by immunoblot analysis (Fig. 6, A and B). In retinal explants, we observed a twofold increase in activation of Akt-1 in response to insulin \( (P < 0.05, \text{Fig. 6C}) \) but no change in Akt-2 or Akt-3 activity (Fig. 6C). These results suggest that insulin preferentially activates specific Akt isoforms in retina. These data also suggest 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 18-h-fasted rats. Interestingly, retina tissue harbored significantly more Akt-1 activity than liver and skeletal muscle (Fig. 6D), demonstrating that the retina maintains higher Akt-1 kinase activity than other insulin-responsive tissues. Taken together with tonic IR\( \beta \) phosphorylation in the basal state, the IR\( \rightarrow \)Akt signaling system in retina appears constantly active compared with other insulin-responsive tissues and may play a significant role in cell survival and metabolism.

**Phospho-Ser\(^{473} \) Akt immunoreactivity increases in ex vivo retina nuclear layers.** To understand which retinal layers respond to insulin, explanted retinas were fixed after insulin or vehicle treatment, and cross sections were immunostained for Akt\(^{\text{Ser473}} \) phosphorylation. Figure 7A shows a histological section of a vehicle-treated retina in which basal phospho-Akt-1 is observed diffusely throughout the retina. With insulin treatment (Fig. 7B), the immunoreactivity for phospho-Akt\(^{\text{Ser473}} \) is increased in the nuclear layers and the innermost portion of the retina, suggesting that numerous retinal cell types are insulin responsive.

**Insulin phosphorylates IRS-2 in retinal explants.** IRS-1 and -2 are substrates for the IR\( \beta \) kinase, and IRS-1 expression has been observed in rat retina (18, 21). Using the same time course as that used for retinal explants, IRS-1 and -2 phosphorylation was investigated by immunoprecipitation and PY immunoblotting. Basal IRS-1 tyrosine phosphorylation was detected, but insulin does not further increase the phosphorylation over the time course (Fig. 8A). 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. 8B), suggesting that IRS-2 may mediate the insulin signal more than IRS-1. This is the first demonstration that insulin phosphorylates IRS-2 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 ERK1/2. Similar to Akt, detection of basal phosphor-
ylation of ERK1/2 was observed, but there was no change with 10 nM insulin treatment by immunoblot analysis. This implies that either the ERK1/2 pathway is not a major mediator of IR signaling in adult retina, or ERK1/2 phosphorylation is already nearly maximal, and further stimulation with insulin is not detectable by immunoblot analysis.

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 by use of in vivo and ex vivo approaches. The major in vivo findings of this work are
that retinal IRβ exhibits constitutive tyrosine phosphorylation and activity that increase with supraphysiological insulin administration via the portal vein and remain stable during feeding and fasting in normal rats. Ex vivo studies reveal that IR activation increases IRβ, IRS-2, and Akt phosphorylation and Akt-1 kinase activity. IRS-1 and ERK1/2 phosphorylation remained stable in response to 10 nM insulin. Furthermore, insulin increased phospho-AktSer473 immunoreactivity in retinal nuclear layers. This is the first demonstration of retinal insulin-signaling characteristics in rats.

A critical observation in this study is that the IRβ and Akt in retina display tonic and elevated tyrosine and Ser473 phosphorylation, respectively, and kinetic activity, despite variable nutritional status and circulating plasma insulin. These results in retina are similar to those described in chicken brain (58). As expected, the kinetics of such activation are delayed compared with IR activation in skeletal muscle, because the transport of insulin across the BRB is slower. Peripherally administered insulin binds to retinal microvasculature (27) and pericytes (26), is detected in the vitreous fluid (57), and crosses the blood-brain barrier of rats (5). This is the first demonstration of exogenous insulin activating retinal IRSs in vivo. The kinetics of retinal IR activation with the higher dose are similar to the transport kinetics described in brain (5). Because of the stability in retinal IR phosphorylation and kinetic activity compared with 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 IRSs 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 IRSs can be activated by both ligand-dependent and ligand-independent mechanisms (51). Budd et al. (11) 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 IRSs 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 (35), 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 (38), 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-IRs (20, 45). If the retina, like the brain, expresses IR-A exclusively (56), 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 we hypothesize that retinal insulin signaling is in a constitutively higher steady state and that 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 BRB is removed. Insulin rapidly induces IRβ 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→IRS-2→Akt-1 signaling cascade in retina that does not further increase ERK1/2 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, whereas Akt-2 is restricted to the classically
insulin-responsive tissues and Akt-3 to neuronal cells and testis (2, 28, 32). Akt, like the IR, exhibits high basal phosphorylation and activity in retina in vivo; however, insulin promoted Akt phosphorylation in vivo and increased AktSer473 immunoreactivity in the retinal nuclear layers, suggesting that 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 retinal tissue (22). Akt nuclear translocation has been reported in hippocampal neurons in a cerebral ischemia reperfusion model (72), suggesting that AktSer473 phosphorylation is a critical event promoting neuronal survival and that cofactors, such as Tc1, may also play a significant role in neuronal survival by aiding Akt translocation (46). Further investigation is required to determine the nature of retinal insulin signaling in plexiform layers (1) 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. On the basis of gene deletion studies, IRS-1 and IRS-2 have distinct functions despite being immediate substrates for the IR (3, 60, 69). This and another study (64) also suggest that IRS-2 is the preferred docking substrate for the IR (3, 60, 69). This and another study (64) also suggest that IRS-1 and IRS-2 in neurons, such as the Trk receptors (70). Therefore, one may predict that IRS-2 gene disruption would disrupt 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 (67). PI3K is downstream of IRS proteins, and it has been detected immunohistochemically (18) in the same retinal layers in which 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 (50) and a role for PI3K in retinal development (48).

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 brain-derived neurotropic factor treatment in vivo reduce ganglion cell death (29, 40). After hypoxia, IGF-I mediates protection of retinal cells (55). In terms of diabetes, we and others (6, 39, 42) have demonstrated increased apoptosis in retinal neurons cultured in excess hexosamines, experimental animal models, and humans; with insulin treatment, retinal apoptosis was decreased in cultured retinal neurons and in the retinal neurons of diabetic animals. 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 (6, 12, 39, 49). Therefore, insulin or other growth factors that activate the antiapoptotic kinase Akt may prove beneficial in preserving retinal cell survival and function.

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DISCLOSURES

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