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# Light activation of the insulin receptor regulates mitochondrial hexokinase. A possible mechanism of retinal neuroprotection

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ABSTRACT

The serine/threonine kinase Akt has been shown to mediate the anti-apoptotic activity through hexokinase (HK)–mitochondria interaction. We previously reported that Akt activation in retinal rod photoreceptor cells is mediated through the light-dependent insulin receptor (IR)/PI3K pathway. Our data indicate that light-induced activation of IR/PI3K/Akt results in the translocation of HK-II to mitochondria. We also found that PHLPPL, a serine/threonine phosphatase, enhanced the binding of HK-II to mitochondria. We found a mitochondrial targeting signal in PHLPPL and our study suggests that Akt translocation to mitochondria could be mediated through PHLPPL. Our results suggest that the light-dependent IR/PI3K/Akt pathway regulates hexokinase–mitochondria interaction in photoreceptors. Down-regulation of IR signaling has been associated with ocular diseases of retinitis pigmentosa, diabetic retinopathy, and Leber Congenital Amaurosis-type 2, and agents that enhance the binding interaction between hexokinase and mitochondria may have therapeutic potential against these ocular diseases.

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## 1. Introduction

Emerging evidence suggests that energy metabolism and cell survival are interrelated processes that are mainly coordinated by the serine/threonine kinase Akt (Robey and Hay, 2005; Whiteman et al., 2002). Several studies suggest that mitochondrial hexokinase (mtHK) is a major downstream effector of Akt- and growth factor-mediated cell survival (Bryson et al., 2002; Gottlob et al., 2001; Majewski et al., 2004; Majewski et al., 2004). Activated Akt has been shown to inhibit HK dissociation from mitochondria, which is an early event following induction of apoptosis (Gottlob et al., 2001). It has also been shown that growth factors increase mitochondrial–HK association in normal cells, an effect that is markedly deregulated in Akt deficient cells (Majewski et al., 2004). In mammalian cells, the HK activity exists both in soluble and membrane fractions (CRANE and SOLS, 1953; Katzen et al., 1970). Membrane activity can be largely accounted for by the specific binding of high affinity HKI and HKII isoforms to the outer mitochondrial membrane (OMM) at mitochondrial contact sites (Wilson, 1995). The binding of HK to OMM is mediated, at least in part, by specific interactions between the OMM voltage-dependent anion channel (VDAC) and hydrophobic N-terminal mitochondrial binding domains found in HKI and HKII, but not the corresponding HKIII and HKIV isoforms (Gelb et al., 1992; Majewski et al., 2004; Pastorino et al., 2002; Polakis and Wilson, 1985). The binding of HK-II to VDAC has been extensively studied and further reduced binding of HK-II to mitochondria lacking VDAC has also been reported (Anflous-Pharayra et al., 2007). HK associates with mitochondria and catalyzes the first committed step in glucose metabolism, the ATP-dependent phosphorylation of glucose to generate glucose-6-phosphate (Glu-6-P) (Wilson, 1995). The dynamic movement of HK between mitochondrial and cytosolic compartments is greatly influenced by physiological concentrations of Glu-6-P, ATP, and inorganic phosphate, and by intracellular pH (Miccoli et al., 1996, 1998; Robey and Hay, 2005).

Akt activation inhibits the opening of the mitochondrial permeability transition pore through the inactivation of glycogen synthase kinase-3β (GSK-3β), whereby it induces the translocation of HK to mitochondria and binding to VDAC (Feng et al., 2005; Pastorino et al., 2005). Phosphorylation of serine-9 of GSK-3β is well known to inhibit its activity (Cross et al., 1995). Thus, one consequence of the action of Akt in mitochondria is to inhibit GSK-3β (Feng et al., 2005; Pastorino et al., 2005). In the absence of Akt activation, GSK-3β becomes active and phosphorylates VDAC on threonine 51, which disrupts the binding of HK to VDAC

*Abbreviations:* IR, insulin receptor; PI3K, phosphoinositide 3-kinase; ROS, rod outer segments; HK-II, hexokinase; VDAC, voltage-dependent anion channel; GSK-3β, glycogen synthase kinase-3β; PHLPPL, PH domain and leucine-rich repeat protein phosphatase-like; PHB1, prohibitin-1; HSP60, heat shock protein 60.

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(Pastorino et al., 2005). In this manner, activated Akt inhibits the mitochondrial cytochrome *c* release and apoptosis (Majewski et al., 2004; Robey and Hay, 2006).

We previously reported that Akt activation is neuroprotective as down regulation of this pathway leads to photoreceptor degeneration (Li et al., 2007, 2008). In photoreceptor cells, Akt activation is light-dependent and regulated through the G-protein coupled receptor activated IR/PI3K pathway (Li et al., 2008; Rajala et al., 2007). In this study, we examined the role of light-dependent IR/PI3K/Akt signaling on HK–mitochondria interaction. Our results indicate that light-induced activation of IR/PI3K/Akt leads to the translocation of HK-II to mitochondria, and this light-dependent translocation of HK-II is significantly reduced in rod photoreceptors conditionally depleted of the insulin receptor gene. Our studies also reveal that the GSK-3β inhibitor enhanced the binding of HK-II to mitochondria, whereas PI3K inhibitor reversed this effect. We also made a novel observation that PHLPPL, a serine/threonine phosphatase (Brognard et al., 2007), potentiates the effect of Akt and thereby enhances the binding of HK-II to mitochondria. Dissociation of hexokinase from mitochondria has been shown to induce apoptosis (Chiara et al., 2008; Galluzzi et al., 2008) and our study suggests a mechanism whereby light activation of the IR regulates mitochondrial hexokinase in photoreceptors, which provides retinal neuroprotection.

## 2. Materials and methods

### *2.1. Materials*

Polyclonal anti-hexokinase II, anti-VDAC, anti-cytochrome *c*, anti-HSP60, anti-PHB1 (prohibitin-1), anti-pAkt (S473), anti-Akt, anti-pGSK-3α/β, anti-GSK-3β, anti-Flag, and monoclonal anti-Myc antibodies, and PI3K inhibitor LY294002 were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Actin antibody was obtained from Affinity BioReagents (Golden, CO). Polyclonal PHLPPL antibody was obtained from Novus Biologicals (Littleton, CO). GSK-3β inhibitor N-(4-Methoxybenzyl)-N′-(5-nitro-1,3-thiazol-2-yl)urea was obtained from Calbiochem (San Diego, CA). Mitochondria Isolation Kit for tissue (Zhang et al., 2010) was obtained from Thermo Fisher Scientific Inc. (Rockford, IL). Human insulin R (rDNA origin) was obtained from Eli Lilly & Company (Indianapolis, IN). All other chemicals were purchased from Sigma (St. Louis, MO).

### *2.2. Animals*

All animal work was performed in strict accordance with the Association for Research in Vision and Ophthalmology statement on the “Use of Animals in Ophthalmic and Vision Research.” All protocols were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center and the Dean A. McGee Eye Institute. A breeding colony of albino Sprague–Dawley (SD) rats is maintained in our vivarium in cyclic light (5 lx; 12 h on/12 h off). Experiments were carried out on both male and female rats (150–200 g). Photoreceptor specific conditional insulin receptor knockout mice (Rajala et al., 2008) and p85α knockout mice (Ivanovic et al., 2011a) on BALB/c background were born and raised in a 60-lux cyclic light (12 h on/off) in our animal facility. Mitochondria were prepared from two independent sets of light- and dark-adapted retinas (either *ex vivo* or *in vivo*). C57BL/6J Ins2$^{Akita}$ heterozygote mice (Jackson Laboratory, Bar Harbor, ME) were bred in the Dean A. McGee Eye Institute Animal vivarium. Diabetic phenotype and genotype were confirmed 4.5 weeks after birth by blood glucose >250 mg/dl (TrueTrack Smart System; AR-MED Ltd, Egham, UK) in a drop of blood from a tail puncture. The disease is 100% penetrant in mice with the Ins2 mutation (Wang et al., 1999). Each set of mitochondrial preparations had at least 20 rats or mice (10 for light- and 10 for dark-adapted animals).

### *2.3. Ex vivo retinal cultures*

For insulin treatment of retinal *in vivo* explants, rats were dark-adapted overnight, killed the next day, and retinas were removed and placed in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA). Insulin (10–1000 nM) or an equal volume of PBS was added and the retinas were incubated at 27 °C for 5 min. To inhibit PI3K activity or GSK-3β activity, retinal *in vivo* explants were incubated in 100 μM LY294002 or 100 μM GSK-3β inhibitor, or an equal volume of DMSO at 27 °C for 30 min, after which half of the retinal *in vivo* explants were exposed to a 300 lux light for 30 min prior to the isolation of mitochondria.

### *2.4. Plasmids and vectors*

Flag-tagged PHLPPL construct has been reported earlier (Kanan et al., 2010). We amplified a fragment of PHLPPL that contains the PP2C domain with (amino acids 130 to 1028) and without (amino acids 148–1028) a mitochondrial targeting signal (MT) using sense (+MT: AGA TCT ATG ATT CGA TTT TAT GGT GGA AAA CC; -MT: AGA TCT CGA ATC CTA CTG TCT GGC ATC) and antisense (GTC GAC TCA AAC CAC CAT TGC CCC CAC GTTG) primers and cloned into Myc-tagged pcDNA3 as a BglII/SalI fragment. We thank Dr. Morris Birnbaum (University of Pennsylvania) for his generous gift of mammalian expression constructs of Akt. The dominant negative Akt1 (K179M) (Zhou et al., 2000) construct was kindly provided by Dr. Mein-Chie Hung, M. D. Anderson Cancer Center, Houston, Texas. The dominant negative Akt1 (K179M) (Addgene plasmid 16243) was obtained from Addgene Inc., Cambridge, MA (http://www.addgene.org/pgvec1).

### *2.5. Cell lines and culture condition*

HEK-293T cells were maintained in DMEM containing 10% (v/v) FBS at 37 °C. Approximately $2.5 \times 10^5$ cells were seeded in each culture dish 12–18 h before transfection. Calcium phosphate-mediated DNA transfection was performed and cells were harvested for experiments ~48 h post-transfection.

### *2.6. Phosphatase assay*

The *in vitro* phosphatase activity assay was conducted based on a protocol previously described (Kanan et al., 2010). Proteins expressed in HEK-293T cells were immunoprecipitated overnight at 4 °C with anti-Flag (PHLPPL) or anti-Myc (PP2C domain) antibodies. The immune complexes were precipitated with protein A-Sepharose at 4 °C for an additional 2 h. Immunoprecipitates were washed in phosphatase assay buffer [100 mM HEPES (pH 7.6), 2 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, and 0.5 mg/ml bovine serum albumin]. The peptide substrate RRLIEDAE$_P$YAARG was added to a final concentration of 200 μM in a total reaction volume of 60 μl in phosphatase assay buffer, and the reaction was allowed to proceed for 1 h at 30 °C. At the end of the reaction, 40-μl aliquots were placed in a 96-well plate, 100 μl of Malachite green phosphatase reagent was added, and absorbance was measured at 630 nm.

### *2.7. Exposure of animals to light stress*

Sprague–Dawley rats were born and raised in a dim cyclic (5 lx) light. Albino rats were exposed to constant light for 3 h at an illuminance level of 5000 lx (Kanan et al., 2010). During light exposure, animals were maintained in transparent polycarbonate cages with stainless-steel wire bar covers. Drinking water was supplied by a bottle attached to the side of the cage, so that there was no obstruction between the light and the animal, and food was placed on a bedding in the bottom of the cage.

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