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Insulin-like Growth Factor 1 Rescues R28 Retinal Neurons from Apoptotic Death through ERK-mediated Bim_{EL} Phosphorylation Independent of Akt

Dejuan Kong^{*,1}, Lijie Gong^{*,1}, Edith Arnold^{1,2}, Sumathi Shanmugam¹, Patrice E. Fort¹, Thomas W. Gardner¹, and Steven F. Abcouwer^{1,#}

¹Department of Ophthalmology and Visual Sciences, University of Michigan Kellogg Eye Center, Ann Arbor, MI

Abstract

Insulin-like growth factor 1 (IGF-1) can provide long-term neurotrophic support by activation of Akt, inhibition of FoxO nuclear localization and suppression of Bim gene transcription in multiple neuronal systems. However, MEK/ERK activation can also promote neuron survival through phosphorylation of Bim_{EL}. We explored the contribution of the PI3K/Akt/FoxO and MEK/ERK/Bim_{EL} pathways in IGF-1 stimulated survival after serum deprivation (SD) of R28 cells differentiated to model retinal neurons. IGF-1 caused rapid activation of Akt leading to FoxO1/3-T32/T24 phosphorylation, and prevented FoxO1/3 nuclear translocation and Bim mRNA upregulation in response to SD. IGF-1 also caused MAPK/MEK pathway activation as indicated by ERK1/2-T202/Y204 and Bim-S65 phosphorylation. Overexpression of FoxO1 increased Bim mRNA expression and amplified the apoptotic response to SD without shifting the serum response curve. Inhibition of Akt activation with LY294002 or by Rictor knockdown did not block the protective effect of IGF-1, while inhibition of MEK activity with PD98059 prevented Bim phosphorylation and blocked IGF-1 protection. In addition, knockdown of Bim expression was protective during SD, while co-silencing of FoxO1 and FoxO3 expression had little effect. Thus, the PI3K/Akt/FoxO pathway was not essential for protection from SD-induced apoptosis by IGF-1 in R28 cells. Instead, IGF-1 protection was dependent on activation of the MEK/ERK pathway leading to Bim_{EL} phosphorylation, which is known to prevent Bax/Bak oligomerization and

[#]Correspondence should be addressed to: Steve Abcouwer, University of Michigan Kellogg Eye Center, 1000 Wall Street, Ann Arbor, MI, 48105; phone (734) 232-8404; sabcouwe@umich.edu.

²Current address is Institute of Neurobiology, Universidad Nacional Autonoma de Mexico, Queretaro, Mexico

*These authors contributed equally and should be considered co-first authors.

Conflict of Interest

The authors declare that they have no conflicts of interest in relation to the contents of this article.

Author Contributions

DK designed and performed experiments, analyzed results, composed figures and wrote portions of the manuscript. LG designed and performed experiments, analyzed results and composed figures. EA compared protective effects of neurotrophins, performed IGF-1 dose-response experiments, selected transfected clones and contributed to siRNA experiments. SS aided in the performance of experiments. PEF contributed to the inception and design of the project and edited the manuscript. TWG contributed to the inception and design of the project and edited the manuscript. SFA conceived and designed the project and wrote most of the manuscript.

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activation of the intrinsic mitochondrial apoptosis pathway. These studies demonstrate the requirement of the MEK/ERK pathway in a model of retinal neuron cell survival and highlight the cell specificity for IGF-1 signaling in this response.

Keywords

Neuroprotection; insulin-like growth factor 1; PI3K; Akt; MAPK; ERK; Bim

1. Introduction

Insulin-like growth factor 1 (IGF-1) is a pleiotropic growth factor that acts by binding to the IGF type 1 receptor (IGF1R). IGF1R acts as a receptor tyrosine kinase (RTK) and G-protein coupled receptor (GPCR) hybrid. According to the classic model of IGF1R RTK there are two main pathways downstream of IGF1R-PI3K/Akt and MAPK/ERK (Girnita et al., 2014). The PI3K/Akt pathway is activated following binding of insulin receptor substrates (IRS) to activated IGF-1R, while both PI3K/Akt and MAPK/ERK pathways are downstream of Src homology 2 (SH2) containing proteins (Shc) bound to the activated receptor.

Numerous models have demonstrated the importance of the IGF-1/IGF1R system in brain development and growth (reviewed in (O'Kusky and Ye, 2012)). IGF-1 stimulates the proliferation, survival and differentiation of numerous neuronal cell types (Joseph D'Ercole and Ye, 2008). The IGF-1/IGF1R system also impacts retinal development. IGF-1 mRNA is highly expressed in mouse retinal ganglion cells (RGC) from just prior to birth until P16 (Lee et al., 1992). IGF-1 protein is highly expressed in both RGCs and Muller glia in the inner retina of neonatal rats and steadily diminishes with age (Hansson et al., 1989). IGF1R phosphorylation is high in the early rat postnatal (P0) retina and progressively decreases toward adulthood (P30) (Maturana-Teixeira et al., 2015). Environmental enrichment of visual acuity in neonatal rats depends on IGF1R function and is mimicked by intravitreal IGF-1 injection (Landi et al., 2009). Intravitreal IGF1 injection also diminishes postnatal culling of retinal ganglion cells (RGC) in rats (Gutierrez-Ospina et al., 2002).

IGF-1 acts as an essential neurotrophic factor. IGF-1 signaling prevents neuron death following brain injury and cerebral ischemia (Guan et al., 2003; Madathil and Saatman, 2015; Russo et al., 2005), supports the survival of neurons *in vitro* (Bozyczko-Coyne et al., 1993), and protects neuronal cells from apoptotic death in models of serum deprivation (SD) (Lu et al., 2008; Yamada et al., 2001; Zheng et al., 2002), trophic factor withdrawal (Ambacher et al., 2012; Brewster et al., 2006) and glutamate toxicity (Liu et al., 2012; Vincent et al., 2004). IGF-1 protects rat RGCs from apoptotic death following optic nerve crush (Homma et al., 2007) and axotomy (Kermer et al., 2000). IGF-1 also protects RGCs from elevated intraocular pressure-induced death in a mouse model of glaucoma (Ma et al., 2015). These protective effects extend to photoreceptors, as intravitreal IGF-1 injection also prevents retinal degeneration in the rd10 mouse model of retinitis pigmentosa (Arroba et al., 2011). IGF-1 deficiency is also associated with prematurity and insulin-deficient diabetes, both of which cause retinal neurovascular degenerations (Bereket et al., 1996; Lofqvist et al., 2006). Indeed, systemic IGF-1 treatment reduces retinal neurodegeneration in diabetic rats

without affecting the metabolic effects of diabetes (Seigel et al., 2006) and IGF-1 therapy is currently under evaluation as a treatment for retinopathy of prematurity (ClinicalTrials.gov Identifier: NCT01096784).

The neurotrophic effects of IGF-1 have been largely attributed to activation of the PI3K/Akt pathway (Dudek et al., 1997; Leininger et al., 2004; Miller et al., 1997; Ryu et al., 1999; Zheng and Quirion, 2004; Zhong et al., 2004). The protective effects of Akt are in large part due to inhibition of nuclear localization of Forkhead box O (FoxO) transcription factors (FoxO1 and FoxO3a), thus preventing expression of pro-apoptotic FoxO target genes (Zhang et al., 2011). Principal among these pro-apoptotic genes is Bcl-2 like 11 (Bcl2l11) encoding the BH3-only protein Bim. Bim acts upstream of Bax/Bak-mediated mitochondrial cytochrome-c release and apoptosome formation (Putchu et al., 2001). Bim and other BH3-only proteins stimulate apoptosis by sequestering the pro-survival Bcl2-family proteins (Bcl-2, Bcl-XL, Mcl-1), thus allowing Bax/Bak oligomerization and mitochondrial outer membrane pore formation (Willis et al., 2007). Several studies have suggested that Bim proteins can also directly bind and activate Bax and Bak (reviewed in (Sionov et al., 2015)). Trophic factor withdrawal activates Bim transcription through two FoxO binding DNA elements in the Bim promoter (Gilley et al., 2003; Gilley and Ham, 2005). Thus, trophic factor deprivation of neurons causes inactivation of PI3K/Akt signaling leading to FoxO activation, Bim expression and intrinsic apoptotic death (Gilley et al., 2003).

Cells express Bim as three major splice variants referred to as Bim short (Bim_S), long (Bim_L) and extra-long (Bim_{EL}) (Sionov et al., 2015). While activation of the PI3K/Akt pathway suppresses Bim gene transcription, activation of MAPK/ERK pathway suppresses the pro-apoptotic functions of Bim_{EL} and promotes its degradation. Bim_S exhibits a relatively unregulated pro-apoptotic character and is not normally expressed. The two longer forms are more ubiquitous, but contain dynein light chain binding domains that sequester them to the microtubule-associated dynein motor complex, thus inhibiting their apoptotic function in the absence of pro-apoptotic stimuli (Puthalakath et al., 1999). Bim_{EL} also contains a unique region (amino acids 42-97 in mouse and 42-101 in human) that is missing in Bim_S and Bim_L and provides regulation of Bim_{EL} by the MAPK/ERK pathway (Hubner et al., 2008; Ley et al., 2005). Phosphorylation of serine residues within this region (in particular Ser65 of mouse and Ser69 of human (Luciano et al., 2003)) by Erk1/2 promotes phosphorylation of neighboring residues by ribosomal S6 kinases (Rsk1/2), binding to beta-transducin repeat containing E3 ubiquitin protein ligase (β TrCP) F-box protein, ubiquitination and degradation by the 26S proteasome (Dehan et al., 2009). In addition, phosphorylation of Bim_{EL} at Ser65 promotes its dissociation from Bcl-XL and Mcl-1, thus allowing their binding and inhibition of pro-apoptotic Bax and Bak (Ewings et al., 2007). It has also been reported that phosphorylation of Bim_{EL} caused it to dissociate from Bax (Harada et al., 2004).

Although the MAPK/ERK pathway can affect apoptosis through modulation of Bim protein function and expression, a contribution of this mechanism in IGF-1-mediated rescue of neurons from trophic factor withdrawal has not been demonstrated. Therefore, we hypothesized that the ability of IGF-1 to protect retinal neurons from trophic factor withdrawal-induced apoptosis was largely dependent on the PI3K/Akt/FoxO pathway rather

than the MAPK/ERK/Bim_{EL} pathway. To test this, we employed the R28 rat retinal neuronal precursor cell line (Seigel, 2014). R28 cells can be induced toward neuronal differentiation by plating on laminin and treating with a cell-permeable cAMP analogue (Barber et al., 2001). Several previous studies have utilized this cell line to examine the induction of cell death following trophic factor withdrawal, as modeled by SD, as well the rescue from apoptosis by IGF-1 (Barber et al., 2001; Seigel et al., 2000; Wu et al., 2004). In contrast to expectations, we found that although IGF-1 caused a pronounced activation of the PI3K/Akt/FoxO pathway, pharmacological inhibition of Akt activation, prevention of Akt activation by Rictor knockdown, and co-silencing of FoxO1 and FoxO3a expression had little effect on the rescue from SD-induced apoptosis. In contrast, knockdown of Bim expression protected cells from SD, while pharmacological inhibition of ERK1/2 activation prevented rescue by IGF-1. Thus, for R28 retinal neuronal cells, the MAPK/ERK/Bim_{EL} pathway is largely responsible for IGF-1's ability to provide neurotrophic support.

2. Materials and Methods

2.1. Reagents

Apo-ONE Homogeneous Caspase-3/7 Assay kit and CytoTox96 Non-Radioactive Cytotoxicity Assay kit were purchased from Promega (Madison, WI). Recombinant human IGF-1 was from R&D Systems (Minneapolis, MN). Laminin and 8-(4-Chlorophenylthio) adenosine 3',5'-cyclic monophosphate (pCPT-cAMP) were obtained from Sigma (St. Louis, MO). LY294002 and PD98059 were from EMD Millipore Chemicals (Billerica, MA). NE-PER Nuclear and Cytoplasmic Extraction Reagents were from Pierce (Rockford IL). The qRT-PCR master mix and TaqMan primer/probes were purchased from Applied Biosystems (Grand Island, NY). FoxO1, FoxO3 and Bim siRNA were from Dharmacon (Lafayette, CO). All antibodies except beta-actin were from Cell Signaling (Danvers, MA). Beta-actin antibody was from Sigma.

2.2. Cell lines and culture conditions

R28 retinal neuronal-like cells were kindly provided by Dr. Gail Seigel (Ross Eye Institute, SUNY, Buffalo, NY). A short tandem repeat (STR) marker profile of R28 genome was produced by IDEXX Bioresearch (Columbia, MO) and confirmed the Sprague-Dawley rat genotype of R28 cells (Supplemental data). Cell passages 47 to 60 were used for experiments. R28 cells and derived clones were maintained in DMEM with low glucose (Invitrogen, Grand Island, NY) supplemented with 5% FBS and 1% Penicillin-streptomycin and maintained in a 5% CO₂ containing humidified incubator at 37 °C. For serum deprivation (SD) experiments the cells were treated as previously described (Abcouwer et al., 2008). Prior to experiments, cells were maintained in differentiating conditions by plating on laminin-coated (1 µg/cm²) tissue culture plastic in culture media containing 1% FBS and 250 µM pCPT-cAMP and incubated for 18–22 h. For SD, media were removed, cells were washed with PBS, fed with media with or without 5% FBS, and treated with IGF-1 or other indicated reagents.

For siRNA knockdown of gene expression, R28 cells were seeded in differentiating conditions and incubated for 18–24h and then transfected with 25 nM (except where

otherwise indicated) small interfering RNA (siRNA) or non-targeted (NT) control siRNA developed by the supplier (Dharmacon) using siRNA siQuest transfection reagent (Mirus, Madison, WI) in antibiotic-free media. Media containing siRNA were removed after 24-hour transfection and the cells were treated as indicated in each experiment.

Clones of R28 cells overexpressing mouse FoxO1 protein were created by transfection of R28 cells with modified mouse FoxO1 expression plasmids or empty vector (EV) control plasmid and then selected with puromycin. For construction of the FoxO1 expression plasmid, pSELECT-HA-mFOXO1, the mouse FOXO1 ORF fused to hemagglutinin (HA)-tag was obtained from pCMV5-HA-FOXO1 (Addgene, catalogue #12142), originally constructed by Nakae and coworkers (Nakae et al., 2001) and cloned into pSELECT-puro (Invivogen #psetp-mcs). The mouse FoxO1 ORF was amplified and SalI and NheI restriction enzyme sites were introduced by PCR using Phusion Hot-Start II polymerase enzyme (Thermo Fisher) with upstream primer 5'-AACGGTCGACCGCCATGTACCCATACGATGTTCCGGATTACGCTGCCGAAGC-3' and downstream primer 5'-ATCACGCTAGCGCATCTTTGGACTGCTCCTC-3'. The resulting PCR product was inserted into the NheI and SalI sites of pSELECT-puro, sequenced and compared to mouse FoxO1 (NCBI NM_019739.3). Sequence identity was confirmed except for a conservative A->G mutation at base 474, a non-conservative A->G mutation at base 1121 and a non-conservative T->C mutation at base 2321 of NM_019739.3. Surprisingly, these mutations were confirmed to exist in the original pCMV-HA-FOXO1 plasmid obtained from Addgene. The non-conservative mutations result in a K->R substitution at amino acid 219 and a L->P substitution at amino acid 619 of mFoxO1 (NCBI NP_062713.2). The mutations at bases 1121 and 2321 were reversed to wild type sequence by site-directed mutagenesis (Quick-Change Lightning kit, Agilent) with the following primers: For correcting the mutation at 1121, forward primer 5'-TCTGTCCCTTCACAGCAAGTTTATTCGAGTGCAGA-3' and reverse primer 5'-TCTGCACTCGAATAAACTTGCTGTGAAGGGACAGA-3' were used. For correcting the mutation at 2321 forward primer 5'-CATCATTCGGAATGACCTCATGGATGGAGATACCT-3' and reverse primer 5'-AGGTATCTCCATCCATGAGGTCATTCCGAATGATG-3' were used. The corrections were confirmed by sequencing. R28 cells were transfected with pSELECTmFOXO1 and pSELECT-puro using LipoFectamine 2000 (Thermo-Fisher) and colonies selected and maintained in 2 µg/mL puromycin-containing media.

2.3. Caspase-3/7 assay

Caspase-3/7 activity was measured by using Apo-ONE Homogeneous Caspase-3/7 Assay kit from Promega as described previously (Abcouwer et al., 2008). Briefly, the cells were plated under differentiating conditions in black-walled 96-well tissue culture plates with clear bottoms and relative caspase activities measured with a fluorescence plate reader, with excitation at 485nm and emission at 530nm. Relative caspase activities are reported as endpoint relative fluorescence density compared to a control group. Relative caspase activity was then normalized to protein content or to cell number. To obtain protein-normalized caspase activities, the cells were lysed after measuring fluorescence by repeated trituration in 50µl/well of RIPA buffer, followed by incubation for 30 minutes and centrifugation, and

cellular protein concentrations were measured with the BCA protein assay kit (Pierce, Rockford, IL).

2.4. Lactate dehydrogenase (LDH) release assay

LDH activities were measured by using CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to manufacturer's instructions with cells plated under differentiating conditions in 96-well tissue culture plates. After treatments, cell culture media were transferred to a new plate for LDH assay and adherent cells were lysed with provided lysis buffer in fresh media. Collected media and cell lysates were centrifuged and 50 µl of supernatants were used in LDH colorimetric detection reactions. Absorbances at 492 nm were measured after the addition of stop solution. Absorbance values of released LDH in the media were divided by combined absorbance values for total LDH activity in each well (LDH of the media plus LDH in the cell lysate) and reported as fractional extracellular LDH activity.

2.5. Nuclear and cytoplasmic fractionation

Nuclear protein and cytoplasmic protein were separated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) according to manufacturer's instructions. The concentrations of proteins in nuclear and cytoplasmic fractions were measured by BCA protein assay reagent (Pierce) and 10µg of each protein used for Western blot assay. Fractionation controls included a 14-3-3 (pan) antibody (Cell Signaling, catalogue #8312) detecting all known forms of mammalian 14-3-3 proteins and a histone H4 antibody (Cell Signaling, catalogue #2935).

2.6. Western Blotting

R28 cells were harvested and lysed in cold RIPA buffer (Millipore) with protease and phosphatase inhibitor cocktail (Thermo-Fisher). Proteins were extracted by incubating samples on ice for 1 hour with occasional trituration, followed by 20 min centrifugation at 14,000 xg. Protein concentrations were determined by BCA protein assay (Pierce) and equal amounts (20 to 50 µg) of total proteins from each sample were electrophoretically separated on NuPAGE 4–20% acrylamide gradient gels (Invitrogen). Proteins were then transferred onto nitrocellulose membrane. Blots were probed with primary antibodies overnight at 4°C. HRP-conjugated secondary antibodies and SuperSignal Pico or Femto chemoluminescent substrates (Thermo-Fisher) were used to visualize the immuno-detected bands. Densitometry was carried out using a FluorChem imager and AlphaView SA software (ProteinSimple, San Jose, CA).

2.7. Immunofluorescence and confocal microscopy

For confocal microscopy R28 cells were seeded under differentiating conditions on laminin-coated glass coverslips prior to various treatments. After treatments, cells were then fixed with 4% paraformaldehyde (PFA) for 10 minutes, washed with PBS 3 times, permeabilized with PBS containing 0.2% Triton X-100 and blocked in 5% normal goat serum in PBS with 0.05% triton X-100. The cells were incubated overnight at 4°C with FoxO1 antibody (Cell Signaling, catalogue #2880) diluted 1:100 in blocking solution, rinsed 3X in PBS with

0.05% Triton X-100, incubated in the dark for 1 hour with AlexaFluor594-conjugated secondary antibody (Invitrogen) diluted 1:1000 in blocking solution and then washed 3X in PBS with 0.05% triton X-100. The coverslips were mounted with Prolong Gold mounting media containing DAPI (Invitrogen) and images acquired using FluoView 500 confocal microscope (Olympus). Single channel images for each wavelength were acquired.

2.8. Quantitative Real-time PCR

Total RNA was extracted from R28 cells with RNeasy Plus mini kit (Qiagen) according to the manufacturer's protocol. Total RNAs were reverse transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad). Duplex qRT-PCRs were carried out using 1 μ l of RT reaction, Taqman universal PCR master mix (Applied Biosystems), gene-specific primers with FAM-labeled probes (Applied Biosystems) as listed in Table S1, along with β -actin primers with VIC-labeled probe (primer limited formulation, Applied Biosystems). Reactions were performed and monitored using a CFX384 real time PCR system (Bio-Rad). Normalized relative gene expressions were calculated using the Ct method.

2.9. Statistical analysis

Experiments presented in this study are representative of three or more repetitions. Data are shown as the mean value $\pm$ Standard deviation. A two-tailed Student's t-test was used for comparisons between two groups. One-way ANOVA was used for comparisons of three or more groups. Values of $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Protection of R28 cells from SD-induced death by IGF-1

SD induced a progressive, time dependent apoptotic death of R28 cells shown by increasing caspase-3/7 activity (Fig. 1A) and LDH release (Fig. 1B). Cleavage of caspase-9 and caspase-3 could be observed within 4 h of SD (Fig. 1C). We screened several trophic factors, including brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF), IGF-2, insulin, and vascular endothelial growth factor (VEGF) for the ability to inhibit SD-induced caspase-3/7 activation (Fig. 1D). Each of these neurotrophic factors was tested at a concentration often found in the literature. IGF-I (50 ng/ml) and bFGF (50 ng/ml) exhibited the greatest protection. IGF-1 inhibited the activation of caspase-3/7 induced by 4 h of SD in a dose-dependent fashion from 25 ng/ml to 100 ng/ml (Fig. 1E) and at 10 ng/ml abrogated the release of LDH after 18 h of SD (Fig. 1F).

3.2. Activation of PI3k/Akt and MEK/ERK pathways by IGF-1

To examine the mechanisms by which IGF-1 protected R28 cells from SD-induced death, the phosphorylation of signaling molecules in PI3K/Akt/FoxO and MAPK/ERK pathways was followed over 80 min (2A and B) and 24 h (2C) time courses of IGF-I treatment (100 ng/mL). IGF-1 induced rapid (10 min) and sustained (24 h) phosphorylation of Akt at the two key activation sites: T308 residue in the kinase domain and S473 residue in the C-terminal domain (Fig. 2A and Fig. 2C). This coincided with dramatic phosphorylation of the 40 kDa proline-rich Akt substrate (PRAS40) at residue T246 (Fig. 2B) and of the Akt substrates FoxO1/3a at T32/T24 (Fig. 2B and Fig 2C). An increase in phosphorylation of

glycogen synthase kinase-3 alpha and beta (GSK3 α/β) at the Akt substrate residues S21/9 was only evident for the lower band corresponding to GSK3 β S21 (Fig. 2A). Treatment of R28 cells with IGF-1 also caused a rapid and sustained phosphorylation of ERK1/2 at T202/Y204 residues that was maximal at approximately 15 min and still apparent at 8 h (Fig. 2A and Fig. 2C). ERK1/2 activation was further evidenced by increased phosphorylation of cAMP response element binding protein (CREB) at S133 (Fig. 2A). Phosphorylation of Bim_{EL} on S65 also peaked at approximately 15 min and diminished by 8 h after IGF-1 treatment (Fig. 2B and Fig. 2C). Thus, IGF-1 activated both PI3K/Akt/FoxO and MAPK/ERK/Bim_{EL} signal transduction pathways, with the former demonstrating relatively greater persistence.

3.3. IGF-1 prevented SD-induced FoxO1 and FoxO3 nuclear translocation and induction of Bim mRNA expression

Phosphorylation of FoxO1 and FoxO3 proteins by Akt promotes their binding to 14-3-3 proteins in the cytoplasm, thus preventing nuclear translocation and transcriptional activation of FoxO target genes (Tzivion et al., 2011). To quantify FoxO nuclear localization in response to IGF-1 treatment, R28 cells were serum-deprived for 4 h and then treated with or without 100 ng/mL IGF-1 for 90 min, while in this instance 10% FBS was added to control cells. Immunofluorescence staining showed that FoxO1 was largely localized to the cytoplasm of R28 cells treated with FBS. Serum deprivation induced an obvious FoxO1 nuclear translocation that IGF-1 treatment reversed (Fig. 3A). Nuclear and cytoplasmic protein fractionation confirmed these results, showing that SD increased the nuclear/cytoplasmic ratio of FoxO1 protein and that this was abrogated by IGF-1 (Fig. 3B and Fig. 3C). Similar results were obtained for FoxO3 (Fig. 3B). Because Akt has been reported to translocate into the nucleus in response to growth factor treatment of neuronal cells (Ahn, 2014), fractionation of Akt protein was also examined. Akt was overwhelmingly cytoplasmic, but detectable in the nuclear fractions. While the cytoplasmic versus nuclear localization of Akt did not change with SD or IGF-1 treatment, T308 and S473 phosphorylation of nuclear Akt could only be observed in the IGF-1 treated samples.

The long-term (18 h) effects of SD, 10% FBS and IGF-1 treatment on expression of Bim mRNAs was also examined. SD caused a significant 2-fold increase in Bim mRNA, relative to FBS-treated cells, which IGF-1 treatment abrogated (Fig. 3D). These results suggest that FoxO1 and FoxO3 nuclear localization stimulates Bim transcription, consistent with the literature (Sionov et al., 2015).

3.4. FoxO1 overexpression promoted apoptosis without increasing sensitivity to SD

Because SD inactivates Akt and Akt activity determines the proportions of FoxO1 protein in the cytoplasm and nucleus, we hypothesized that over-expression of FoxO1 protein would increase the sensitivity of R28 cells to SD and/or increase the apoptotic response. Four clonal populations of R28 cells overexpressing FoxO1 were generated by transfection with a plasmid containing FoxO1 full length ORF fused with HA-tag. Four control clones containing empty vector plasmid (EV) were obtained as controls. [It should be noted that a widely used FoxO1 cDNA clone was found to contain two non-conservative mutations compared the mouse FoxO1 mRNA reference sequence (see Materials and Methods)].

Western blotting confirmed HA tag expression and overexpression of FoxO1 protein (Fig. 4A). Expression of Bim proteins was slightly, but not significantly increased in FoxO1 clones, while pan 14-3-3 protein levels were not altered. Immunofluorescence staining indicated that total FoxO1 was localized to the cytoplasm in cells cultured with 10% FBS, while SD (0.1% FBS) induced FoxO1 nuclear translocation (Fig. 4B). Note that complete serum deprivation (0% FBS) was not used when serum depriving these clones because of an exaggerated death response of the FoxO1 over-expressers. Surprisingly, clones overexpressing FoxO1 exhibited 4- to 6-fold elevated caspase-3/7 activity in the presence of 10% FBS, while SD increased caspase-3/7 activity in a manner proportional to that in EV clones (Fig. 4C and 4E). Overexpression of FoxO1 increased LDH release by 50–70% under all FBS conditions (Fig. 4D). Comparison of FBS dose-response curves of caspase-3/7 activity demonstrated an exaggerated response to SD, with a 2.6-fold greater maximal effect in FoxO1 over-expressing clones (Fig. 4E left panel). However, serum-response curves showed an identical response when the data sets were each normalized to their respective mean basal caspase-3/7 activities at 10% FBS (Fig. 4E right panel). Thus, FoxO1 determined the magnitude of the R28 cell apoptotic response to SD without increasing the cells sensitivity to SD.

3.5. IGF-1 rescued R28 cells from SD-induced apoptosis in a PI3K/Akt-independent and MEK/ERK-dependent manner

In order to examine the contributions of PI3K/Akt and MEK/ERK pathways to the protective effects of IGF-1, the effects of pharmacologic inhibition of PI3K and MEK were compared. Unexpectedly, pretreatment with the PI3K inhibitor LY294002 at 10 μ M and 25 μ M did not prevent the ability of IGF-1 to block the increase of caspase-3/7 activity in response to SD (Fig. 5A), even though these concentrations of LY294002 effectively inhibited phosphorylation of Akt, FoxO1 and FoxO3 in response to IGF-1 (Fig. 5B). LY294002 pretreatment also failed to affect the ability of IGF-1 to block pro-caspase-3 cleavage (Fig. 5B). In contrast, pretreatment with the MEK1/2 inhibitor PD98059 at 25 μ M significantly blocked the protective effects of IGF-1 on caspase-3/7 activity (Fig. 5A) and pro-caspase-3 cleavage (Fig. 5B). This concentration of PD98059 effectively prevented ERK1/2 activating phosphorylation and Bim_{EL}-S65 phosphorylation in response to IGF-1 (Fig. 5B). These results suggest that IGF-1 protected R28 cells from SD-induced apoptosis in a manner dependent on MEK/ERK activation but not dependent on PI3K/Akt activation.

The rapamycin-insensitive companion of mechanistic target of rapamycin (Rictor) is a key component of the mechanistic target of rapamycin complex 2 (mTORC2) that regulates Akt stability and activity. MTORC2 mediates the co-translational phosphorylation of T450 residue in the turn motif of Akt as well as phosphorylation of the S473 residue, respectively inducing Akt stability and activity (Foster and Fingar, 2010; Oh et al., 2010). To test the effects of blocking Akt activation by a genetic means, the expression of Rictor was effectively silenced in R28 cells by transfection with Rictor siRNA (Fig. 5C–E). Transfection with a non-targeted siRNA served as control. Diminishing Rictor expression by approximately 75% resulted in significant inhibition of Akt phosphorylation at T450, S473 and T308 following treatment with 100 ng/ml IGF-1 (Fig. 5D and F) consistent with S473 as a direct Rictor target and the subsequent phosphorylation of T308 by 3-phosphoinositide-

dependent protein kinase 1 (Sarbasov et al., 2005). Rictor knockdown did not increase either basal or SD levels of caspase-3/7 activation. Most importantly, consistent with the results obtained with LY294002, knockdown of Rictor expression and resultant inhibition of Akt activation did not alter the protective effects of IGF-1 on caspase-3/7 activation in response to SD (Fig. 5G).

3.6. SD-induced apoptosis of R28 cells is mediated by Bim but not FoxO1/3 proteins

The previous results suggested that in this model IGF-1 protects from SD-induced apoptosis by activating MAPK/ERK rather than PI3K/Akt, and that modifications of Bim_{EL}, rather than FoxO1/3 proteins, are key to the protective effects of IGF-1. If so, then silencing Bim expression should recapitulate the protective effects of IGF-1 treatment on serum-deprived R28 cells, while silencing FoxO1 should have little effect. To test this hypothesis, the effects of silencing FoxO1 and Bim expression on SD-induced apoptosis of R28 cells were compared. FoxO1 and Bim expression were effectively inhibited using pools of targeted siRNAs (Fig. 6A). Silencing of Bim expression nearly abrogated caspase-3/7 activation, whereas silencing of FoxO1 expression had no effect (Fig. 6B). Similarly, transfection with control non-targeted siRNA had no effect. To validate these results and avoid potential off-target effects of siRNA, we transfected R28 cells with the 4 individual members from each pool of siRNAs targeting FoxO1 and Bim. For FoxO1 all of the individual siRNAs caused highly effective silencing of expression (Fig. 6C), while for Bim, 3 out of 4 siRNAs were highly effective (Fig. 6D). None of the individual or pooled siRNAs targeting FoxO1 had a significant effect on caspase-3/7 activation and LDH release during SD (Fig. 6E and Fig. 6F). In contrast, all of the siRNAs targeting Bim effectively inhibited activation of caspase-3/7, while only the least effective siRNA (Bim-8) failed to significantly inhibit LDH release (Fig. 6E and Fig. 6F). These results demonstrate that Bim is necessary for SD-induced apoptosis of R28 cells and suggest that IGF-1 treatment protects R28 cells from SD induced apoptosis by promoting MAPK/ERK activation and phosphorylation of Bim_{EL}.

It is possible that silencing of FoxO1 expression had no effect because the negative effects of FoxO3 alone are sufficient to mediate SD-induced apoptosis of R28 cells. To test this hypothesis the effects of co-silencing the expression of these two proteins were examined. Transfection of individual and combined siRNAs targeting FoxO1 and FoxO3 produced the desired effects on expression of mRNA (Fig. 7A) and protein (Fig. 7B and Fig. 7D). The effects of silencing FoxO1 and FoxO3 expression on caspase-3/7 activation were examined following either 4 h (Fig. 7C) or 24 h (Fig. 7E) of SD. Individual and combined knockdown of Foxo1/3 expression had no significant effects of caspase-3/7 activation after 4 h of SD (Fig. 7C). However, combined knockdown, but not individual knockdown, of FoxO1/3 expression had a small but significant inhibitory effect on caspase-3/7 activation after 24 h SD (Fig. 7E). These results, together with the results of FoxO1 over-expression (Fig. 4), suggest that FoxO1/3 proteins can play a role in SD-induced apoptosis of R28 cells; however, less considerable than that served by Bim_{EL}.

4. Discussion

This study shows, for the first time, the specific role of MAPK/ERK dependent Bim signaling on retinal neuronal survival mediated by IGF-1 after trophic factor deprivation. IGF-1 acts as an essential neurotrophic factor during development and following neuronal injury (Guan et al., 2003; Joseph D'Ercole and Ye, 2008; Madathil and Saatman, 2015; O'Kusky and Ye, 2012; Russo et al., 2005). Several studies have demonstrated that IGF-1 protects neuronal cells from trophic factor-induced death modeled with SD (Lu et al., 2008; Yamada et al., 2001; Zheng et al., 2002). However, few studies have examined the contributions of PI3K/Akt and MAPK/ERK pathways to the neuroprotective effects of IGF-1, and most attribute these effects exclusively to PI3K/Akt signaling (Dudek et al., 1997; Zheng et al., 2000). For example, using cultures of rat hippocampal neurons (HN) and PC-12 cells, Zheng, Kar and Quirion (Zheng et al., 2002) demonstrated that IGF-1 induced FoxO3 phosphorylation in an Akt-dependent fashion and prevented FoxO3 nuclear translocation and cell death following neurotrophin withdrawal. A previous study demonstrated that IGF-1 protected R28 cells from SD-induced death, but did not examine mechanism (Seigel et al., 2000). A previous study from Barber and colleagues demonstrated that R28 cells were rescued from SD-induced apoptotic death by both insulin and IGF-1 (Barber et al., 2001). In that study both LY294002 and wortmannin prevented IGF-1's ability to prevent the appearance of pyknotic nuclei after 24 h of SD. These studies did not include treatment with inhibitors alone, and we speculate that the results were due to long-term effects of PI3K inhibition. The present study suggests that, at least in the short-term, protection of R28 retinal neuronal cells from SD-induced death is dependent on MAPK/ERK activation and mediated through phosphorylation of Bim_{EL}. An obvious major limitation of this study is reliance on the *in vitro* R28 cell system. The results should guide future *in vivo* studies to determine the relative importance of MAPK/ERK-dependent Bim signaling on neuronal survival in the retina and other neural tissues.

There are few examples of the protective effects of IGF-1 acting in a manner independent of PI3K/Akt: Using a SD model Yamada and co-workers found that inhibition of the PI3K/Akt pathway with LY294002 did not prevent neuroprotection of cortical neurons by IGF-1 (Yamada et al., 2001). Protection of mouse cortical neurons from ceramide-induced apoptosis by IGF-1 was completely blocked by inhibition of MEK with U0126 (Willaime-Morawek et al., 2005). Protection of INS-1 pancreatic beta cells from dexamethasone-induced death by IGF-1 was inhibited by the MEK inhibitor PD98059 but not by PI3K inhibition with LY94002 (Avram et al., 2008). Other studies have shown that PI3K/Akt activation was not totally responsible for the protective effects of IGF-1: LY94002 and U0126 each only partially blocked the ability of IGF-1 to rescue rat RGC from hypoxia-induced apoptosis (Yang et al., 2013). Both PI3K-Akt and MAPK-ERK pathways were necessary for IGF-1's ability to rescue rat motor neurons from glutamate-induced apoptosis, as combining LY94002 and PD98059 was necessary to block IGF-1's protective effect (Vincent et al., 2004). Likewise, protection of rat dorsal root ganglion cells from glutamate-induced death by IGF-1 was not inhibited by PD98059 or LY94002, but was inhibited by a combination of the two (Liu et al., 2012).

Another important concept is that PI3K/Akt/FoxO1/3 pathway and the MAPK/ERK/Bim_{EL} pathways are complementary, with the latter mediating rapid neuroprotection and the former promoting long-term neuroprotection. A potential explanation of IGF-1's distinct neurotrophic capabilities is its ability to activate both of these pathways. IGF-1-mediated activation of PI3K/Akt and MAPK/ERK signaling can each affect Bim_{EL} expression and function; PI3K/Akt acts by preventing induction of Bim transcription by FoxO proteins while MAPK/ERK acts by phosphorylating Bim_{EL} on Ser65 by blocking its pro-apoptotic function and promoting its degradation (Sionov et al., 2015). Thus, IGF-1 can have rapid protective effects while hindering Bim gene transcription to promote long-term survival.

An important caveat of this study is that the influences of insulin-like growth factor binding proteins (IGFBPs) on the response to IGF-1 were not examined. IGFBPs bind IGF-1 with high affinity and can inhibit or enhance its action (Bach, 2015). IGFBPs also play important roles in retinal pathophysiology (Nguyen et al., 2013). Subjecting the cells to serum-starved conditions necessarily removed IGFBPs that are present in FBS (Grigoriev et al., 1994), thus removing their negative and positive influences on IGF-1 action. In addition, we did not determine possible IGFBP secretion of R28 cells, which may have influenced the concentration of unbound IGF-1 and IGF-1 action.

Given IGF-1's role in protection of neurons from stress-induced death, this factor might be used for retinal neuroprotection. For example, diabetes causes chronic retinal neurodegeneration with loss of RGC and decreased Akt activity, which precedes the vascular pathologies that characterize clinical diabetic retinopathy (Abcouwer and Gardner, 2014). Bim protein levels are also elevated in retinas from diabetic patients (Valverde et al., 2013). One might speculate that chronically lower levels of Akt activity increase the likelihood of neuronal cell death by increasing Bim expression and amplifying the intrinsic apoptotic pathway response during stress. Indeed, systemic IGF-1 treatment reduces retinal neurodegeneration in diabetic rats without affecting the metabolic effects of diabetes (Seigel et al., 2006). Taken together, the results of this study emphasize the complex signal transduction pathways that underlie neuronal survival in response to stress and provide new insights into potential avenues for neuroprotective strategies in conditions such as glaucoma and diabetic retinopathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used

Akt, protein kinase B, PKB

Ak mouse thymoma kinase

bFGF

basic fibroblast growth factor

BCA

bicinchoninic acid

Bcl-2

B-cell lymphoma 2

Bcl2l11

Bcl-2 like 11

Bcl-XL

Bcl-extra large

Bim_S

Bim short

Bim_L

Bim long

Bim_{EL}

Bim extra-long

BDNF

brain-derived neurotrophic factor

cAMP

cyclic AMP

CREB

cAMP response element binding protein

cDNA

complementary DNA

pCPT-cAMP

8-4-Chlorophenylthio adenosine 3',5'-cyclic monophosphate

EV

empty vector

ERK

extracellular signal-regulated kinase

FBS

fetal bovine serum

FoxO

Forkhead box O

GPCR

G-protein coupled receptor

GSK3 α/β

glycogen synthase kinase-3 alpha and beta

HA

hemagglutinin

IGF-1

insulin-like growth factor 1

IGF1R

IGF type 1 receptor

IRS

insulin receptor substrates

LHD

lactate dehydrogenase

MAPK

mitogen-activated protein kinase

Mcl-1

myeloid cell leukemia 1

mTORC2

mechanistic target of rapamycin complex 2

NT

non-targeted

PFA

paraformaldehyde

PBS

phosphate buffered saline

PCR

polymerase chain reaction

PI3K

phosphoinositide 3-kinase

PRAS40

40 kDa proline-rich Akt substrate

qRT-PCR

quantitative reverse-transcriptase polymerase chain reaction

RIPA

radio-immunoprecipitation assay

Rictor

rapamycin-insensitive companion of mechanistic target of rapamycin

RTK

receptor tyrosine kinase

RGC

retinal ganglion cell

Rsk1/2

ribosomal S6 kinase 1 and 2

RT

reverse transcriptase

SD

serum deprivation

STR

short tandem repeat

siRNA

small interfering RNA

VEGF

vascular endothelial growth factor

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Research highlights

- IGF-1 protected R28 retinal neuron cells from serum-deprivation induced death
- IGF-1 activated both PI3K/Akt/FoxO and MAPK/ERK/Bim_{EL} pathways
- Inhibition of ERK1/2 activation, but not Akt, prevented rescue by IGF-1
- Silencing Bim expression protected cells from serum deprivation-induced apoptosis
- Co-silencing of FoxO1 and FoxO3a expression did not block apoptosis

Figure 1. IGF-1 protected R28 retinal neuronal cells from SD-induced apoptotic death

(A) Differentiated R28 cells were incubated in serum free media (SD) for indicated times and caspase-3/7 activity measured and compared to control cells maintained with 5% FBS (n=3). (B) At time zero differentiated R28 cells were switched to serum free media and LDH activity in media and cells was measured after the indicated times. The fraction of total LDH was calculated as described in Materials and Methods (n=3). (C) At time zero differentiated R28 cells were switched to serum free media and media containing 10% FBS, and cells were harvested and lysed at the indicated times. Cleavage of pro-caspase-9 and pro-caspase-3 was examined by western blotting. (D) R28 cells were treated with serum free media containing the indicated concentrations of growth factors for 4 h, and then assayed for caspase-3/7 activity. Caspase activity was normalized to that in control cells treated with 5% FBS (n=3). (E and F) R28 cells were treated with serum free media containing the indicated concentrations of IGF-1. (E) After 4 h caspase-3/7 activity was measured and normalized to control cells with 5% FBS (n=3). (F) After 18 h LDH activity in media and in cells was measured (n=4–10). The fractions of total LDH in media were calculated and normalized to control samples with 5% FBS. (Significance between SD and 5% FBS or IGF-1 treatment groups were calculated by one-way ANOVA: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001).

Figure 2. IGF-1 activated PI3K/Akt and MAPK/ERK pathways in R28 cells

R28 cells were differentiated in 1% FBS medium overnight and then incubated in serum free media with and without IGF-1, lysates obtained at the indicated times and western blotting performed. **(A)** IGF-1 induced phosphorylation of Akt and ERK, as well as the Akt substrate GSK3 and the ERK1/2 pathway substrate CREB. **(B)** IGF-1 induced phosphorylation of the Akt substrates PRAS40, FoxO1 and FoxO3, as well as the ERK1/2 substrate Bim. **(C)** Comparison of long-term Akt and ERK1/2 activation in response to IGF-1 treatment.

Figure 3. IGF-1 prevented FoxO protein translocation and upregulation of Bim mRNA expression in response to SD

Differentiated R28 cells were deprived of serum for 4 hours and treated with 10%FBS or 100ng/ml IGF-1 for 90 min. **(A)** Effect of SD and IGF-1 on FoxO1 cellular localization. Cells were fixed and FoxO1 cellular localization was detected by immunofluorescent staining. Cells were counterstained with DAPI to highlight nuclei. **(B)** Effect of SD and IGF-1 on FoxO1 and FoxO3 nuclear localization. Cells were lysed, nuclei were separated from cytoplasm and fractionated proteins examined by western blotting. An antibody to all mammalian forms of 14-3-3 proteins was used as a cytoplasmic protein control, and an antibody to histone H4 was used as a nuclear protein control. **(C)** Quantification of FoxO1 nuclear/cytoplasmic ratios obtained by western blotting (n=2). **(D)** Quantification of mRNA expression for differentiated R28 cells were treated with 10% FBS and SD with or without 100ng/ml IGF-1 for 18 hours. Cells were harvested, RNA was extracted and qRT-PCR used to quantify relative Bim mRNA levels (n=5). Note that the Bim primer-probe used does not differentiate between Bim splice forms. (**** p<0.0001 tested by one-way ANOVA).

Figure 4. Overexpression of FoxO1 increased caspase activation in response to SD without increasing the sensitivity to SD

Clonal populations of R28 cells overexpressing FoxO1-HA-tag fusion protein and harboring an empty vector (EV) plasmid were obtained by transfection and antibiotic selection. **(A)** Western blotting demonstrating expression of HA-tag and greatly increased FoxO1 protein. Numbers indicate clone designations. Note the slightly decreased mobility of the FoxO1-HA fusion protein compared to native FoxO1. Bim protein is slightly increased in FoxO over-expressers, whereas levels of 14-3-3 proteins are unchanged. **(B)** Immunofluorescent staining showing FoxO1 nuclear translocation in response to SD (0.1% FBS) for two FoxO1-HA clones. Note that the white areas are due to saturation of the photo-detector. **(C)** Effect of SD for 4 h on caspase-3/7 activities for parental R28 cells (WT), 2 FoxO1-HA clones and 2 EV control clones. Note that FoxO1 overexpression increased caspase-3/7 activity under SD condition and serum-fed conditions (n=6). **(D)** Effect of incubation for 18 h with the indicated concentrations of FBS on mean extracellular LDH activity for 2 FoxO1-HA clones and EV control clones (n=4). **(E)** Caspase-3/7 activity was measured in 4 FoxO1 clones and 4 EV control clones incubated in various percentages of FBS for 4 h. In the left panel all caspase activities are normalized to the mean of EV control clones in 10% FBS. In the right panel caspase activities for each group of clones were normalized to the mean values for that group in 10% FBS, so that graphed values represent the increases relative to 10% FBS. (* p<0.05, and **** p<0.0001 by Student's T-test).

Figure 5. IGF-1 protected R28 cells from SD-induced apoptosis in an ERK-dependent and PI3K-independent manner

(A) Differentiated R28 cells were treated with the indicated concentrations of the PI3K inhibitor LY294002 and the MEK inhibitor PD98059 prior to (1 h) and during SD with 100 ng/mL IGF-1 for 4 hours, followed by evaluation of caspase-3/7 activity. Caspase activity was normalized to that in cells treated with 5% FBS ($n=5$). (B) R28 cells were treated as in (A), followed by western blotting to evaluate phosphorylation of Akt, FoxO1, FoxO3, ERK and Bim, as well as cleavage of pro-caspase-3. (C) R28 cells were transfected with the indicated concentrations of siRNA pool targeting Rictor or a non-targeted (NT) control siRNA for the indicated times, followed by RNA isolation and evaluation of relative Rictor mRNA levels by qRT-PCR ($n=2$). (D) R28 cells were transfected with 25 nM Rictor or NT siRNA and then subjected to differentiating conditions. At 24 h after transfection, cells were subjected to SD for 4 h and then treated with or without 100ng/ml of IGF-1 for 20 minutes, and western blotting used to determine the effect of Rictor knockdown on Akt activating phosphorylations. (E and F) Quantification of relative Rictor and phospho-Akt (p-Akt) protein levels by western blotting (as shown in (D)). All protein amounts were normalized to beta-actin and expressed as relative to NT without IGF-1 treatment group ($n=6$). (G) R28 cells were transfected with 25 nM Rictor or NT siRNA and then subjected to differentiating conditions. At 24 h after transfection, cells were subjected to SD with or without 100ng/ml of IGF-I for 4 h, when caspase-3/7 activity was measured. Mean caspase values were

normalized to control group treated with 5% FBS (n=4). (ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by one-way ANOVA or student's T-test).

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Figure 6. Bim, but not FoxO1, was necessary for SD-induced apoptosis of R28 cells

R28 cells were transfected with siRNAs targeting FoxO1, Bim or non-targeted (NT) siRNA and then subjected to differentiating conditions. At 24h after transfection, the cells subjected to SD for 4 h or 24 h. (A) The effect of siRNA pools on FoxO1 and Bim protein expression was evaluated by western blotting. (B) Effect of siRNA pools targeting FoxO1 and Bim on caspase-3/7 activity induced by 4 h of SD (n=4). (C) The effect of individual siRNAs targeting FoxO1 on mRNA expression at 24 h after transfection was evaluated by qRT-PCR (n=3). (D) The effect of individual siRNAs targeting Bim on mRNA expression at 24 h after transfection evaluated by qRT-PCR. Note that the siRNA designated Bim-8 was relatively ineffective (n=3). (E) Effects of individual and pooled siRNAs targeting FoxO1 and Bim on caspase-3/7 activation in response to 4 h of SD (n=4). (F) Effects of individual and pooled siRNAs targeting FoxO1 and Bim on LDH release in response to 24 h of SD. (ns = not significant, **** p<0.0001 by one-way ANOVA or student's T-test).

Figure 7. Co-silencing expression of FoxO1 and FoxO3 had a minimal effect on SD-induced caspase activation

R28 cells were transfected with siRNAs targeting FoxO1, FoxO3, a combination of FoxO1 and FoxO3 siRNAs, or non-targeted (NT) siRNA and then subjected to differentiating conditions. **(A)** The effects of siRNAs targeting FoxO1 and FoxO3 on mRNA expression of FoxO1 (left panel) and FoxO3 (right panel) at 24 h after transfection was evaluated by qRT-PCR (n=3). **(B–E)** At 24 h after transfection, the cells were subjected to SD for 4 h **(B–C)** or 24 h **(D–E)**. The effects of siRNAs on FoxO1 and FoxO3 protein expression after 4 h **(B)** and 24 h **(D)** of SD was evaluated by western blotting. The effects of FoxO1 and FoxO3 RNA silencing and SD on caspase-3/7 activation after 4 h **(C, n=4)** and 24 h **(E, n=4)** of SD was evaluated. Note the combined knockdown of FoxO1 and FoxO3 had a slight but significant effect on caspase activation, but only after 24 h of SD. (ns = not significant, * p<0.05, and **** p<0.0001 by one-way ANOVA or student's T-test).