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Short communication

RAGE and Diaph1 deficiency affect retinal cytoarchitecture both in physiological and long-term hyperglycemic settings in diabetic mice – a preliminary study

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Abstract

Retinal complications in diabetes are a leading cause of vision loss in adults. Long-term hyperglycemia triggers molecular changes that damage retinal neuronal tissues and blood vessels, leading to degeneration, inflammation, and vision impairment. Studies show that the Receptor for Advanced Glycation End-Products (RAGE) and its cytosolic ligand, Diaph1 are both implicated in the pathogenesis of diabetic complications; however, their role in the development of retinal damage is not yet clear. We hypothesized that the deletion of either RAGE or Diaph1 would be beneficial and prevent or delay damage to the retinal structure in diabetes. Wild-type, RAGE and Diaph1 knockout mice were used in the study. The mice were randomly selected and divided into control and diabetic groups. Diabetes was induced with Streptozotocin injections, and six months after diabetes induction all mice were sacrificed, samples collected and processed for morphometric analysis. Our analysis revealed a reduction in retinal depth across all layers between control and diabetic samples. The effect of RAGE or Diaph1 deletion on retinal structure differed, indicating that both proteins may play independent if not interchangeable roles in retina function and structure, highlighting their potential role in the pathogenesis of diabetic retinal complications.

Keywords: cytoarchitecture, diabetes, Diaph1, receptor for advanced glycation end-products, retina

Introduction

The retina is a highly specialized and metabolically active tissue, with limited ability to regenerate in humans. Retinal complications in diabetes mellitus are a leading cause of vision loss in adults aged 20 to 70 in developed countries (Cheung et al. 2010).

Our study focuses on one of the proinflammatory receptors, namely RAGE, and its intercellular ligand Diaph1. RAGE, a receptor for AGEs (advanced glycation end-products), is a signal transduction receptor, whose activation triggers an increase in proinflammatory molecules, oxidative stressors and cytokines (Schmidt et al. 2000). The upregulation of RAGE is associated with many proinflammatory disorders such as nephropathy, retinopathy, neuropathy, cardiovascular disorders, Alzheimer's, multiple sclerosis, rheumatoid arthritis, osteoporosis and cancer (Dong et al. 2022). In retina, RAGE expression remains low under normal conditions but increases during prolonged hyperglycemia due to the accumulation of AGEs, upregulating its expression in Muller cells and Ganglion cells (Wang et al. 2008) and triggering a cascade of detrimental metabolic signaling pathways promoting inflammation, increase of free oxygen species (ROS), angiogenesis, dysfunction of the blood-retina barrier, and vascular leakage via the Vascular Endothelial Growth Factor (VEGF) and Glial Fibrillary Acidic Protein (GFAP) upregulation of Angiopoietin 2 (ANG2) (Takagi 2003, Lu et al. 2023).

Diaph1, protein diaphanous homolog 1, belongs to the Rho-GTPase formins (Mao 2011), known for its regulatory role in structural modification of actin, microtubulin and related cytoskeleton proteins, thus affecting cellular morphology, motility and secretion (Lu et al. 2015). Studies on Diaph1 demonstrated that in the retina under normal physiological conditions, the presence of Diaph1 is required for proper ciliogenesis and the function of photoreceptor cilia by nucleating actin and stabilizing microtubules necessary for vesicle trafficking to the basal body, thus enabling proper photoreceptor functioning. Conversely, Diaph1 depletion resulted in cilia elongation, bulb formation and defective ciliation (Palander and Trimble 2020). Studies show that RAGE and Diaph1 interact with each other, with RAGE binding to Diaph1 via its cytosolic tail (Rai et al. 2012) and together they play a primary role in the pathogenesis of diabetes and its related complications. Since the time of the discovery, major progress has been made in discerning the role of RAGE-Diaph1 signaling in neuroinflammation and hyperglycemia; however, despite the progress made in some aspects of diabetes and neuroinflammatory disorder studies, little is known about the role of RAGE-Diaph1 signaling in diabetic complications affecting the retina and the development of diabetic retinopathy.

Considering the lack of data and in order to dissect the effect of either genotype on retinal cytoarchitecture in normo- and hyperglycemic environment, we studied retinas obtained from Wild-type, RAGE and Diaph1 knockout control and diabetic mice.

Materials and Methods

Animals

Six mice per each genotype: wild-type (WT), RAGE (RKO) and Diaph1 (DKO) knockout were used in the study. Mice from the same batch were assigned together to a set time-point group and divided into non-diabetic and diabetic groups to minimize intra-group variations and ensure homogeneity of results. Diabetes was induced with Streptozotocin (STZ, 50 mg/kg in citrate buffer, intraperitoneal injections for 5 consecutive days) (Juranek et al. 2013) at the 8th week of age as per earlier studies (Juranek et al. 2013, Daffu et al. 2015). RAGE and Diaph1 knockouts were made to order at the International Institute of Molecular and Cell Biology in Warsaw, Poland, using the Crisper Cas9 technique. The Crisper Cas technique, unlike the previously used classical knockout technique is more precise and minimizes the risk of genetic flaws (Lee et al. 2018).

To verify Diaph1 null/null status, Diaph1 knockout F1 offspring was genotyped using customized Diaph1-specific primers: forward – GCATTGCTGTCT CTTACACA and reverse – TCAACTTAGGAGAC CACACA against reference WT mice. The presence of three Diaph1 amplicons at 504 bp, 313 bp and 201 bp verified the deletion. To verify AGER (RAGE encoding gene) null/null status, RAGE knockout F1 offspring was genotyped using AGER-specific primers: forward – GGTGGTCAGAACATCACAGC, reverse – CTAACCAGACTGGGCCCTC against reference WT mice. The presence of two AGER/RAGE amplicons at 380 bp and 278 bp verified AGER deletion. All animal experiments were conducted following the national guidelines and the relevant national laws on the protection of animals. The study was approved by the Local Ethics Committee of Experiments on Animals in Olsztyn (Poland; decision no. 57/2019). Following sacrifice, eyes were prefixed in 1% paraformaldehyde (Merck, Saint Louis, MO, USA) and 2.5% glutaraldehyde (Polisciences, Warrington, PA, USA) in 0.2 M phosphate buffer (pH 7.4) for 30 mins at 4°C. The samples were then washed, postfixed in 2% osmium tetroxide for 2 hours at room temperature, dehydrated,

embedded in Epon 812 and cut at a microtome. Semithin sections were stained with toluidine blue according to standard protocols and morphometric analysis was performed across five retinal layers using CaseViewer software (3DHISTECH Ltd.); four sections in four replicates per animal were quantified. Statistical analysis and correlation data between control and experimental conditions were determined by GraphPadPrism software using ANOVA (one-way or with time as a repeated measure, parametric data) followed by post hoc tests of significance.

Results and Discussion

The results of our study revealed that the deletion of either RAGE or Diaph1 affects retinal cytoarchitecture both in normal and hyperglycemic environments. We demonstrated for the first time that the deletion of either component of the RAGE-Diaph1 tandem impacts the structure of the retina across its layers, underscoring the critical role of these proteins in retina structure and function both in physiological and pathological conditions. We observed depth reduction across all retinal layers in diabetic samples for all genotypes studied; however, the extent of the observed reduction differed between genotypes; in general, the thickness and cytoarchitecture of the retinal layers were better persevered both in RKO and DKO diabetic samples, indicating that the deletion of either of these proteins might be beneficial for hyperglycemia affected retinas. Surprisingly, however, we also noted depth increase across all but the photoreceptor layer, in the control samples indicating that RAGE or Diaph1 deficiency likely plays a role in the structure and function of the developing and mature retina in physiological, non-diseased settings. On average, except for, the photoreceptor layer, retinal layers were thicker both in RAGE and Diaph1 deficient mice, the increase in depth being more pronounced in DKO as compared to WT and RKO samples. The most significant difference between genotypes was noted for DKO in the outer plexiform layer, which is involved in signal transmission from photoreceptors to bipolar cells of the inner layer (Fig. 1). Our previous studies showed that while RAGE has been upregulated in diabetic neuropathy, Diaph1 levels were reduced in the diabetic peripheral nerve and this reduction was independent of genotype (WT or RAGE deficient); it should be noted that we showed that the decreased Diaph1 level as well the observed impairment of its transport might be correlated with diabetes triggered glycation of cytoskeletal proteins such as actin, contributing to a lower expression of Diaph1 in diabetic peripheral nerve, rather than to its increase (Juranek et al. 2010, Juranek et al. 2013). It is also plausible to speculate that the role of Diaph1 in retinal cytoarchitecture is linked to, and correlates tightly with both the level of RAGE and actin available for binding. The increase in binding between RAGE and Diaph1 at the cost of, or in the absence of, available actin and other cytoskeletal ligands may trigger a cascade of detrimental changes leading to deformation of the retinal structure and its dysfunction. In recent years, some progress has been made in uncovering hyperglycemia triggered molecular pathways leading to eye and optic nerve damage; a detailed report, reviewing the plausible involvement of RhoA, Diaph1 and profilin1 in diabetic retinopathy indicated that RhoA/Diaph1/profilin1 signaling was involved in retinal endothelial dysfunction in diabetic patients (Lu et al. 2015) via its interactions with Angiopoietin-like protein-4 (ANGPTL4) and Rho/ROCK signaling (Lu et al. 2018). The results of experimental studies have also been validated by genomic analysis, reporting upregulation of ROCK2, AGER (gene encoding RAGE) and ARHGAP22 (Rho family GTPase protein) among others, and various pathways including, but not limited to, axonal growth, eye development, cell and subcellular compartment movement and response to external stimuli along with more typical pathways such as those related to glucose metabolism, inflammation or apoptosis (Sharma et al. 2019, Kumari et al. 2020), thus highlighting the significance of our preliminary results and validating the importance of further examination of the RAGE-Diaph1 signaling pathway in the diabetic eye. The primary limitation of our work is the small number of animals used. Consequently, a larger-scale study with an increased number of animals has already been designed to address this limitation. Another limitation is the lack of ophthalmoscopic or functional data i.e visual acuity tests to assess differences, if any, in eye vision between mice of different genotypes; this limitation has been also addressed in our ongoing studies. Despite these constraints, the observed trends and statistically significant differences in retinal layers between genotypes in both control and diabetic groups underscore the necessity for further investigation into the role of RAGE-Diaph1 signaling in diabetic retinopathy.

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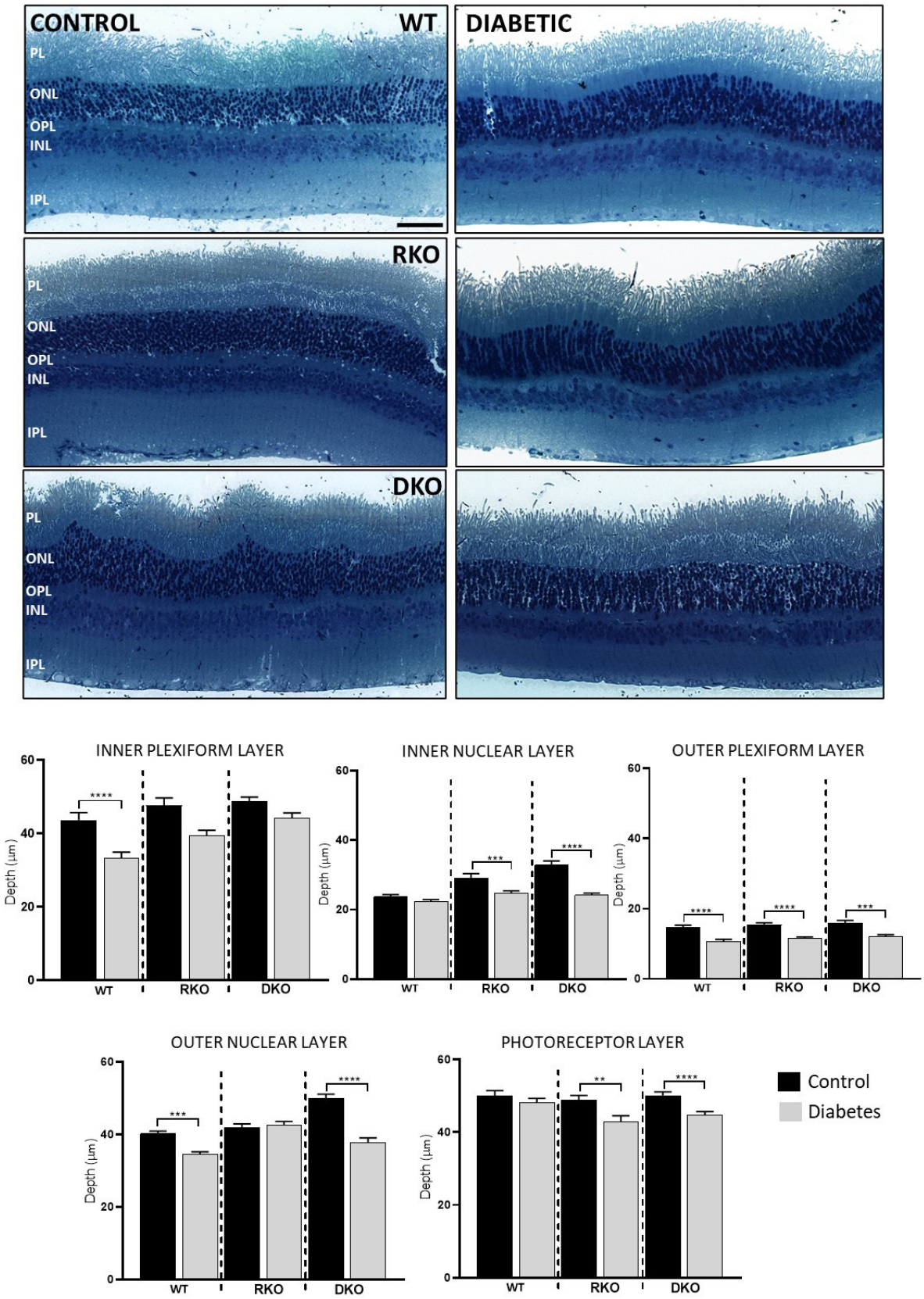

Fig 1. Representative images of toluidine blue staining of mouse retinal semi-thin sections. PL – photoreceptor layer, ONL – outer nuclear layer, OPL – outer plexiform layer, INL – inner nucleal layer, IPL – inner plexiform layer. Scale bar – 50 µm. Below: comparative analysis of differences between control and diabetic samples across genotypes. OPL was the only layer that demonstrated reduction in thickness across all genotypes in diabetic samples. Asterisks (*) denotes statistically significant differences, $p \leq 0.005$ **, $p \leq 0.001$ ***, $p \leq 0.0001$ ****.

Author Declarations

Ethics approval

The study was approved by the Institutional Ethics Committee at the University of Warmia and Mazury in Olsztyn (decision no. 57/2019, dated July 17, 2019).

Use of generative artificial intelligence (AI)

AI was not used to prepare the manuscript or its figures.

Conflict of interest

Authors declare no conflict of interest

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