p58IPK is an Endogenous Neuroprotectant for Retinal Ganglion Cells

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p58IPK is an endoplasmic reticulum (ER)-resident chaperone playing a critical role in facilitating protein folding and protein homeostasis. Previously, we have demonstrated that p58IPK is expressed broadly in retinal neurons including retinal ganglion cells (RGCs) and loss of p58IPK results in age-related RGC degeneration. In the present study, we investigate the role of p58IPK in neuroprotection by in vitro and in vivo studies using primary RGC culture and two well-established disease-relevant RGC injury models: retinal ischemia/reperfusion (I/R) and microbead-induced ocular hypertension. Our results demonstrate that in both in vivo models, p58IPK−/− mice exhibit significantly increased RGC loss compared to wild type (WT) mice. In vitro, p58IPK-deficient RGCs show reduced viability and are more susceptible to cell death induced by the ER stress inducer tunicamycin (TM). Overexpression of p58IPK by adeno-associated virus (AAV) significantly diminishes TM-induced cell death in both WT and p58IPK−/− RGCs. Interestingly, we find that loss of p58IPK leads to reduced mRNA expression, but not the protein level, of mesencephalic astrocyte-derived neurotrophic factor (MANF), a neurotrophic factor that resides in the ER. Treatment with recombinant MANF protein protects R28 retinal neural cells and mouse retinal explants from TM-induced cell death. Taken together, our study suggests that p58IPK functions as an endogenous neuroprotectant for RGCs. The mechanisms underlying p58IPK’s neuroprotective action and the potential interactions between p58IPK and MANF warrant future investigation.

Keywords: p58IPK, retinal ganglion cells, retinal ischemia/reperfusion, ocular hypertension, X-box binding protein 1

INTRODUCTION

Accumulating evidence suggests that disturbed protein homeostasis in the endoplasmic reticulum (ER), or ER stress, is a significant contributing factor to neurodegeneration in the central nervous system including the retina (Zhang et al., 2014). Prolonged ER stress activates pro-apoptotic genes such as C/EBP homologous protein (CHOP) resulting in apoptosis, while reducing ER stress alleviates cell death of retinal neurons, such as retinal ganglion cells (RGCs), induced by a variety of disease-provoking insults (Hu et al., 2012; Jing et al., 2012; Chen et al., 2014; Huang et al., 2015). In addition, ER stress reduces the production of neurotrophic factors but increases the secretion of pro-inflammatory cytokines, which further exacerbate neuronal death (Zhong et al., 2012; Kim et al., 2014; Cai et al., 2016). Thus, restoring ER protein homeostasis may provide a new strategy to prevent and treat retinal neurodegeneration.
The ER chaperone p58\textsuperscript{IPK} is an important component of the ER protein folding system and is upregulated by the highly conserved unfolded protein response (UPR) genes XBP1 and ATF6 (Lee et al., 2003; van Huizen et al., 2003). Activation of p58\textsuperscript{IPK} facilitates protein folding, reduces ER stress, and promotes cell survival (Rutkowski et al., 2007). In pancreatic β cells, deletion of p58\textsuperscript{IPK} results in CHOP-mediated apoptosis and reduced insulin production (Huber et al., 2013). In a previous study, we demonstrated that p58\textsuperscript{IPK} is present throughout the retina and mice lacking p58\textsuperscript{IPK} display increased susceptibility to retinal injury caused by glutamate toxicity and age-related cell death (Boriushkin et al., 2015). In R28 retinal neural cells, p58\textsuperscript{IPK} overexpression suppresses ER stress and improves cell survival under conditions of oxidative stress (Boriushkin et al., 2015). Furthermore, p58\textsuperscript{IPK} acts as a potent inhibitor of NLRP3 inflammasome and reduces IL-1β secretion in bone marrow macrophages (Boriushkin et al., 2016). These findings suggest that p58\textsuperscript{IPK} may function as a neuroprotectant and play an important role in maintaining the viability of retinal neurons such as RGCs in pathogenic conditions related to ischemic retinopathy and glaucoma.

In the present study, we tested this hypothesis in two commonly used RGC injury models induced by retinal ischemia/reperfusion (I/R) or ocular hypertension and explored the potential mechanism of p58\textsuperscript{IPK} neuroprotection and its interaction with mesencephalic astrocyte-derived neurotrophic factor (MANF), an ER stress responsive neurotrophic factor.

**MATERIALS AND METHODS**

**Animals**

The generation and maintenance of p58\textsuperscript{IPK} knockout mice were previously described elsewhere (Ladiges et al., 2005; Boriushkin et al., 2015). All experimental procedures were performed in compliance with protocols approved by the Institutional Animal Care and Use Committees at the State University of New York at Buffalo.

**Isolation and Culture of Mouse RGCs**

RGCs were isolated from retinas of postnatal day 6 neonatal mice using the Miltenyi Biotec magnetic cell sorting (MACS) system following a published protocol (Huang et al., 2003; Jiao et al., 2005). Immediately after isolation, cells were incubated with adeno-associated virus (AAV)-GFP or AAV-p58\textsuperscript{IPK} (Vector Biolabs, Malvern, PA, USA) at 10\textsuperscript{4}–12 GC/ml following standard procedures approved by the Institutional Biosafety Committee at the University at Buffalo. After 24 h, transduced cells were treated with 1 µg/ml tunicamycin (TM) or a vehicle control (0.05% DMSO) for an additional 16 h. Cell viability was examined using the live/dead cytotoxicity assay (Molecular Probes) following the manufacturer’s protocol. Images were analyzed for live cells (green) and dead cells (red), blind to treatment or genotype (see Supplementary Material).

**Retinal Ischemia/Reperfusion (I/R) Mouse Model**

The anterior chamber of anesthetized mice was canulated with a 30g needle attached to a reservoir of sterile PBS. Retinal ischemia was induced by elevating the reservoir to generate a hydrostatic pressure of 90 mmHg for 60 min. After 7 days, mice were sacrificed and retina whole-mounts were prepared for Tuji immunostaining to visualize RGCs (see Supplementary Material).

**Microbead-Induced Ocular Hypertension Mouse Model**

Microbead injection was performed as described previously (Chen et al., 2011). Briefly, the anterior chamber of anesthetized mice was canulated and 2 µl PBS containing 10 µm diameter polystyrene, fluorescent microspheres at 1.5 × 10\textsuperscript{7} beads/ml (Fluospheres, Invitrogen) was injected. Intraocular pressure (IOP) was recorded by bounce tonometer (Iconic). Two weeks after microbead injection mice were sacrificed and retina whole-mounts were prepared for Tuji immunostaining (see Supplementary Material).

**R28 Cell Treatment and Analysis**

R28 cells were cultured as described previously (Boriushkin et al., 2015). At approximately 60%–70% confluence, cells were pretreated with 50 ng/ml recombinant human MANF (hMANF; PeproTech) or vehicle for 16 h and then treated with 5 µg/ml TM or vehicle for 24 h. Cells were labeled using the live/dead cytotoxicity assay, photographed, and analyzed (see Supplementary Material).

**Immunohistochemistry and Morphological Analysis**

Mouse eye cryosections were prepared for immunohistochemistry as described previously (McLaughlin et al., 2018). The primary antibodies used were: Ribeye (Synaptic Systems 192003, 1:800); PKCa (Santa Cruz sc-8393, 1:400); Pax6 (DSHB Pax6-s, 1:25); and calretinin (Millipore Mab1658, 1:800). The secondary antibodies used were: Texas-red conjugated goat-anti-rabbit (ThermoFisher T6391, 1:800); Alexa594 conjugated goat-anti-mouse (ThermoFisher A11005, 1:800). Photomontages were assembled with Adobe Photoshop.

**Quantitative RT-PCR**

Total RNA was isolated with Trizol (Invitrogen) per manufacturer instructions. cDNA was made from 500 ng of total RNA using Bio-Rad cDNA kit followed by quantitative RT-PCR (qPCR) with Bio-Rad iQ Sybr Green Supermix. The following primers were used: MANF f1: 5’-CAC CAG CCA CTA TTG AAG AAG A-3’ with MANF r1: 5’-AGC ATC ATC TGT GTCCAA-3’; MANF f2: 5’-AAA GAG AAT CCG TTG TGC TAC T-3’ with MANF r2: 5’-CCA GGA TCT TCT TCA GCT CTT T-3’; 18sF 5’-GTA ACC GTG TGA ACC CCA TT-3’ with 18sR 5’-CCA TCC AAT CGG TAG TGAG-3’.
Statistical Analysis

The quantitative data were expressed as mean ± SD. Statistical analyses were performed using Student’s t-test for two-group comparisons and one-way ANOVA with Bonferroni post hoc test for three groups or more. Statistical differences were considered significant at a p value of less than 0.05.

RESULTS

p58\textsuperscript{IPK} Deficiency Exacerbates RGC Loss Induced by Retinal I/R

Retinal I/R is a widely used model for investigating the mechanisms and treatment of inner retinal neuron injury caused by acute ischemia. Seven days after I/R, RGC numbers were examined by immunostaining for Tuj1, a putative RGC marker, in retinal whole mounts. We found a 36.6% RGC loss in WT mice and a 52.0% RGC loss in p58\textsuperscript{IPK}−/− mice (Figure 1B). This indicates that I/R induced a significantly higher rate of RGC loss (a 42% increase) in p58\textsuperscript{IPK}−/− mice than in WT, suggesting a protective role of p58\textsuperscript{IPK} in RGCs.

Loss of p58\textsuperscript{IPK} Increases RGC Damage in Microbead-Induced Ocular Hypertension

We used a second model that is highly relevant to glaucoma to validate the role of p58\textsuperscript{IPK} in RGC protection. We found no significant difference in IOP elevation after microbead injection in p58\textsuperscript{IPK}−/− mice and WT mice (Figure 1C). Two weeks after induction, WT retinas with increased IOP exhibited a 20% loss of RGCs; the percentage of RGC loss increased significantly to over 35% in p58ipk−/− mice (Figure 1D). This again
indicates that a loss of p58IPK results in an increase in RGC death.

**Overexpression of p58IPK Protects RGCs From ER Stress-Induced Cell Death**

Next, we examined whether overexpressing p58IPK by AAV is sufficient to protect primary mouse RGCs isolated from p58IPK−/− and WT mice. Under control conditions, we found that the percentage of RGCs survival in culture is sufficient to protect primary mouse RGCs isolated from p58IPK−/− mice. Two sets of independent primers specific for mesencephalic astrocyte-derived neurotrophic factor (MANF) reveal a mean significant 50% reduction in mRNA level in p58IPK−/− mice compared to infection with AAV-GFP for both WT and p58IPK−/− mice. Mean ± SD, n = 3 independent experiments. *p < 0.05 (Student’s t-test). Western blot analysis (bottom) of whole retina lysate from WT or p58IPK−/− mice reveal that the level of MANF protein is not statistically different for whole retina (n = 6 mice per group). (C) R28 cells in culture exposed to 16-h pre-treatment with 50 ng/ml MANF or vehicle, followed by 5 µg/ml TM or control treatment for 24 h. Cells are labeled with the live/dead cytotoxicity assay as in (A). Quantification of the survival of R28 cells reveals that approximately 60% of R28 cells survive TM treatment in the absence of MANF. With MANF pre-treatment the survival percentage is significantly increased to over 85%. Mean ± SD, n = 4 independent experiments; *p < 0.05, **p < 0.01 (one-way ANOVA with Bonferroni post hoc test). Scale bar = 100 µm.

**Reduced Retinal mRNA Expression of MANF in p58IPK−/− Mice**

MANF is a member of a newly identified ER-localized neurotrophic factor family and is upregulated during ER stress (Glembotski et al., 2012). We investigated whether MANF expression is altered in p58IPK−/− retinas. Using qPCR, we found that MANF mRNA expression was significantly reduced in the retina of p58IPK−/− mice (Figure 2B). However, the protein level appeared to be insignificantly different (Figure 2B). To further explore whether there is a reciprocal regulation between p58IPK and MANF, we transduced R28 cells with adenovirus to overexpress p58IPK and then treated the cells with TM to induce ER stress. We found that both MANF and p58IPK levels were significantly increased after TM treatment (Supplementary Figure S1A). Overexpression of p58IPK did not alter MANF protein expression in unstimulated cells, but significantly.
Reduced the induction of MANF by TM (Supplementary Figure S1A).

**Recombinant MANF Protects R28 Cells or Retinal Explants From ER Stress Induced Cell Death and Caspase-3 Activation**

To examine whether MANF is important for neuronal survival under ER stress, we pretreated R28 cells with 50 ng/ml recombinant hMANF protein or vehicle (0.1% PBS) followed by treatment with 5 μg/ml TM, or vehicle, for 24 h. We find TM treatment induces cell death in 43% of R28 cells in the absence of MANF treatment (Figure 2C). With MANF pretreatment, this number was reduced to 16%, which represents a recovery to 91% of the survival rate of control R28 cells treated with vehicle (Figure 2C). In addition, MANF treatment significantly reduced TM-elicited caspase-3 activation in *ex vivo* cultured retinal explants (Supplementary Figure S1B).

**DISCUSSION**

The findings from the present study provide strong evidence for an endogenous role of p58<sup>IPK</sup> in protection of RGCs in conditions of ER stress, acute ischemia and ocular hypertension. Previous studies, including our own, have implicated p58<sup>IPK</sup> in a broad range of neurodegenerative diseases and diabetes (Ladiges et al., 2005; Boriushkin et al., 2014, 2015, 2016; Han et al., 2015; McLaughlin and Zhang, 2015). Clinically, patients bearing loss-of-function mutations in the DNAJC3 gene, which encodes p58<sup>IPK</sup>, suffer from diabetes and multisystemic neurodegeneration (Synofzik et al., 2014). In animal models, loss of p58<sup>IPK</sup> results in increased ER stress and oxidative stress, decreased β cell function, progressive cell death and diabetes in male p58<sup>IPK</sup> KO mice (Ladiges et al., 2005; Han et al., 2015). In contrast to the relatively thoroughly studied role of p58<sup>IPK</sup> in diabetes, how p58<sup>IPK</sup> regulates neuronal function remains unexplored. Herein, we examined the impact of p58<sup>IPK</sup> deficiency on RGC survival in disease-relevant models. Our data clearly indicate an important role of p58<sup>IPK</sup> in RGC survival during acute retinal ischemia and in ocular hypertension. Notably, we used young adult p58<sup>IPK</sup> KO mice that demonstrate no significant morphological defects in the retina compared to their age-matched littermate controls. In addition, most of our experiments were carried out in female mice at 2 months of age, which do not experience increased blood glucose levels as some male p58<sup>IPK</sup> KO mice do with age.

Our *in vitro* results suggest a therapeutic potential of overexpressing p58<sup>IPK</sup> or enhancing its function for neuroprotection of RGCs, which warrants an *in vivo* study in disease models. Another interesting finding is that there is likely a reciprocal regulation between p58<sup>IPK</sup> and MANF in retinal cells undergoing ER stress. Recent studies show that MANF protects CNS neurons (Voutilainen et al., 2017) and markedly improves RGC survival in a rat glaucoma model (Gao et al., 2017). Our data support these findings and demonstrate that MANF inhibits ER stress-induced cell death and caspase-3 activation in retinal cells and explants. In R28 cells, both p58<sup>IPK</sup> and MANF are upregulated during ER stress; interestingly, overexpressing p58<sup>IPK</sup> reduces ER stress-stimulated MANF upregulation. This suggests a potential interaction between the two chaperones, which both bind to GRP78 and regulate the UPR, although it remains unclear whether MANF secretion is affected by p58<sup>IPK</sup> manipulation. Future studies will investigate how MANF and p58<sup>IPK</sup> are regulated in RGCs and whether overexpression of p58<sup>IPK</sup> and MANF would synergistically promote RGC survival and function.

**AUTHOR CONTRIBUTIONS**

TM designed and performed the experiments, analyzed the data and drafted the manuscript. ND and JL performed the experiments, analyzed the data and revised the manuscript. JW and SZ conceived and designed the study, reviewed all the data, drafted and revised the manuscript. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2018.00267/full#supplementary-material
regulation of aerobic exercise. *Brain Behav. Immun.* 57, 347–359. doi: 10.1016/j.bbi.2016.05.010

Chen, C., Cano, M., Wang, J. J., Li, J., Huang, C., Yu, Q., et al. (2014). Role of unfolded protein response dysregulation in oxidative injury of retinal pigment epithelial cells. *Antioxid. Redox Signal.* 20, 2091–2106. doi: 10.1089/ars.2013.5240

Chen, H., Wei, X., Cho, K.-S., Chen, G., Sappington, R., Calkins, D. J., et al. (2011). Optic neuropathy due to microbead-induced elevated intraocular pressure in the mouse. *Invest. Ophthalmol. Vis. Sci.* 52, 36–44. doi: 10.1167/iolsj.09-5115

Gao, F.-J., Wu, J.-H., Li, T.-T., Du, S.-S., and Wu, Q. (2017). Identification of mesencephalic astrocyte-derived neurotrophic factor as a novel neuroprotective factor for retinal ganglion cells. *Front. Mol. Neurosci.* 10:76. doi: 10.3389/fnmol.2017.00076

Glembocksi, C. C., Thuerauf, D. J., Huang, C., Vekich, J. A., Gottlieb, R. A., and Doroudgar, S. (2012). Mesencephalic astrocyte-derived neurotrophic factor protects the heart from ischemic damage and is selectively secreted upon sarco/endoplasmic reticulum calcium depletion. *J. Biol. Chem.* 287, 25893–25904. doi: 10.1074/jbc.m112.356345

Han, J., Song, B., Kim, J., Kodali, V. K., Pottekat, A., Wang, M., et al. (2015). Antioxidants complement the requirement for protein chaperone function to maintain β-cell function and glucose homeostasis. *Diabetes* 64, 2892–2904. doi: 10.2337/db14-1357

Hu, Y., Park, K. K., Yang, L., Wei, X., Yang, Q., Cho, K.-S., et al. (2012). Differential effects of unfolded protein response pathways on axon injury-induced death of retinal ganglion cells. *Neuron* 73, 445–452. doi: 10.1016/j.neuron.2011.10.026

Huang, C., Wang, J. J., Ma, J. H., Jin, C., Yu, Q., and Zhang, S. X. (2015). Activation of the UPR protects against cigarette smoke-induced RPE apoptosis through up-regulation of Nrf2. *J. Biol. Chem.* 290, 5367–5380. doi: 10.1074/jbc.m114.603738

Huang, X., Wu, D. Y., Chen, G., Manji, H., and Chen, D. F. (2003). Support of retinal ganglion cell survival and axon regeneration by lithium through a Bcl-2-dependent mechanism. *Invest. Ophthalmol. Vis. Sci.* 44, 347–354. doi: 10.1167/iovs.02-0198

Huber, A.-L., Lebeau, J., Guillaumot, P., Petit, V., Malek, M., Chiloux, J., et al. (2013). p58IPK-mediated attenuation of the proapoptotic PERK-CHOP pathway allows malignant progression upon low glucose. *Mol. Cell* 49, 1049–1059. doi: 10.1016/j.molcel.2013.01.009

Jiao, J., Huang, X., Feit-Leithman, R. A., Neve, R. L., Snider, W., Darby, D. A., et al. (2005). Bcl-2 enhances Ca2+ signaling to support the intrinsic regulatory capacity of CNS axons. *EMBO J.* 24, 1068–1078. doi: 10.1038/sj.emboj.7600589

Jing, G., Wang, J. J., and Zhang, S. X. (2012). ER stress and apoptosis: a new mechanism for retinal cell death. *Exp. Diabetes Res.* 2012:69589. doi: 10.1155/2012/695898

Kim, S., Joe, Y., Jeong, S. O., Zheng, M., Back, S. H., Park, S. W., et al. (2014). Endoplasmic reticulum stress is sufficient for the induction of IL-1β production via activation of the NF-κB and inflammasome pathways. *Innate Immun.* 20, 799–815. doi: 10.1177/1753425913508593

Ladies, W. C., Knoplaugh, S. E., Morton, J. K., Korth, M. J., Sopher, B. L., Baskin, C. R., et al. (2005). Pancreatic β-cell failure and diabetes in mice with a deletion mutation of the endoplasmic reticulum molecular chaperone gene P58IPK. *Diabetes* 54, 1074–1081. doi: 10.2337/db04-1074

Lee, A. H., Iwakoshi, N. N., and Glimcher, L. H. (2003). XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol. Cell. Biol.* 23, 7448–7459. doi: 10.1128/mcb.23.21.7448–7459.2003

McLaughlin, T., Falkowski, M., Park, J. W., Keegan, S., Elliott, M., Wang, J. J., et al. (2018). Loss of XPB1 accelerates age-related decline in retinal function and neurodegeneration. *Mol. Neurodegener.* 13:16. doi: 10.1186/s13024-018-0250-z

McLaughlin, T., and Zhang, S. X. (2015). The neuroprotective potential of endoplasmic reticulum chaperones. *Neural Regen. Res.* 10, 1211–1213. doi: 10.4103/1673-5374.162696

Rutkowski, D. T., Kang, S.-W., Goodman, A. G., Garrison, J. L., Taunton, J., Kats, M. G., et al. (2007). The role of p58IPK in protecting the stressed endoplasmic reticulum. *Mol. Biol. Cell* 18, 3681–3691. doi: 10.1091/mbc.e07-03-0272

Synofzik, M., Haack, T. B., Kopajtich, R., Gorza, M., Rapaport, D., Greiner, M., et al. (2014). Absence of BiP co-chaperone DNAJC3 causes diabetes mellitus and multisystemic neurodegeneration. *Am. J. Hum. Genet.* 95, 689–697. doi: 10.1016/j.ajhg.2014.10.013

van Huizen, R., Martindale, J. L., Gorospe, M., and Holbrook, N. J. (2003). P58IPK, a novel endoplasmic reticulum stress-inducible protein and potential negative regulator of eIF2α signaling. *J. Biol. Chem.* 278, 15558–15564. doi: 10.1074/jbc.m121074200

Voutilainen, M. H., De Lorenzo, F., Stepanova, P., Back, S., Yu, L.-Y., Lindholm, P., et al. (2017). Evidence for an additive neurorestorative effect of simultaneously administered CDN1 and GDNF in hemiparkinsonian rats: implications for different mechanism of action. *eNeuro 4:E NEURO.0117-16.2017*. doi: 10.1523/eneuro.0117-16.2017

Zhang, S. X., Sanders, E., Fliesler, S. J., and Wang, J. J. (2014). Endoplasmic reticulum stress and the unfolded protein responses in retinal degeneration. *Exp. Eye Res.* 125, 30–40. doi: 10.1016/j.exer.2014.04.015

Zhong, Y., Li, J., Chen, Y., Wang, J. J., Ratan, R., and Zhang, S. X. (2012). XBP-1 regulates a muller cell-derived inflammatory cytokine production in diabetes. *Diabetes* 61, 492–504. doi: 10.2337/db11-0151

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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