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Pigment Epithelium-Derived Factor Protects Cone Photoreceptor-Derived 661W Cells from Light Damage Through Akt Activation

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Abstract

Pigment epithelium-derived factor (PEDF) can delay and prevent the death of photoreceptors *in vivo*. We investigated the survival activity of PEDF on cone photoreceptor-derived 661W cells in culture, the presence of PEDF receptor (PEDF-R) in these cells and the activation of prosurvival Akt. Cell death was induced by light exposure in the presence of 9-*cis* retinal. Cell viability assays showed that PEDF increased the number of 661W cells exposed to these conditions. Western blots showed that PEDF-treated 661W cells had a higher ratio of phosphorylated Akt to total Akt than untreated cells. The PEDF receptor PEDF-R was immunodetected in the plasma membrane fractions of 661W cells. The results demonstrated that PEDF can protect 661W cells against light-induced cell death and suggest that the binding of PEDF to cell surface PEDF-R triggers a prosurvival signaling pathway.

Keywords

PEDF; PEDF-R; Photoreceptor; 661W cells; Akt phosphorylation; Survival

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102.1 Introduction

Pigment epithelium-derived factor (PEDF) has potent retinal survival properties [1–3]. It is a natural glycoprotein (50-kDa) that is highly expressed in the retinal pigment epithelium (RPE) [4–5] and is secreted into the interphotoreceptor matrix [6]. PEDF is involved in the maintenance and promotion of photoreceptor and retinal neuron cell survival [1–2]. It also plays a role in the prevention of neovascular invasion [1, 3], [7–9]. Studies in animal models for inherited and light-induced retinal degeneration, retinal ischemia, and degeneration of spinal cord motor neurons have established a role for PEDF as a neurotrophic factor in the retina and CNS [10–15]. PEDF protects cultured cells of retinal origin from death induced by ischemia and cytotoxic agents [15–19]. In neurovascular ocular diseases such as age-related macular degeneration, diabetic retinopathy, and neuroretinal dystrophies, PEDF levels are reduced [20–22]. Although numerous studies have demonstrated the importance of PEDF as a neuroprotective factor, specific mechanisms of PEDF action are yet to be elucidated.

One of the proposed mechanisms is thought to be initiated via interaction with high-affinity receptors on cell surfaces. Pigment epithelium-derived factor (PEDF-R) is a phospholipase A (PLA) enzyme with high affinity for PEDF, whose activity is enhanced by PEDF binding [23, 24]. The PEDF-R sequence has four transmembrane domains and intracellular and extracellular regions. In the rat retina, active PEDF-R is detected in the plasma membranes of RPE cells and in the inner segments of the photoreceptors. Thus the location of PEDF and PEDF-R indicates that ligand and receptor are available to interact in the retina.

To investigate the molecular mechanism of action of PEDF in response to light-induced damage of photoreceptors, we used the 661W cell line, a mouse retinal tumor-derived cone-specific cell line expressing cone pigments, transducin, and arrestin [25]. These cells have been used as an in vitro model for studying light-induced cell death in cone photoreceptors [26].

102.2 Materials and Methods

102.2.1 Cell Culture

661W cells were cultured in DMEM with 10 % Fetal Bovine Serum (FBS) and 1 % antimycotic-antibiotic cocktail. The media was also supplemented with 4.3 μg/ml hydrocortisone, 0.04 μg/ml progesterone, 32 μg/ml putrescine, and 4 % β-mercaptoethanol. Cells were kept at 37° C with 5 % CO_2.

102.2.2 Cell Viability Assays

Three thousand cells were plated per well of a 96-well tissue culture plate and allowed to grow for 16 h in the dark. Then, the media was replaced with regular media, media containing 10 μM 9-*cis* retinal, or media with 9-*cis* retinal and PEDF. After 4 h, the plates were placed in a light box (~ 22,000 lx) at 30° C, either covered or not covered to obtain data from samples in the dark and in the light for the indicated time period. Cell viability measurements were made with a CellTiter-Glo viability assay kit (Promega) following the manufacturer's instructions, except that the extract mixture with the CellTiter-Glo reagent

was pipetted up and down 2–3 times and transferred to a 96-well opaque-walled plate. The luminescence intensity was measured using an automated plate reader (Envision, Perkin Elmer, MA).

102.2.3 Western Blot and Protein Analysis

Cells were harvested at various times with 1X SDS sample buffer and passed through a needle to shred DNA. The samples were boiled for 10 min, cooled on ice, and loaded on a 10–20 % tricine polyacrylamide gel for SDS-PAGE [27]. After immunotransfer, the nitrocellulose membrane was incubated sequentially in blocking solution for 1 h at room temperature, rabbit anti-phospho-Akt diluted 1:1,000 (Cell Signaling Technology, Inc., cat #9271) for 16 h at 4° C, HRP-conjugated goat anti-rabbit IgG secondary antibody (1:5,000) for 1 h at room temperature, and finally SuperSignal West Dura Extended Duration Substrate (Pierce) as per the manufacturer's protocol. Washes were with TBS-T (50 mM Tris HCl pH 7.4, 150 mM NaCl plus 0.1 % Tween-20, TBS-T) and stripping was with Restore™ Western Blot Stripping Buffer (Pierce). For total Akt detection, the stripped and blocked membrane was incubated with mouse anti-Akt diluted 1:1,000 (Cell Signaling Technology, Inc., cat #2967), followed with HRP-conjugated goat anti-mouse IgG (1:5,000). For PEDF-R detection, primary and secondary antibodies were 0.25 μg/ml anti-PEDF-R (R&D systems, cat# AF5365) and HRP-conjugated donkey anti-sheep IgG diluted 1:20,000 (SIGMA, cat# A3415) in 1 % BSA/TBS-T, respectively. Blots were exposed to X-ray films to visualize chemiluminescent immunoreactive bands.

102.2.4 Membrane Fractionation

Confluent 661W cells (90 %) were harvested and lysed with RIPA buffer (Pierce) to obtain total cell lysates or subjected to biochemical fractionation to separate cytosolic and membrane fractions [23]. Protein concentration was determined with BCA Protein Assay (Pierce).

102.3 Results

102.3.1 PEDF Promotes Survival Activity on 661W Cells

Cells were treated with 9-*cis* retinal with or without PEDF before and during light exposure (22,000-lux). Figure 102.1 shows that when exposed to light, only 33 % and 17 % of the cells were viable after 2 and 3 h, respectively, as compared to cells kept in the dark (100 %). However, in the presence of PEDF, the viable cell numbers increased and had a significant recovery (approximately 100 %) with 10 nM PEDF. The results indicate that PEDF protected these cells from light-induced death.

102.3.2 PEDF Promotes Akt Activation

To identify prosurvival pathways for PEDF protection of 661W cells, we investigated the activation of Akt, a known survival target. Cells were damaged by light and 9-*cis* in the presence and absence of PEDF, and phosphorylated Akt (pAkt) levels were determined in cell lysates. The level of pAkt was normally high in the dark and dropped with light exposure (Fig. 102.2). Cells treated with 10 nM PEDF and exposed to light for 1 and 2 h had significant increases of pAkt relative to those without PEDF (Fig. 102.2). However, by

the third hour, no pAkt was detected with or without PEDF. The results show that PEDF induced intracellular signaling events in the Akt pathway in 661W cells and further support our previous findings on photoreceptor protection by PEDF.

102.3.3 PEDF-R in 661W Cells

Previous studies have shown that PEDF-R is a transmembrane protein present in plasma membranes [23]. Western blots of 661W total lysates and cell membrane fractions with antibody to PEDF-R show the presence of a single immunoreacting band that migrates as an 80-kDa protein (Fig. 102.3). The results demonstrate the presence of PEDF-R in 661W cells and imply its cell surface location and availability to interact with extracellular PEDF ligands.

102.4 Discussion

It was previously determined that 661W cells undergo apoptosis when exposed to light in the presence of 9-*cis* retinal [26]. In this study, we successfully demonstrate that PEDF prevents 661W cell death induced by light damage and that PEDF activates Akt, a known survival target.

While light exposure lowers the activated Akt levels with time, PEDF promotes Akt activation as early as 1 h post-addition and in agreement with the idea of pAkt being a precursor for survival. Interestingly, photoreceptor 661W cells contain PEDF-R on their surfaces indicating the availability of a PEDF receptor that could interact with a prosurvival signaling pathway in these cells.

A distinct signal transduction mechanism leading to the neurotrophic action of PEDF has not yet been established, but is implied by the following observations: (1) The phosphatidylinositol 3-kinase (PI3K)/Akt signaling is a critical downstream regulator for neuronal survival [28], (2) Several survival factors, including PEDF and docosahexaenoic acid (DHA), promote neuronal survival through the activation of Akt [28, 29], and (3) PEDF enhances the PLA activity of PEDF-R to liberate free-fatty acids, such as DHA, in the retina [23]. Our findings infer that PEDF interaction with PEDF-R to release DHA could serve to promote Akt as a prosurvival mediator, which would establish a signal transduction pathway in photoreceptor cell survival by PEDF.

Abbreviations

PEDF	Pigment epithelium-derived factor
PEDF-R	PEDF receptor
RPE	Retinal pigment epithelium
FBS	Fetal bovine serum
Akt	The serine/threonine kinase
PI3K	Phosphatidylinositol 3-kinase

DHA	Docosahexaenoic acid

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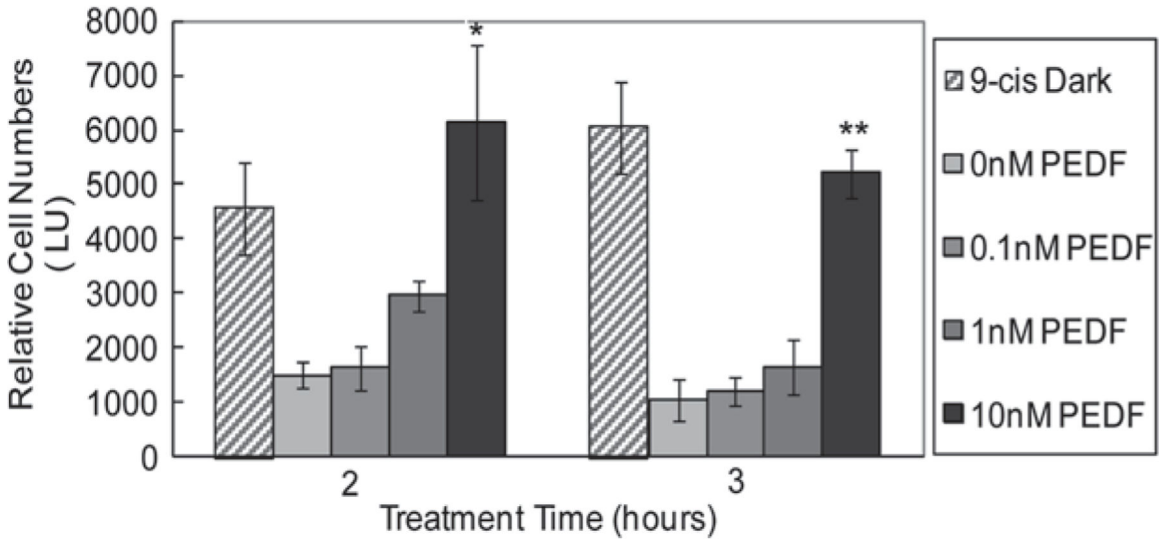

Fig. 102.1.

Effect of *PEDF* on 661W Cell Viability. The plot shows relative 661W cell numbers after treatments with indicated *PEDF* concentrations in the presence of 9-*cis* as a function of time (h) of light exposure. Control, 9-*cis dark*. Each point is the average of four replicate wells ± SD. (* $p < 0.002$ for 2 h, ** $p < 0.00001$ for 3 h)

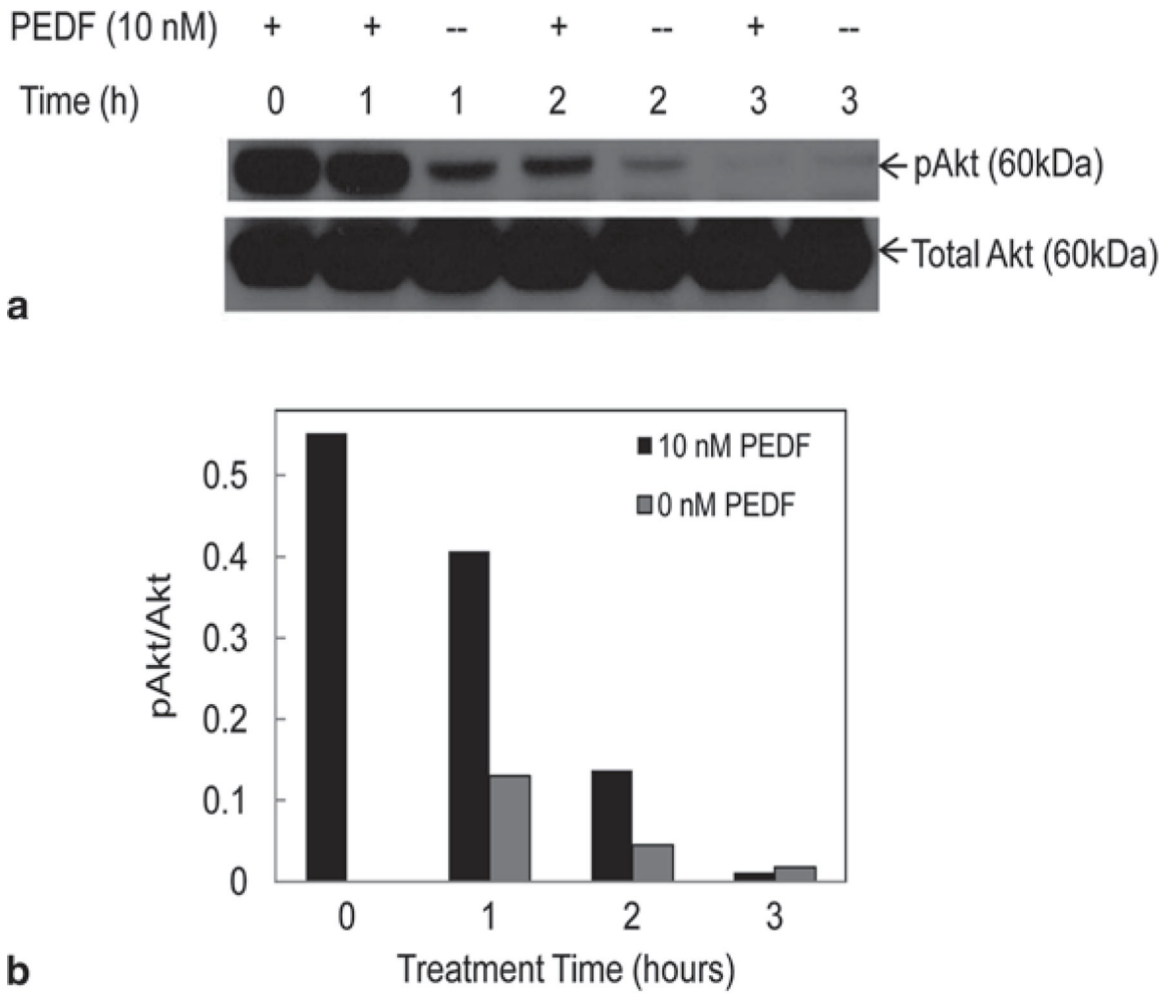

Fig. 102.2.

PEDF activates Akt in 661W cells. **a** 661W cells were treated with or without *PEDF* in the presence of 9-*cis* and exposed to light for the indicated times. Western blots of total lysates with antibodies to *pAkt* and total *Akt* are shown. **b** Plot showing the ratio of *pAkt* and *Akt* band intensities from (**a**) using UN-SCAN-IT software

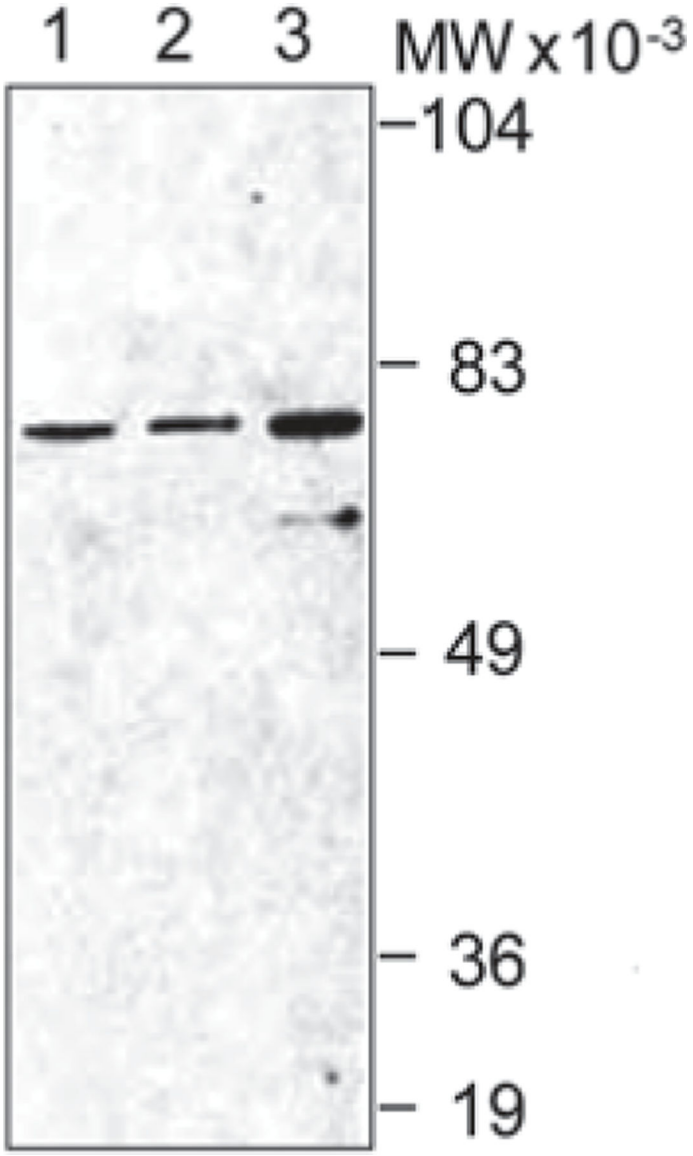

Fig. 102.3.

PEDF-R in 661W cells. Total RIPA lysate and membrane fractions from 661W cells were analyzed by western blotting for PEDF-R. Lane *1*, total lysate (20 μg protein); lanes *2* and *3*, membrane fractions isolated with NP-40 and CHAPS, respectively (5 μg protein). Molecular weight markers are shown to the right