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Cytoskeletal components enhance the autophosphorylation of retinal insulin receptor

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ABSTRACT

Insulin receptor (IR) signaling provides a trophic signal for transformed retinal neurons in culture, and we recently reported that deletion of IR from rod photoreceptors resulted in stress-induced photoreceptor degeneration. Retinal insulin receptor has a high basal level autophosphorylation compared to liver and the reasons for higher autophosphorylation are not known. In the current study we report a novel finding that cytoplasmic actin associates with and activates the retinal IR *in vivo*. Similar to insulin, actin also induced autophosphorylation at tyrosines 1158, 1162 and 1163 in the catalytic loop of IR. Our studies also suggest that globular actin activates the retinal IR more effectively than does filamentous actin. Retinal IR kinase activity has been shown to decrease in hyperglycemia and we found a decreased binding of actin to the IR under hyperglycemia. This is the first study which demonstrates that cytoplasmic actin regulates autophosphorylation of the retinal IR.

1. Introduction

The actions of insulin are initiated by its binding to the IR, a disulfide-bonded heterotetrameric membrane protein [1–3]. Insulin binds to two asymmetric sites on the extracellular α subunits, causing conformational changes that lead to autophosphorylation of the membrane-spanning β subunits and activation of the receptor's intrinsic tyrosine kinase [4,5]. The IR transphosphorylates several immediate substrates on tyrosine residues, including IR substrate (IRS) proteins [6]. These events lead to the activation of downstream signaling molecules. Retinal cells contain specific high-affinity receptors for insulin [7]. We have shown previously that light causes increased tyrosine phosphorylation of the retinal IR and this activation leads to the activation of phosphoinositide 3-kinase (PI3K) *in vivo* [8]. The light effect is localized to the photoreceptor neurons [8]. Very recently we have demonstrated that photobleaching of rhodopsin regulates the tyrosine phosphorylation of the retina IR [9]. IR signaling provides a trophic signal for transformed retinal neurons in culture [10], and we recently reported that deletion of IR from rod photoreceptors resulted in stress-induced photoreceptor degeneration [11]. Further, it has been reported that the retinal IR has high basal constitutive activity which is independent of circulating insulin [12,13]. A significant decrease of retinal IR kinase activity has been reported after 4 weeks of hyperglycemia in STZ-treated rats [14]; however, the mechanism of this constitutive IR activation remains unknown. The high basal IR kinase activity [12] and light-induced activation of the IR [8] led to the hypothesis that novel ligands or regulators of the IR exist in the retina. Consistent with this hypothesis that ligand-independent activation of IR has been reported previously [15–18]. Two compounds capable of stimulating IR autophosphorylation by acting on the cytoplasmic domain have been reported [15,16]. One of these (L783,281) modestly elevates IR autophosphorylation in the absence of insulin [19], whereas the other (TLK16988) potentiates receptor autophosphorylation in the presence of insulin, suggesting two different mechanisms of action [18]. Structural and biochemical evidence for an autoinhibitory role for tyrosine 984 in the IR has been reported [20]. Substitution of tyrosine 984 in the β sheet-αC cleft with alanine resulted in increased basal level of IR phosphorylation [20]. Based on these results, it has been proposed that compounds that bind in the β sheet-αC cleft and displace tyrosine 984 should partially activate the IR [20]. Furthermore, regulation of IR kinase activity by phosphatidylinositol in the absence of insulin has also been reported [17]. These studies suggest that the

Abbreviations: IR, insulin receptor; PI3K, phosphoinositide 3-kinase; IRβ, IR beta subunit; PAGE, polyacrylamide gel electrophoresis; STZ, streptozotocin; ROS, rod outer segments; IPs, immunoprecipitates; G-actin, globular actin; F-actin, filamentous actin; SDM, site-directed mutagenesis.

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cytoplasmic domain of the IR can be autophosphorylated independent of insulin.

In this study we report a novel finding that actin endogenously associates with and activates the retinal IR *in vivo*. Similar to insulin, actin induced the autophosphorylation of tyrosines 1158, 1162 and 1163 in the catalytic loop of IR. Our studies also suggest that G-actin more effectively activates the retinal IR than F-actin. Our studies demonstrate for the first time that actin may be one of the physiological regulators of the retinal IR.

2. Materials and methods

2.1. Materials

Polyclonal anti-IRß and monoclonal anti-PY-99 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-insulin/IGF-1-like growth factor-1 receptor ($pYpYpY^{1158, 1162, 1163}$) phosphospecific (pIR) antibody was obtained from Biosource (Camarillo, CA). Anti-His antibody was obtained from Cell Signaling (Beverly, MA). [γ^{32}P]ATP was obtained from New England Nuclear (Boston, MA). Actin antibody was obtained from Affinity BioReagents (Golden, CO). Human recombinant β-insulin receptor kinase-GST fusion was obtained from Calbiochem (San Diego, CA). Skeletal muscle actin was obtained from Sigma (St. Louis, MO). TNT-T7 quick coupled transcription/translation reagents were obtained from Promega (Madison, WI). All other reagents were of analytical grade and from Sigma.

2.2. Animals

All animal work was in strict accordance with *the NIH Guide for the Care and use of Laboratory Animals* and the Association for Research in Vision and Ophthalmology on the Use of Animals in Vision Research. All protocols were approved by the IACUC at the University of Oklahoma Health Sciences Center and the Dean McGee Eye Institute. C57BL/6 mice (4–6 weeks old, male) were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in our vivarium in cyclic light (12 h on; 12 h off; ~300 lux). Mice were acclimated with a 12-h light/dark schedule and fed standard laboratory food and water *ad libitum* until the induction of hyperglycemia.

2.3. Generation of hyperglycemic mice

Hyperglycemia was induced by a series of two injections. At 8 weeks, mice were weighed and given an intraperitoneal injection (100 mg/kg) of streptozotocin (STZ) in freshly dissolved citrate buffer (10 mmol, pH 4.5). Control animals were given an intraperitoneal injection of citrate buffer only. At 12 weeks, mice were weighed and blood glucose levels were analyzed. Mice with blood glucose levels >250 mg/dl (TrueTrack Smart System; AR-MED Ltd, Egham, UK) were considered hyperglycemic. Mice were euthanized by CO_2 asphyxiation and cervical dislocation. Retinas were immediately removed after euthanization and frozen in liquid nitrogen.

2.4. Retinal tissue preparation

Either rats or mice were killed by CO_2 asphyxiation followed by cervical dislocation. Retinas were quickly removed by a technique called "winkling" [21]. A deep cut is made across the corneal surface and curved forceps are placed behind the eyeball on either side of the optic nerve head. The forceps are squeezed and brought forward, forcing the contents of the eye to be extruded through the hole in the cornea. The retina can be recovered relatively intact and placed immediately into ice-cold buffer or snap frozen with liquid nitrogen. Retinas were homogenized by hand in lysis buffer [1% NP 40, 20 mM HEPES (pH 7.4), 2 mM EDTA, phosphatase inhibitors (100 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM $NaVO_3$, and 1 mM molybdate), and protease inhibitors (10 μM leupeptin, 10 μg/ml aprotinin and 1 mM PMSF)]. The lysates were kept on ice for 10 min followed by centrifugation at 4 °C for 20 min.

2.5. Insulin receptor autophosphorylation

Retinal lysates were immunoprecipitated with anti-IRβ antibody. Analysis of IR autophosphorylation was carried out as previously described [17]. Briefly, IR immunoprecipitates were incubated either in the presence or absence of 1 μM human insulin, assay buffer [HEPES (pH 7.4), 10 mM $MnCl_2$, 10 mM $MgCl_2$, and 0.65% n-octyl-β-D-glucopyranoside] and ATP to a final concentration of 100 μM. The reaction was incubated at room temperature for 60 min. Sodium phosphate buffer has been shown to stabilize the phosphotyrosine in proteins compared to that of sample buffer containing Tris [22,23]. Therefore, sodium phosphate sample buffer [0.062 M phosphate, pH 7.0, 10%, w/v, glycerol, 2% SDS, 0.001% bromophenol blue and 5% 2-mercaptoethanol] was added to the immunoprecipitates and the suspension boiled at 100°C for 5 min. The samples were run on 10% SDS-PAGE followed by Western blot analysis with either anti-pIR or anti-phosphotyrosine (PY-99) antibodies.

2.6. Peptide mass fingerprinting (PMF)

The IR was immunoprecipitated with anti-IRβ antibody from retinal lysates and the bound proteins were resolved on SDS-PAGE followed by Gel Code blue staining. The visualized 42 kDa protein on the gel was subjected to in-gel digestion as previously described [24,25]. Mass spectra were obtained using a MALDI-TOF MS (Voyager Elite, Applied Biosystems, Foster City, CA). The PMF search was performed by MASCOT (http://www.matrixscience.com) using the NCBInr database.

2.7. Cloning of beta actin from mouse retina

Retinal actin was obtained by PCR of reverse transcribed retinal RNA using 5′ and 3′ oligonucleotides, designed based on mouse actin (sense: AAG CTT CAT CAT CAT CAT CAT CAT ATG GAT GAC GAT ATC GCT GCG CTG; antisense: GAA TTC CTA GAA GCA CTT GCG GTG CAC G). We added a histidine tag to the N-terminal end of the actin. The cDNA encoding full-length mouse actin was cloned into a TOPO vector and sequenced. The cDNA insert was excised from the TOPO vector as a HindIII/EcoRI fragment and cloned into a pCDNA3 mammalian expression vector.

2.8. Site-directed mutagenesis of actin

Site-directed mutagenesis (SDM) was carried out with the Quickchange site-directed mutagenesis kit (Stratagene Inc., LaJolla, CA) using a PTC 200 programmable thermal controller (MJ Research, Inc., Watertown, MA). The reaction mixture contained SDM buffer [200 mM Tris–HCl (pH 8.8), 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% Triton X-100, 1 mg/ml nuclease-free bovine serum albumin, 1 mM deoxynucleotide mix (dATP, dCTP, dTTP and dGTP), 50 ng of vector and 125 ng of sense and antisense primers with mutations] in a total volume of 50 μl, followed by the addition of 2.5 units of pfu DNA polymerase. The primers used in the SDM are as follows: S14C (sense: GTC GAC AAC GGC TGC GGC ATG TGC AAA GCC; anti-sense: GGC TTT GCA CAT GCC GCA GCC GTT GTC GAC) and R62D (sense: GAG GCC CAG AGC AAG GAC GGT ATC CTG ACC; antisense: GGT CAG GAT ACC GTC CTT GCT CTG GGC CTC). The extension parameters of SDM were as follows: initial denaturation at 95 °C for 30 s, followed by 16 cycles at 95 °C for 30 s, 55 °C for 1 min and at