DOI: 10.1167/iovs.19-26761

δ Opioid Receptor Agonism Preserves the Retinal Pigmented Epithelial Cell Tight Junctions and Ameliorates the Retinopathy in Experimental Diabetes

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Submitted: January 31, 2019
Accepted: August 5, 2019

Citation: Lopes de Faria JM, Duarte DA, Simó R, et al. δ opioid receptor agonism preserves the retinal pigmented epithelial cell tight junctions and ameliorates the retinopathy in experimental diabetes. *Invest Ophthalmol Vis Sci*. 2019;60:3842–3853. https://doi.org/10.1167/iovs.19-26761

Purpose. Outer blood retinal barrier breakdown is a neglected feature of diabetic retinopathy (DR). We demonstrated that the agonism of the δ opioid receptor (DOR) by epicatechin preserves the tight junction proteins in ARPE-19 cells under diabetic conditions. Presently, we aimed to evaluate the possible role of the DOR on the maintenance of tight junction of RPE layer and on the early markers of experimental DR.

Methods. DR markers and external retinal tight junction proteins were evaluated in CL57B diabetic mice submitted to intravitreous injection of short hairpin RNA (shRNA)-DOR (10^8 transducing units [TU]/mL) treated or not with DOR agonist (0.05 g/animal/d of epicatechin in drinking water) for 16 weeks. The presence of DOR in human retina from postmortem eyes from diabetic and nondiabetic donors were also performed.

Results. DOR is present in RPE layer and in neuro retina. The treatment with DOR agonist prevented the upregulation of the early markers of retinopathy (glial fibrillary acidic protein, VEGF) and the downregulation of pigment epithelium-derived factor, occludin, claudin-1, and zonula occludens-1 tight junction expressions. The silencing of DOR in retina of diabetic mice partially abolished the protective effects of epicatechin. In human retina specimens, DOR is present throughout the retina, similarly in nondiabetic and diabetic donors.

Conclusions. This set of experiments strongly indicates that the DOR agonism preserves RPE tight junctions and reduces the early markers of retinopathy in model of diabetes. These novel findings designate DOR as a potential therapeutic tool to treat DR with preservation of the RPE tight junction proteins.

Keywords: diabetic retinopathy, diabetes, δ opioid receptor, retinal pigmented epithelial

Diabetic retinopathy (DR) is one of the most common causes of irreversible blindness worldwide. In the United States, the prevalence of DR is expected to increase substantially by 2020 due to the increasing rates of diabetes over time with the aging of the population.[1] DR often causes visual disabilities during the working ages, thus incurring significant human, social, and economic costs on countries around the world.[2] The major outcomes of DR, including visual impairments, consist of advanced forms of proliferative DR and clinically significant macular edema, which can occur together or not. Data show that 30% (confidence interval: 25.9%–35.5%) of type 2 diabetes patients with nonproliferative retinopathy experience visual threatening due to diabetic maculopathy.[3]

The RPE is a monolayer of polarized, pigmented cells of neuroectodermal origin located between the neural retina and the choroid and helps in maintaining the structural and functional integrity of the retina.[4] The RPE constitutes the outer blood retinal barrier (BRB), wherein the apical side is directed toward the neural retina and the basal side is directed toward the Bruch's membrane, followed by choroid. The functionality of RPE, apart from imparting a physical barrier, is to maintain retinal homeostasis by a series of secretory factors.[5] Moreover, the RPE plays a major role in the absorption of light; protection against photo-oxidation; regulation of nutrients, ions, and water between the neural retina (apical side) and the choriocapillaris (basal side); exchange of 11-cis retinal and all-trans-retinol between the photoreceptors and RPE in the visual cycle; phagocytosis of shed photoreceptor outer segments; and secretion of growth factors such as the pigment epithelium-derived factor (PEDF),[6] vascular endothelial growth factor (VEGF),[7] transforming growth factor-b (TGF-b),[8] insulin-like growth factor-I (IGF-I),[9] brain-derived growth factor (BDNF),[10] and connective tissue growth factor (CTGF).[11,12] More recently, the role of the outer BRB in the pathogenesis of DR, specifically diabetic macular edema, has gained more attention. Proteomics of diabetic human donor eyes with no retinopathy has revealed alterations in the proteins involved in membrane dynamics, metabolic events, and cytoskeletal structure.[13] A previous ARPE-19 cell-based proteomic study showed different profile of the secreted proteins from the RPE cells that are functionally associated with cytoskeleton adhesion/junction when exposed to high glucose (HG) conditions.[14]

Recently, we have demonstrated that the process by which tight junctions claudin-1 and occludin decrease in ARPE-19 cell

and primary porcine RPE monolayers exposed to diabetes milieu conditions occurs through caveolin 1 (CAV-1) endocytosis.[15] The HG condition leads to a profound imbalance in paracellular resistance and permeability, thus mimicking the outer BRB breakdown in vivo.[15] The CAV-1 endocytosis process depends on the upregulation of inducible nitric oxide synthase (iNOS) expression by TNF-α. The increased iNOS generates massive nitric oxide production, which results in S-nitrosylation of CAV-1.[15] Treatment with epicatechin abolished these translational modifications of CAV-1 through direct interaction with the δ-opioid receptor (DOR) in the RPE cells.[15] These are the first observations that epicatechin protects the integrity of the RPE barrier through DOR agonism, thus preserving the retina from the deleterious effects of HG-mimics diabetes.

The anti-angiogenic PEDF is a member of the serine protease inhibitor family, which is characterized by anti-angiogenic, neuroprotective, and anti-inflammatory properties. In a healthy eye, PEDF is secreted from the RPE and maintains the retinal as well as the choriocapillaris structure by inhibiting endothelial cell proliferation.[16] PEDF is predominantly secreted from the apical surface of the RPE. In the postnatal period, PEDF-deficient mice have shown enhanced retinal vascularization; this implies the modulating role of PEDF in the early stage of postnatal retinal vascularization.[17,18] PEDF expression is downregulated in human RPE cells cultured in HG levels, as well as in the vitreous of proliferative DR and diabetic macular edema patients.[19] Studies have also shown that PEDF-34 has neuroprotective and regenerative capacity in optic nerve crush rat models.[20] Therefore, more studies are needed to explore the role of PEDF as a potential therapeutic tool to treat diabetic macular edema.

The aim of the present study was to investigate whether the DOR agonist protects the retina, specially the outer tight junction proteins in an experimental model of DR. This study is the first to demonstrate that DOR agonism protects the RPE maintaining the expression of tight junctions accompanied by a decrease in VEGF and an increase in PEDF retinal levels. Collectively, these observations provide a new approach to treat DR.

Materials and Methods

Animal Model and Experimental Design

The study was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all procedures involving animals were approved by the Ethics Committee on the Use of Animals for the State University of Campinas under regulation number 3986-1. The mice were housed in a temperature-controlled room (22°C) with a 12-hour:12-hour light to dark cycle and had free access to food and water.

Protocol 1. Treatment With DOR-Agonist in Drinking Water. Experimental diabetes was induced in 8-week-old C57BL/6JUnib male mice from the Multidisciplinary Center for Biological Research by five consecutive daily intraperitoneal injections of streptozotocin (50 mg/kg; Sigma-Aldrich Corp., St. Louis, MO, USA); the control animals received vehicle alone. Thirty days after, the animals presenting a blood glucose of ≥15 mmol/L, measured by Accu-Chek (Roche, Mississauga, ON, Canada), were considered diabetic and randomized to receive or not the DOR agonist (high-purity ≥98% (−)-epicatechin; Sigma-Aldrich Corp.) in drinking water. Based on previous studies,[21,22] the oral dose of epicatechin used in this study was 0.2% (0.2 g/100 mL) in drinking water (0.05 g/animal/d).

Protocol 2. Efficiency of the Different Short Hairpin RNA (shRNA) Constructs. As previously described,[23] 1 μL solution containing 10^8 transducing units (TU)/mL virus particles were intravitreally injected in using the Hamilton syringe with a 30G needle. Eight-week-old C57BL/6JUnib mice were randomized to receive an intravitreal injection of one of the three different DOR shRNA construct (TRCN0000027801, TRCN0000027842, and TRCN0000027797) and a nontarget genes shRNA construct (Empty vector) as a negative control (Sigma-Aldrich Corp.). After 1 month, the animals were submitted to electroretinogram (ERG) to test the possible retinal toxicity and then euthanized, and retinas were collected to test the efficiency of DOR silencing.

Protocol 3. Treatment With DOR-Agonist in Drinking Water in Mice Submitted to Intravitreous Injection of shRNA to the Retinal DOR Gene. The diabetic mice received an intravitreous injection of empty vector in the left eye and DOR shRNA construct (TRCN0000027797) in the right eye, in the presence or absence of the DOR agonist in drinking water (0.05 g/animal/d). After 12 weeks of treatment, the animals were submitted to ERG and then euthanized; the eye globes and retinas were collected for subsequent experiments.

Full-Flash ERG

Retinal function was measured using the UTAS-E3000 system (LKC Technologies, Inc., Gaithersburg, MD, USA), as previously described.[24–27] The measurements were taken after overnight dark adaptation. An intensity-response was recorded using a ganzfeld flash with an intensity of 0.25 cd.s/m^2 at 0 to 250 or 0 to 4000 milliseconds. Recordings were amplified and digitized using a 24-bit A/D converter band passed from 0.3 to 300 Hz with a 50-Hz notch filter. The quantitative analyses were performed using the *b*-waves and oscillatory potential amplitude expressed in microvolts (μV) and *c*-waves deflection expressed in area under the curve.

In Vitro Assay

The human retinal pigment epithelial cell line (ARPE-19) was cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium, 10% of fetal bovine serum (FBS; Cultlab, Campinas, Brazil), and 1% of penicillin and streptomycin. ARPE-19 cells at passage 15 to 17 were used for this study. Cell culture at 70% confluence were serum starved with 1% of FBS for 15 hours and exposed to normal glucose (NG; 5.5 mM) or HG (30 mM), or HG plus D-Ala(2)-Deltorphin II (HG+D2; 25 nM; HY-P1013, MedChemExpress, Monmouth Junction, NJ, USA), or HG plus D-Ala(2),D-Leu(5)]enkephalin (HG+DADLE; 50 nM; #E7131, Sigma-Aldrich, Corp.) for 24 hours in the presence or absence of the DOR antagonist Naltrindole (Nalt; 10 μM; 111469-81-9, Cayman Chemical, Ann Arbor, MI, USA).

Transepithelial Electrical Resistance (TER) Measurements

ARPE-19 cells were cultured in a Transwell-Clear Polyester Membrane Insert (HTS, Costar; Corning, Inc., Corning, NY, USA). At day 22, the respective treatments were performed as described above. After 24, 48, 120, and 196 hours, the voltmeter (Millicell-ERS; Millipore, Burlington, MA, USA) was used to evaluate the TER expressed in ohms and corrected by area of the polyester membrane (ohms/cm^2) subtracted from the blank (polyester membrane only).[14]

Western Blot Analysis

The protein extracts obtained from total cell lysate were subjected to SDS-PAGE in a Bio-Rad slab gel apparatus (Mini-PROTEAN Tetra cell; Bio-Rad, Hercules, CA, USA) and

electrophoretically transferred to a nitrocellulose membrane. The membranes were incubated with primary antibodies for phospho-NF-κB (Ser536) 1:1000 (Cell Signaling Technology, Danvers, MA, USA), NF-κB 1:250 (Santa Cruz Biotechnology, Dallas, TX, USA) followed by the appropriated secondary antibodies. As internal control for protein loadings, the membranes were hybridized against vinculin 1:1000 (Cell Signaling Technology). Immunoreactive bands were visualized using the enhanced chemiluminescence method (Super Signal CL-HRP Substrate System; Pierce, Rockford, IL, USA). The membranes were scanned with a digital photo documentator (ImageQuant LAS 500, GE, Boston, MA, USA) and analyzed quantitatively with ImageJ Software (National Institutes of Health, Bethesda, MD, USA). At least three independent experiments were carried out.

Human Samples

Human postmortem eyes were obtained from diabetic ($n = 7$) and nondiabetic donors ($n = 7$) matched by age). To be included in this study, an ophthalmoscopic examination documenting the absence of microvascular abnormalities associated with DR or only mild nonproliferative DR must have been performed within 2 years prior to the individual's death. The demographic characteristics of donors are presented in the Supplementary Table S1. The study was performed following the tenets of the Declaration of Helsinki for research involving human tissue. The time elapsed from death to eye enucleation was 3.4 ± 1.9 hours. After enucleation, one eye from each donor was snap-frozen in liquid nitrogen and stored at –80°C in the Blood and Tissue Bank of Vall d'Hebron University Hospital (Barcelona, Spain) until the eye was assayed. The other eye was fixed in 4% paraformaldehyde and embedded in paraffin for the immunofluorescence study. The neuroretina and RPE were harvested before mRNA or protein extracts were obtained. Briefly, the vitreous and neuroretina were removed, and the RPE layer was then carefully peeled off from Bruch's membrane using forceps (Dumont No 5, Sigma-Aldrich Corp., Madrid, Spain) under the dissecting microscope (Olympus SZ61, Barcelona, Spain).

Human Protein Extraction and Western Blotting

Protein extracts from isolated RPE and neuroretina samples were prepared by homogenization with radio immunoprecipitation assay (RIPA) buffer, and protein concentrations were determined using a bicinchoninic acid assay (BCA) kit (Thermo Fisher Scientific, Rockford, IL, USA). Twenty micrograms of protein was separated by SDS-PAGE acrylamide gels and transferred to nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA). The membranes were incubated in primary antibody against DOR (1:250 Merck, Darmstadt, Germany), and immunoreactive bands were visualized using enhanced chemiluminescence (Millipore, Madrid, Spain). For densitometric analysis, we used a GS-800 calibrated densitometer (Bio-Rad Laboratories, Madrid, Spain) and Quantity One software version 4.6.2 (Bio-Rad Laboratories).

Immunohistochemistry

The immunohistochemistry was performed as previously described.[24] The retinal sections were incubated with anti-GFAP and anti-VEGF (1:250, Santa Cruz Biotechnology) overnight at 4°C. The slides were then incubated with the appropriate secondary antibodies. The negative controls consisted in omitting the primary antibody. The semi-quantitative analyses were performed using the Leica Application Suite (Wetzlar, Germany) using two images per retinal section of at least three nonconsecutive retinal slides (100-μm apart) per animal and three to five animals per group. At the end, at minimum six retinal images/animal were considered for the analyzes.

Immunofluorescence

The immunofluorescence was performed as previously described.[25] The tissues were incubated with anti-claudina-1, occludin, and zonula occludens-1 (ZO-1; Invitrogen, Carlsbad, CA, USA) and anti-human DOR (Merck) followed by the appropriate secondary antibody. The slides were examined under a scanning microscope (LSM510 Zeiss, Jena, Thuringia, Germany). The negative controls consisted in omitting the primary antibody. For the semi-quantitative analyses, at least two images of each retinal section and three nonconsecutive retinal sections (100-μm apart) per animal and three to five animals per group were included into the analyses using the Image J Software (National Institutes of Health).

RNA Extraction and Quantitative RT-PCR

Real-time PCR was performed as previously described.[26] Briefly, the retina extraction was transferred to 300-μL tubes containing Trizol (Invitrogen). The concentration of RNA was determined using a NanoVue (GE). For cDNA synthesis, we used random primers and a dNTP mix. Subsequently, 4 μL 5X-concentrated enzyme buffer, 1 μL 0.1 M dithiothreitol, and 1 μL Superscript III reverse transcriptase enzyme (Thermo Fisher Scientific) were added. Primers for the DOR (Prrd1, Mm01180757_m1, and NM_000911.3), Occludin (Oclin, Mm00500911_m1), and GFAP (Mm01253033_m1) genes were obtained from Applied Biosystems (Foster City, CA, USA). β-actin gene expression was used as a control (Actb, Mm02619580_g1). The RT-PCR was performed using primers, and β-Actin was used as a constitutive gene.

Statistical Analysis

The results were expressed as the mean ± SD. Comparisons between groups were done using ANOVA followed by Fisher's protected least-significant difference test. The analyses were performed using StatView software (SAS Institute, Inc., Cary, NC, USA). $P < 0.05$ was considered significant.

Results

Physiological Parameters

As expected, the final body weights were lower and the blood glucose levels were higher in the diabetic group compared with the nondiabetic group ($P < 0.0001$) (Table). The water consumption was higher in the diabetic group compared with the nondiabetic group ($P < 0.001$). None of the treatment regimens altered body weight, blood glucose levels, or water consumption among the diabetic animals.

The Expression of the DOR Is Not Altered Either by the Presence of Elevated Blood Glucose or by Treatment With the DOR Agonist

DOR is expressed in the RPE and outer and inner plexiform layers. We found that both DOR protein expression and mRNA levels did not change in the presence of elevated blood glucose treated or not with DOR agonist (Figs. 1B, 1C, respectively).

Table. Physiological Parameters of Study Group

Groups	Blood Glucose, mg/dL	Body Weight, g	Water Intake, mL/d
No diabetic, $n = 6$	154.0 ± 18.3	30.5 ± 2.7	9.9 ± 3.0
Diabetic, $n = 7$	539.8 ± 29.6*	21.1 ± 2.8*	28.3 ± 2.4†
Diabetic + DOR agonist, $n = 6$	552.7 ± 74.2*	20.5 ± 2.8*	27.0 ± 1.6†
Nondiabetic-shRNA, $n = 9$	152.6 ± 38.5	31.4 ± 1.8	8.0 ± 2.0
Diabetic-shRNA, $n = 7$	558.8 ± 39.3*	21.0 ± 1.7*	27.6 ± 2.1†
Diabetic-shRNA + DOR agonist, $n = 6$	594.5 ± 9.4*	20.1 ± 2.3*	27.1 ± 1.5†

* $P < 0.0001$.
† $P < 0.001$.

The DOR Agonist Epicatechin Ameliorates the Early Markers of DR and Protects the RPE Tight Junctions in Experimental Diabetic Mice

The presence of elevated blood glucose exacerbates GFAP and VEGF immunolabeling (Figs. 2A, 2B; $P < 0.0001$) and increases GFAP mRNA levels in retinal tissue (Fig. 2C; $P = 0.007$). Treatment with the DOR-agonist prevents glial reaction and reduces the levels of VEGF in diabetic retina ($P < 0.0001$). PEDF expression, used as a marker of RPE function, was markedly reduced in diabetic retina (Figs. 2A, 2B; $P < 0.0001$). The DOR agonism completely restored retinal PEDF expression to nondiabetic levels ($P < 0.0001$).

The presence of elevated blood glucose increased claudin-1, occludin, and ZO-1 tight junction proteins immunolabeling (Figs. 3A, 3B; $P < 0.03$) and occludin mRNA levels in the RPE layer (Fig. 3C; $P = 0.01$), and the treatment with epicatechin prevented these increments in all tight junctions to nondiabetic levels ($P < 0.01$).

Retinal function was estimated through full-flash ERG. Diabetic mice showed a significant decrease in the amplitudes of the b- and c-waves (Fig. 4; $P < 0.02$). However, the oral treatment with epicatechin was not able to improve these parameters.

Efficiency and Toxicity of the shRNA Constructs

To verify the possible role of the DOR in the beneficial effects of the epicatechin treatment (Figs. 1, 2), mice received an intravitreous injection of shRNA-DOR (10^8 TU/mL, 1μL) with three different shRNA constructs for the DOR gene to determine the most efficient one. None of the constructs altered the a, b, or c-waves of full-flash ERG, indicating the absence of retinal toxicity (Supplementary Figs. S1D–F); the best efficiency was found in the TRCN0000027797 construct, with an approximately 32% reduction in DOR mRNA levels (Supplementary Fig. S1A) and a 29% reduction in retinal protein expression (Supplementary Figs. S1B, S1C).

Silencing of DOR Partially Reversed the Protective Effects of Epicatechin on the Early Markers of DR

Diabetic mice that received an intravitreous injection of shRNA-DOR (TRCN0000027797) and were treated with DOR agonist in drinking water (0.05 g epicatechin/d/animal) for 12 weeks showed partial reversal of the expression of GFAP ($P = 0.001$), VEGF ($P = 0.04$), PEDF ($P = 0.02$) (Fig. 5), claudin-1 ($P = 0.003$), occludin ($P = 0.03$), and ZO-1 ($P = 0.003$) (Fig. 6) compared with the contralateral eyes (intraocularly injected

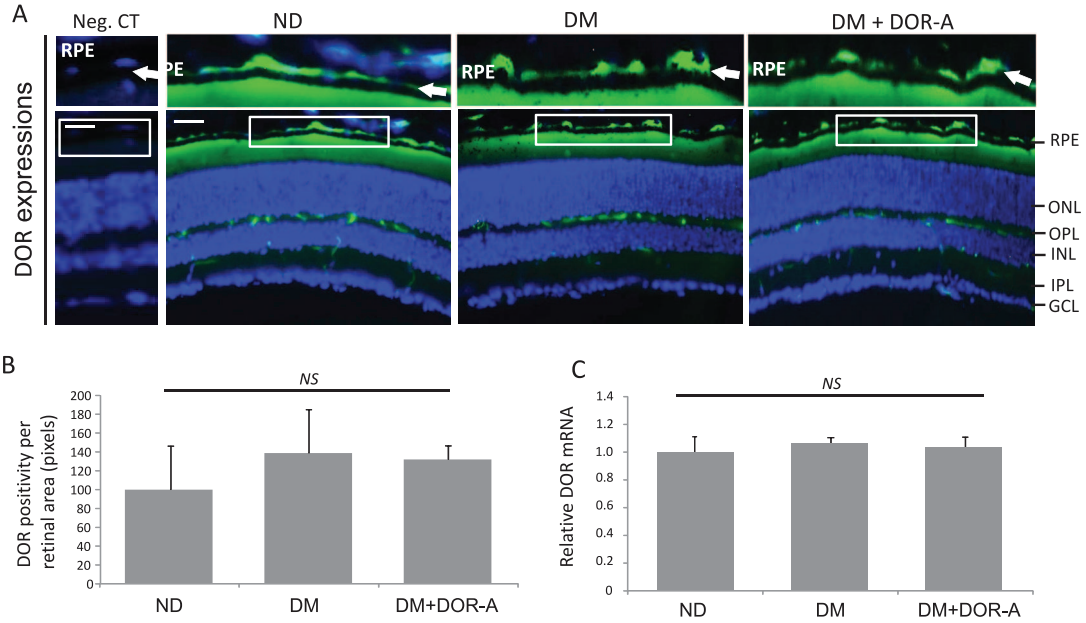

Figure 1. DOR agonist does not affect the protein levels of the DOR receptor. (**A**) Representative photomicrograph of DOR in retinal tissue. DOR immunostaining (*green*) counterstained with the nuclei marker DAPI (*blue*). Magnification: 400×. *Scale bars*: 50 μm. (**B**) Semiquantitative analyses of DOR immunofluorescence. The *bars* represent the mean ± SD for the percentage of DOR-positive per retinal area expressed as a percentage of variation relative to control. (**C**) Relative DOR mRNA expression in retinal tissue. The *bars* represent mean ± SD for the relative expression to control. DM, diabetic; DM+DOR-A, diabetic treated with DOR agonist; ND, no diabetic; NS, no significant statistical difference.

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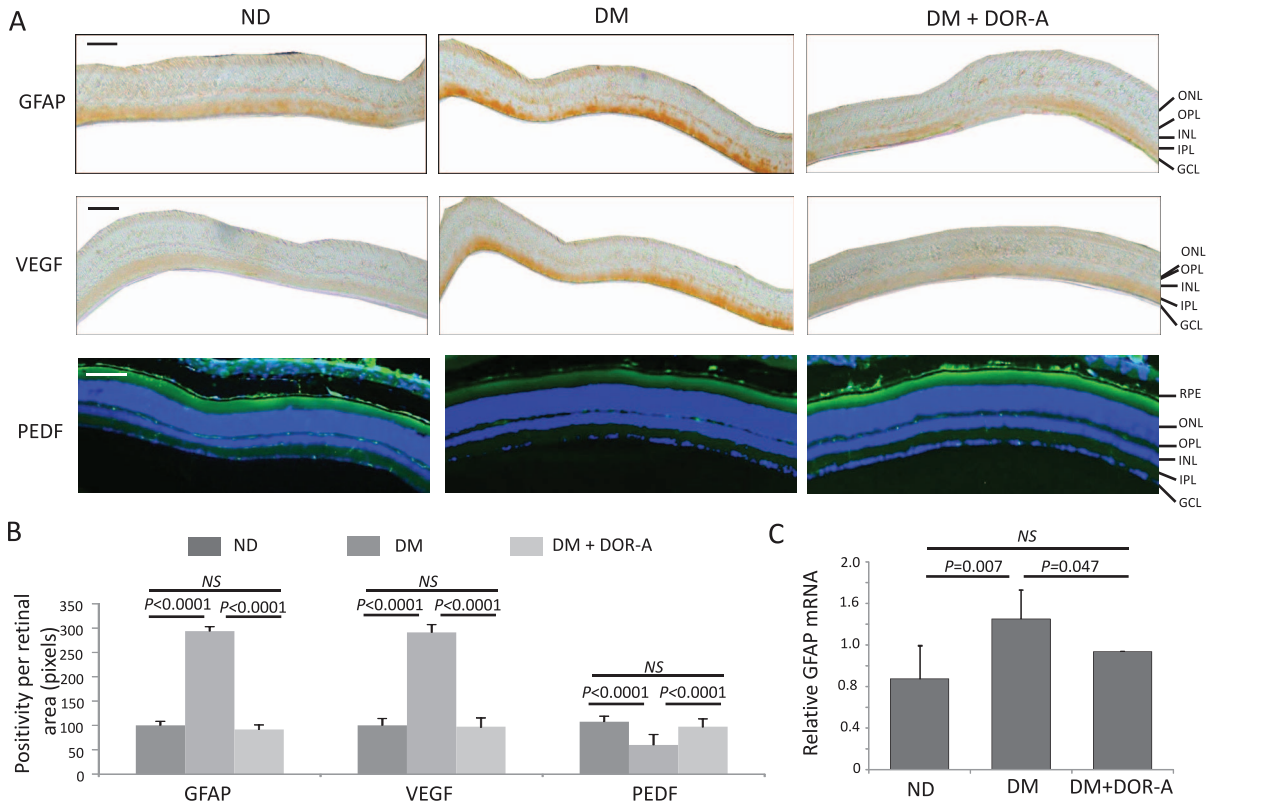

Figure 2. DOR agonist ameliorates early markers of DR and improves PEDF expression in an experimental model of diabetes using mice. (**A**) Representative photomicrograph of glial reactivity revealed by GFAP and retinal dysfunction represented by VEGF and PEDF in retinal tissue. PEDF immunostaining (*green*) counterstained with the nuclei marker DAPI (*blue*). Magnification: 200×. (**B**) Semiquantitative analyses of the GFAP, VEDF, and PEDF immunoreaction. The *bars* represent the mean ± SD for the percentage of GFAP, VEGF, or PEDF-positive per retinal area expressed as a percentage of variation relative to control. (**C**) Relative GFAP mRNA expression in retinal tissue. The *bars* represent the mean ± SD for the relative expression to control. DM, diabetic; DM+DOR-A, diabetic treated with DOR agonist; ND, no diabetic; NS, no significant statistical difference.

with empty lentivirus-green fluorescent protein labeled), which served as a control. These results indicate that epicatechin prevented the early markers of DR and protected the RPE tight junctions in diabetic mice, partially through DOR agonism.

The Activation of DOR-1 But Not 2 Inhibits Inflammatory Signaling in ARPE-19 Cells Exposed to HG Condition Restoring a Barrier Property: a Proof of Concept

In order to confirm whether the stimulation of DOR in RPE could be protective as a possible tool to maintain the barrier property under diabetic milieu conditions, ARPE-19 cells cultured under HG conditions and submitted to treatment with both activators of DOR, type 1 and type 2, in presence or not of unspecific blocker naltrindole for 24 hours. The inflammatory signaling was evaluated through western blot assay and the ARPE-19 barrier function by TER test. As shown (Fig. 7A), the ARPE-19 cells exposed to HG showed a significant increasing in p65-NFkB expression ($P < 0.008$) but the treatment with DOR-2 activator (D-Ala(2)-Deltorphin II) did not interfere in the expression of p65-NFkB, as well as in presence of naltrindole ($P > 0.05$). By the other hand, ARPE-19 under HG and treated with DOR-1 activator (D-Ala(2),D-Leu(5)]enkephalin) (Fig. 7B) showed a significant reduction in p65-NFkB expression ($P = 0.02$) achieving the normal levels. The co-treatment with naltrindole abolished the effect of DOR-1 activation ($P = 0.04$). The anti-inflammatory effect of DOR-1 activation in ARPE-19 also improved the TER feature of ARPE-19 cells barrier. At 48 hours' time point, there was a significant increase in TER in HG conditions ($P \leq 0.008$), which was prevented by the presence of DOR-1 activator up to 196 hours of observation ($P = 0.02$ and $P \leq 0.05$ for 48, 120, and 196 hours, respectively); the presence of naltrindole blocker did not alter the protective effect of DOR-1 activator ($P \geq 0.05$). This last observation could be explained by the nonspecificity of the naltrindole in presence of D-Ala(2),D-Leu(5)]enkephalin), a highly specific DOR-1 activator within this period of time.

To verify whether DOR is present in the human retina, we immunolabeled and quantified the protein and gene expressions of DOR from isolated RPE and neuroretina samples from nondiabetic and diabetic donors. The immunofluorescence images revealed that DOR is widely distributed in RPE and neuro retina (Fig. 8A). We did not find any significant difference between diabetic and nondiabetic donors in terms of protein content (1.2 ± 0.2 vs. 1.4 ± 0.5 arbitrary units [AU] for neuroretina, and 0.8 ± 0.4 vs. 1.0 ± 0.7 AU for RPE) (Fig. 8B) and mRNA levels (1.35 ± 1.27 vs. 2.12 ± 1.44 AU, and 1.98 ± 2.77 vs. 3.23 ± 5.77 AU for RPE) of DOR (Fig. 8C). These observations strengthen the potential translational importance of the DOR agonism as a new therapeutic tool to treat retinopathy in diabetic patients.

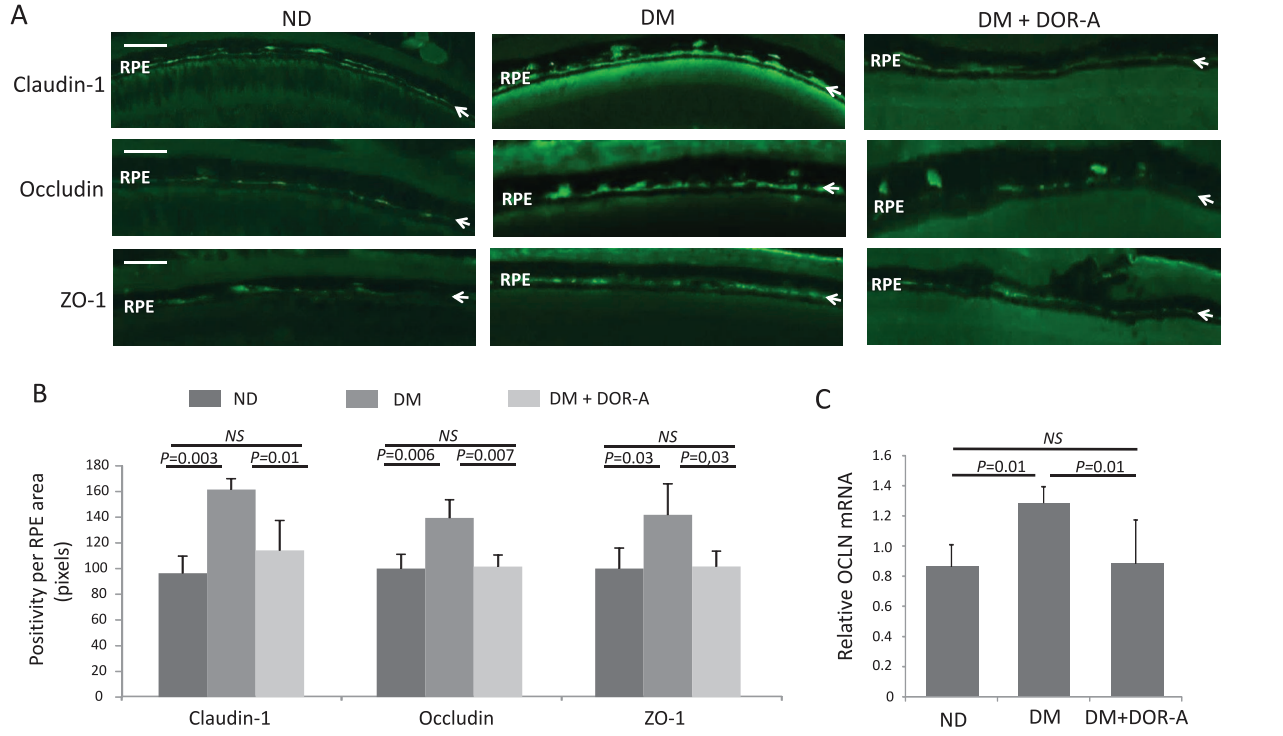

Figure 3. DOR agonist prevents external retinal barrier dysfunction in an experimental model of diabetes using mice. (**A**) Representative photomicrograph of claudin-1, occludin, and ZO-1 in the RPE layer. Magnification: 200×. *Scale bars*: 50 μm. (**B**) Semiquantitative analyses of the claudin-1, occludin, and ZO-1 immunofluorescence. The *bars* represent the mean ± SD for the percentage of claudin-1, occludin, and ZO-1-positive per RPE layer expressed as a percentage of variation relative to control. (**C**) Relative occludin mRNA expression in retinal tissue. The *bars* represent the mean ± SD of the relative expression to control. DM, diabetic; DM+DOR-A, diabetic treated with DOR agonist; ND, no diabetic; NS, no significant statistical difference.

Discussion

This study presents DOR agonism as a potential novel target for the treatment of DR in experimental model of diabetes. We provided evidence that using epicatechin as a DOR agonist efficiently prevents the early diabetic changes present in retinal tissue, such as upregulation of GFAP and VEGF followed by downregulation of PEDF. In addition to those observed changes, diabetic mice treated with epicatechin showed preservation of the outer tight junction proteins claudin-1, occludin, and ZO-1. Epicatechin was shown to be a potential scaffold for the opioid receptor ligands of the C2 and C3 positions of the epicatechin molecule.[28,29] Corroborating with possible role of DOR in these effects, diabetic mice treated with oral epicatechin but submitted to intravitreous injection of shRNA-DOR in its right eye (with an approximately 30% reduction in DOR protein expression), had the beneficial effects partly diminished. These compelling results point out DOR agonism as a possible mechanism by which epicatechin exerts the effects. In this present study, although the DOR agonism protected the retina from the ultrastructural changes related to diabetes, the ERG traces did not reflect this improvement. This observation might be due to relatively short time duration or dose dependent of the oral treatment with epicatechin. Further studies with longer duration or even with different doses are needed in order to understand this finding.

The role of RPE as the outer retinal barrier and its secretory activity in a DR experimental model require further study. The outer barrier transports water and electrolytes in one direction (from the neuroretina to the choriocapillaris) and retinol, glucose, ascorbic acid, and fatty acids in the opposite direction. The glucose depends upon the transporters (GLUT1 and GLUT3), which are diminished in high-glucose conditions.[29] This deleterious effect alters the neuron cell activity, a highly metabolic cell. However, due to the metabolism of neurons, including photoreceptors, a large quantity of water is produced, and it is actively transported by the Na^+-K^+-ATPase, located in the apical membrane, as active transport.[30] The constant elimination of the water from the subretinal space produces an adhesive force between the retina and the RPE that is lost due to the inhibition of Na^+-K^+-ATPase. Studies with cultured bovine RPE cells in high-glucose conditions have demonstrated a loss of Na^+/K^+ ATPase function, which in turn decreases permeability.[31] The impairment of glucose transporter and of Na^+/K^+ ATPase decreases the glucose offered to the neuroretina and weakens the adhesive forces between the retina and the RPE, reducing the permeability.[31] The importance of RPE to DR development depends on the photoreceptor abnormalities present early in the DR pathogenesis.[32–35] Another important feature of increasing outer retinal barrier resistance is the fluid accumulation in the subretinal space, particularly in the macular area,[36–39] which is a hallmark of diabetic macular edema and has important clinical relevance. Thus, the RPE role in DR deserves further investigation and could represent a possible site for novel therapeutic approaches.

In our previous in vitro study with ARPE-19, the cells exposed to HG, a condition that mimics diabetes, showed a significant increase in transepithelial resistance and reduced cell permeability accompanied by increased expression of ZO-1 and decrease of claudin-1 and occludin; the presence of epicatechin abrogated these effects.[14] Differently from the in vitro study using human immortalized cells, the expressions

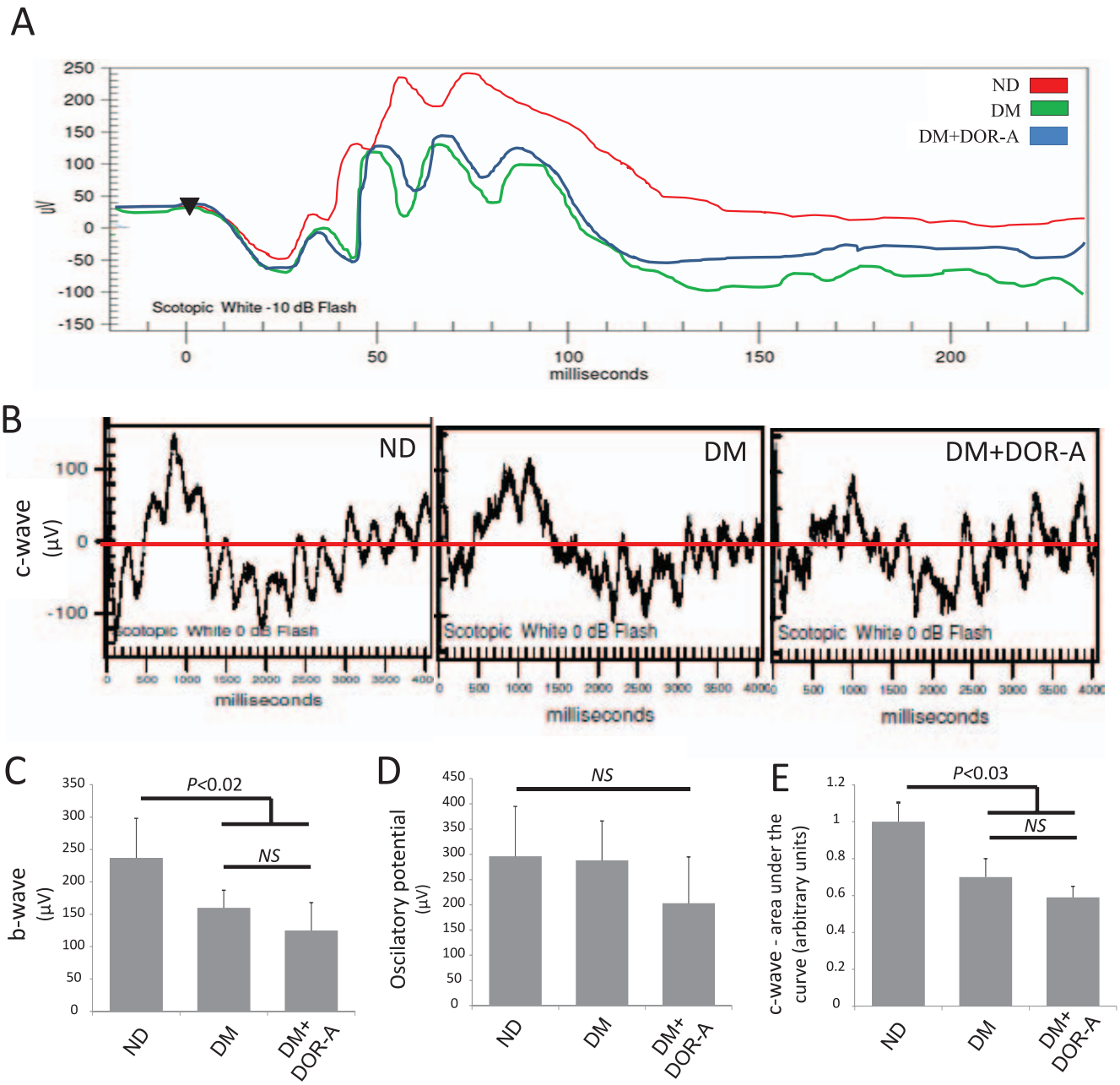

Figure 4. DOR agonist does not improve retinal function in an experimental model of diabetes using mice. (**A**) Representative full-flash ERG waveforms at 0.25 cd.s/m^2 in studied mice. *b*-wave positive deflection generated in part by the Müller and mainly by the bipolar cell potentials. (**B**) Representative waveforms of *c*-waves at a light stimulus intensity of 0 dB; *c*-wave positive deflection generated by the potentials of the RPE cells. (**C–E**) The *bars* represent the mean ± SD for the *b*-waves amplitude and oscillatory potential amplitudes expressed in microvolts (μV) and *c*-waves amplitude (μV) expressed in area under the curve (arbitrary units). DM, diabetic; DM+DOR-A, diabetic treated with DOR agonist; ND, no diabetic; NS, no significant statistical difference.

of claudin-1 and occludin were diminished under HG conditions in tissue of diabetic mice. Previous studies have yielded controversial findings regarding the transepithelial resistance and permeability of the ARPE-19 monolayer exposed to HG in vitro.[15,40,41] Villarroel et al.[40] verified the increase of claudin-1 in ARPE-19 cells exposed to HG; however, at that time, the gene silencing of claudin-1, the status of cell permeability, and resistance were not altered, demonstrating how complex is the functioning of the epithelial cell barrier. Studies with other types of epithelial cells show that stress conditions are mediated by TNFα signaling via NF-κB.[42–45] Other studies have demonstrated anti-inflammatory and anti-oxidative protective effects of activation of DOR.[46–49] A study by Husain et al.[50] showed that in a model of retinal ischemia, TNF-α production was significantly inhibited by morphine, and the combination with naloxone reversed the morphine-induced suppression of TNF-α production in vivo and in vitro. In the present study, the increased expression of the ZO-1, occludin, and claudin-1 tight junction proteins represent one function of the RPE barrier, that is, paracellular diffusion. Besides the paracellular diffusion, other mechanisms are operating to maintain the selectivity and efficiency of the RPE barrier, such as facilitated diffusion and active transport of CL^- and transcytosis.

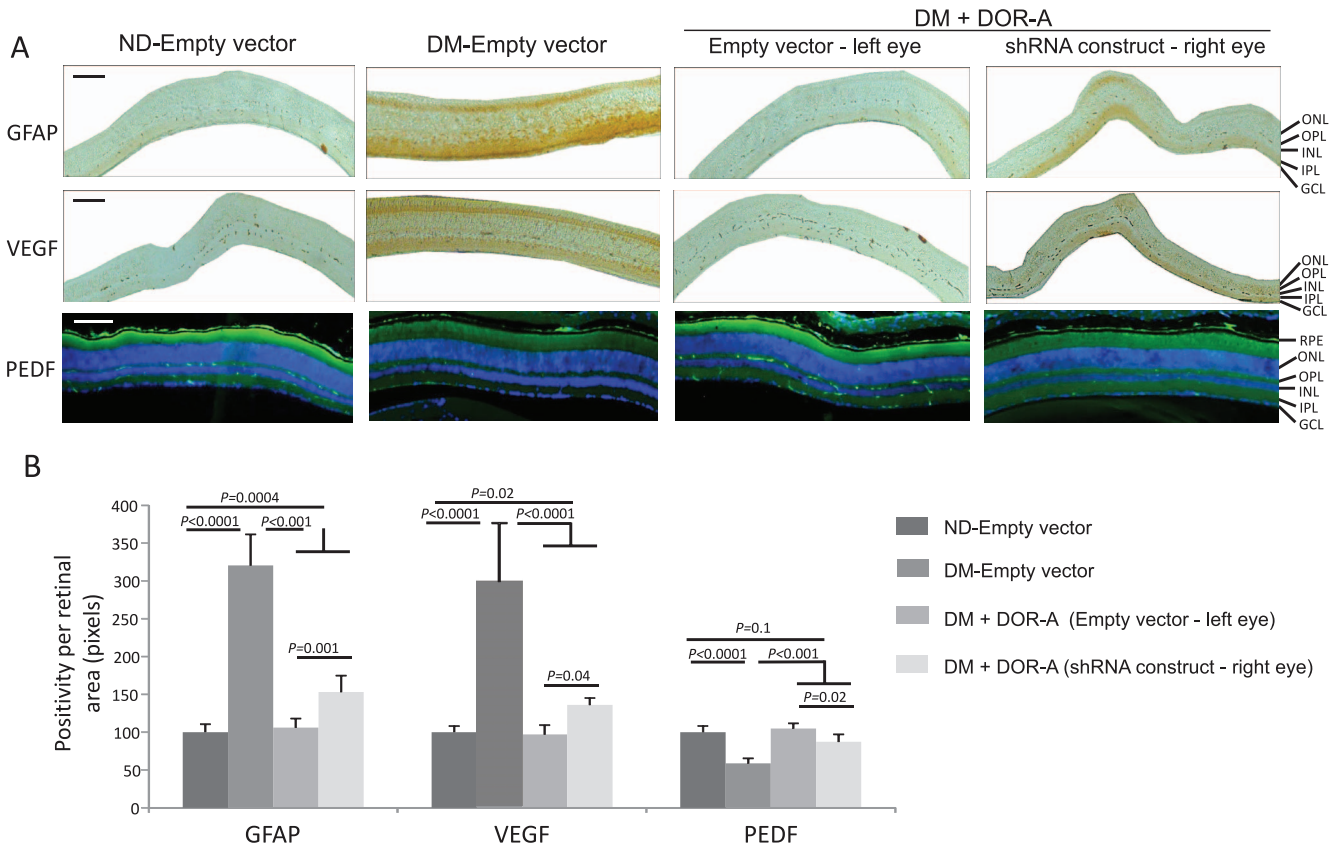

Figure 5. Silencing of DOR partially reverses the effect of its agonist in the improvement of retinal dysfunction in an experimental model of diabetes using mice. (**A**) Representative photomicrograph of glial reactivity revealed by GFAP and retinal dysfunction represented by VEGF and PEDF in retinal tissue. PEDF immunostaining (*green*) counterstained with the nuclei marker DAPI (*blue*). Magnification: 200×. *Scale bars*: 50 μm. (**B**) Semiquantitative analyses of the GFAP, VEDF, and PEDF immunoreaction. The *bars* represent the mean ± SD for the percentage of GFAP, VEGF, or PEDF-positive per retinal area expressed as a percentage of variation relative to control. DM-Empty, diabetic injected with empty virus; DM-Empty+DOR-A, diabetic injected with empty virus and treated with DOR agonist; DM-Si+DOR-A, diabetic injected with DOR silencing and treated with DOR agonist; ND-Empty, no diabetic injected with empty virus; NS, no significant statistical difference.

The profound augment of the tight junctions observed in this study may represent a late RPE barrier dysfunction and possibly represent a compensatory mechanism to rescue the barrier properties under the metabolic condition induced by diabetes. The treatment with epicatechin as a DOR agonist was able to protect the RPE tight junctions in diabetic mice and in presence of DOR silencing, this beneficial effect was mitigated.

DOR is widely expressed in peripheral and central systems, including the brain, heart, liver, and kidney.[51,52] The concept that DOR is a cardio[53–55] and neuro protector[56–58] has been demonstrated and strengthened in recent years. However, the underlying mechanisms of DOR protection remain unclear. We previously demonstrated that the agonism of DOR in in vitro conditions that mimic diabetes preserved the tight junction's maintenance in ARPE-19 cells as a negative regulator of the TNF-α pathway; however, the presence of the DOR blocker naltrindole abolished this effect.[14] In this study, we moved onward and demonstrated that the expression of DOR is present in retinal tissue, including the RPE and neuro retina, and does not change in the presence of elevated blood glucose or with treatment with epicatechin; however, under DOR silencing, the oral treatment with epicatechin was weakened in diabetic retina, suggesting that epicatechin operates partly via DOR agonism.

The epicatechin antioxidant properties are well known. Several studies have shown the protective role of flavonoids on the membrane surface through interactions with the lipid and protein components of these membranes.[28,58,59] Previous studies showed the binding of flavonoids with various receptors: androgen and/or estrogen, IGF-1, epidermal growth factor, aryl hydrocarbon, Fas, VEGF, and laminin.[55] Epicatechin, as well as many other flavonoids, has been identified as a new scaffold for the development of opioid receptor ligands. The biological results suggest that the stereochemistry of the C2 and C3 positions of the flavonoid molecule, including epicatechin, is important for the selectivity of DOR.[27,28] In this study, we showed that intravitreal injection of shRNA-DOR diminished the protective effects of epicatechin on diabetic retinal tissue, thus providing evidence that its protective effect depends on the DOR receptor, at least partly.

In presence of compromised RPE, the monolayer of these polarized cells fails in its directional transport by altering and accumulating subretinal fluid and thus disturbing the photoreceptor segments and neuro retina. Impairment of water transport from the subretinal space to choriocapillaris leads to the accumulation of water in the subretinal space.

The agonism of the DOR using (–)-epicatechin protects the tight junction integrity of the RPE and prevents the early markers of DR. The preservation of RPE function leads to an increase in the endogenous anti-angiogenic factor PEDF, thus counter-balancing the increasing of VEGF levels early on in the pathogenesis of DR. DOR agonism may be used as a new pharmacological therapy for retinal metabolic disorders as DR and disorders in which RPE tight junctions are disarranged.

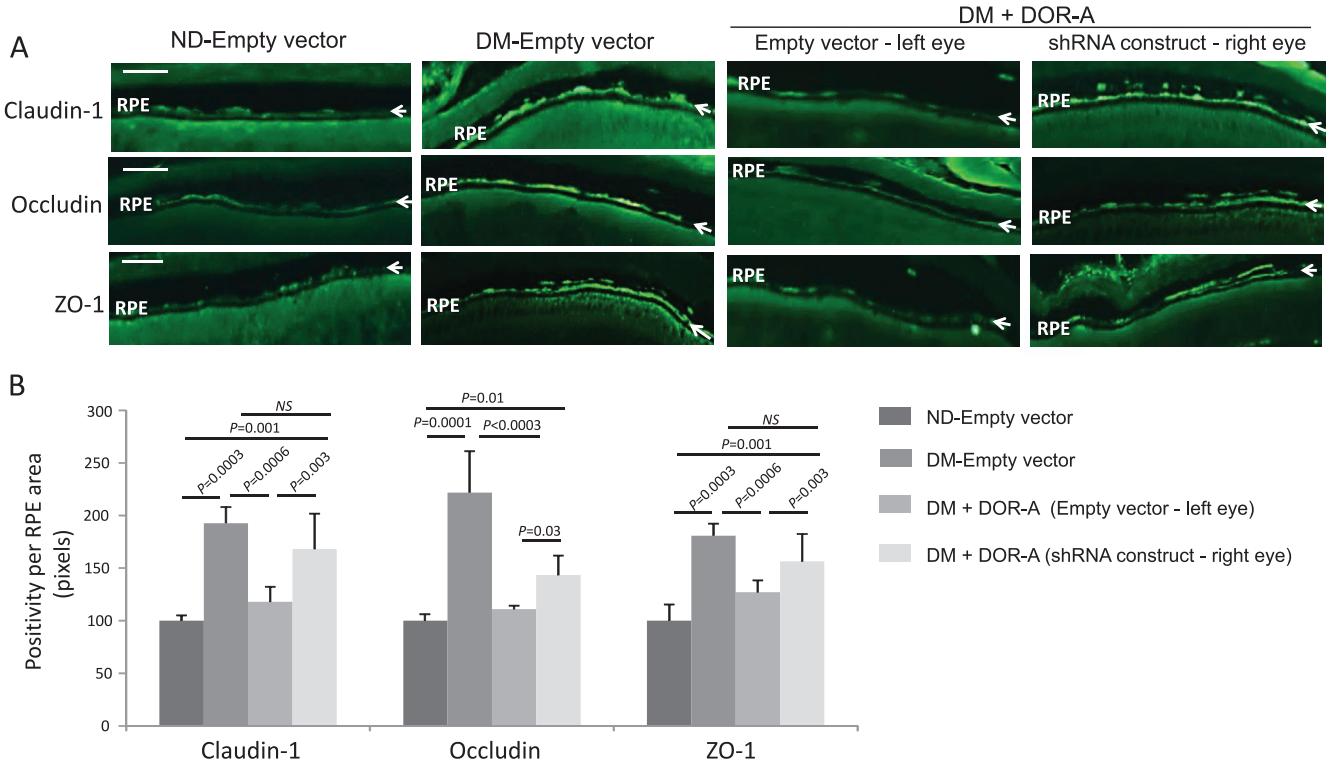

Figure 6. Silencing of DOR partially reverses the effect of its agonist in the improvement of external retinal barrier dysfunction in an experimental model of diabetes using mice. (**A**) Representative photomicrograph of claudin-1, occludin, and ZO-1 in the RPE layer. Magnification: ×200. *Scale bars*: 50 µm. (**B**) Semiquantitative analyses of the claudin-1, occludin, and ZO-1 immunofluorescence. The *bars* represent the mean ± SD for the percentage of claudin-1, occludin, or ZO-1-positive per RPE layer expressed relative to control. DM-Empty, diabetic injected with empty virus; DM-Empty+DOR-A, diabetic injected with empty virus and treated with DOR agonist; DM-Si+DOR-A, diabetic injected with DOR silencing and treated with DOR agonist; ND-Empty, no diabetic injected with empty virus; NS, no significant statistical difference.

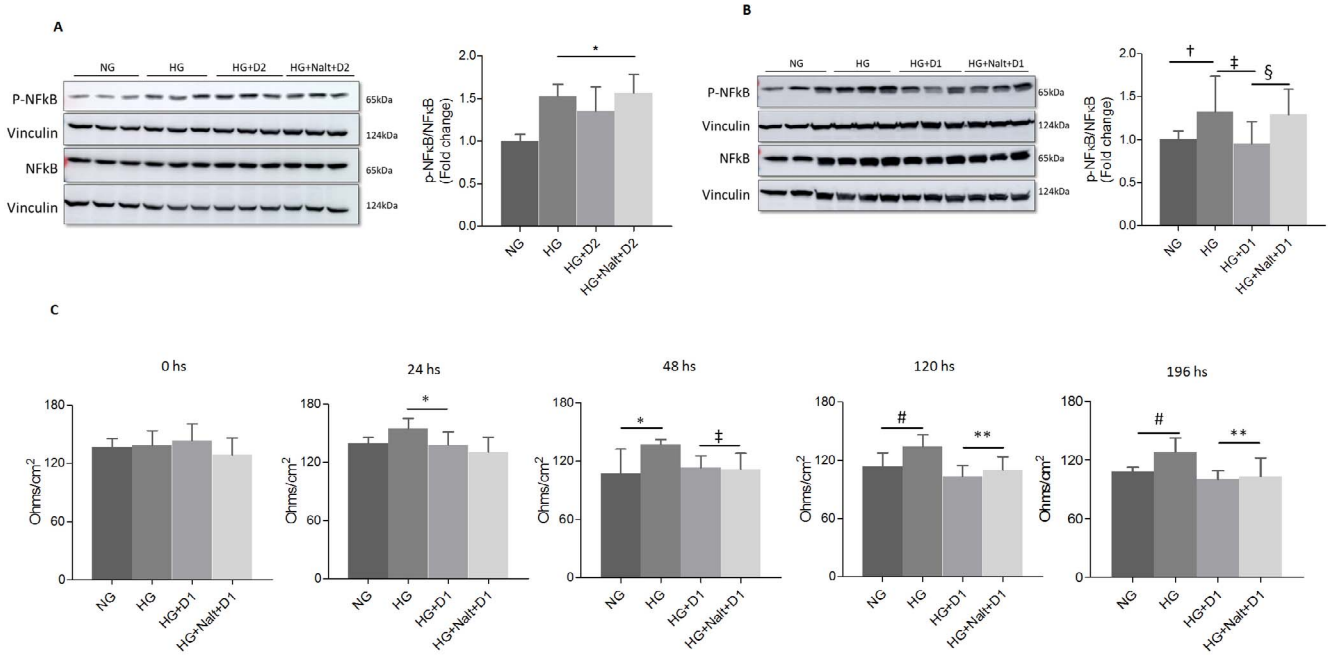

Figure 7. DOR 1 but not DOR 2 is involved in inflammatory pathway in ARPE-19. The phosphorylation of NFκB (p65-NFκB) in total cell lysate was evaluated after 24 hours in ARPE-19 cells exposed to NG, HG, HG+D2, HG+Nalt+D2 in (**A**) or to NG, HG, HG+D1, HG+Nalt+D1, $^{*}P = 0.0008$. (**B**) Protein loading was evaluated re-probing the membranes against vinculin. The *bars* represent mean ± SD. $n = 6$ per group, $\dagger P = 0.05$, $\ddagger P = 0.02$ versus NG, $\S P = 0.04$ vs. HG+D1. (**C**) TER assay. The electrical resistances were measured in ARPE-19 cells exposed to NG, HG, HG+D1, and HG+Nalt+D1 at 0, 24, 48, 120, and 196 hours. The *bars* represent the TER expressed in Ohms/cm^2 (mean ± SD) in each group. $n \geq 5$ per group. $^{*}P \leq 0.05$; $\ddagger P = 0.02$ versus HG; $\#P = 0.02$ versus NG; $^{**}P \leq 0.05$ versus HG. D1, DOR 1 activator, D-Ala(2),D-Leu(5)]enkephalin; D2, DOR 2 activator, D-Ala(2),Deltorphin II; Nalt, Naltrindole (a nonspecific DOR blocker); NG, normal.

Investigative Ophthalmology & Visual Science

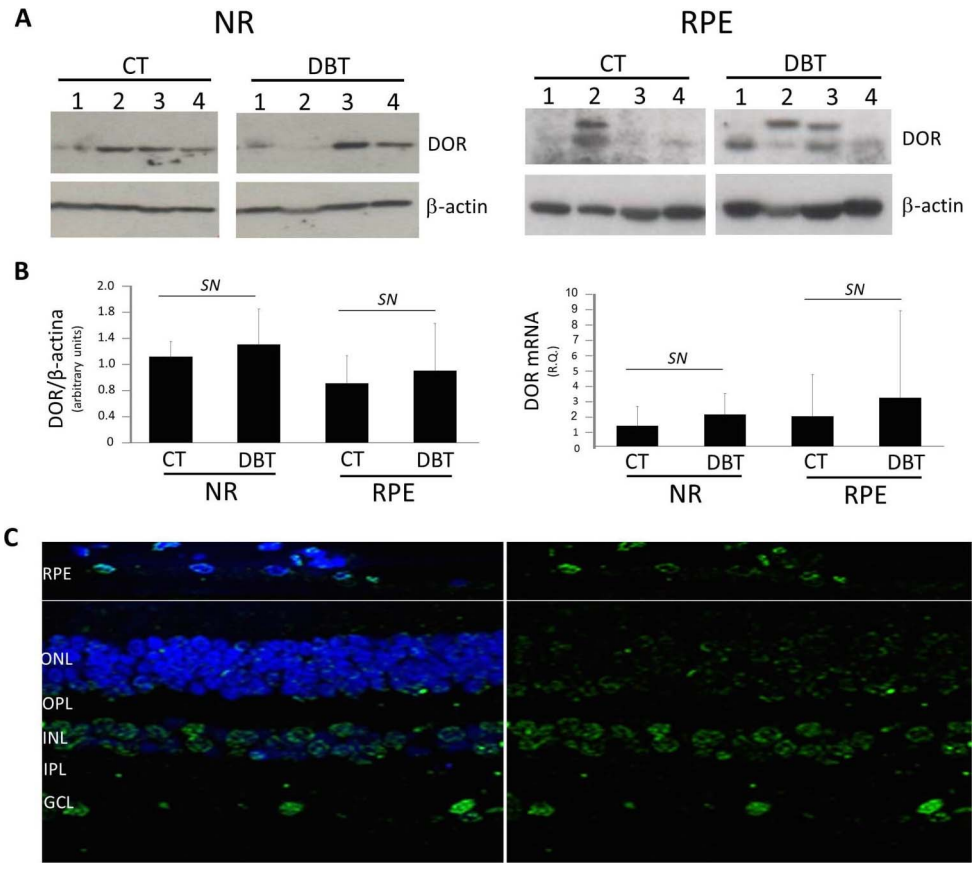

Figure 8. DOR expression in the RPE and neuroretina from human retinal donors. (**A**) DOR expression in human retinas from a representative nondiabetic donor. DOR immunostaining (*green*) in retinal paraffined sections counterstained with DAPI (*blue*). Confocal microscopy (oil immersion, 60×). *Scale bars*: 40 μm. (**B**) DOR representative images of Western blotting. DOR band values were obtained by densitometry and intensities normalized by b-actin bands. *Bars* represent the mean ± SD of the intensity ratio. (**C**) Real-time quantitative PCR of DOR mRNA. *Bars* represent the mean ± SD of the values obtained in the control versus diabetic human eye donors studied. DOR mRNA levels were normalized with β-actin. $N = 14$. GCL, ganglion cell layer; INL, inner nuclear layer; NR, neuroretina; NS, no significant statistical difference; ONL, outer nuclear layer; OPL, outer plexiform layer.

Acknowledgments

The authors thank individuals from the Renal Pathophysiology Laboratory, Investigation on Diabetes Complications, Faculty of Medical Sciences (FCM), and UNICAMP: Antônio R. Calixto for the RT-PCR experiments; Daniele C. Nascimento and Ester de S. A. Mendes for immunofluorescence assays; and Aline M. Faria and André G. de Oliveira for their technical assistance.

Supported by the European Foundation for Study of Diabetes and Sanofi in the Collaborative Clinical Diabetes Research between European and Non-European Countries Programme. DAD received a scholarship from FAPESP (2015/23258-9).

Disclosure: **J.M. Lopes de Faria**, None; **D.A. Duarte**, None; **R. Simó**, None; **M. García-Ramirez**, None; **M.N. Dátilo**, None; **F.C. Pasqualetto**, None; **J.B. Lopes de Faria**, None

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