Biochemical Pharmacology 168 (2019) 341–351

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Aflibercept regulates retinal inflammation elicited by high glucose *via* the PlGF/ERK pathway

Francesca Lazzara[a,1], Annamaria Fidilio[a,1], Chiara Bianca Maria Platania[a,1], Giovanni Giurdanella[a], Salvatore Salomone[a,b], Gian Marco Leggio[a,b], Valeria Tarallo[c], Valeria Cicatiello[c], Sandro De Falco[c,d], Chiara Maria Eandi[e,f], Filippo Drago[a,b], Claudio Bucolo[a,b,*]

[a] Department of Biomedical and Biotechnological Sciences, School of Medicine, University of Catania, Catania, Italy
[b] Center for Research in Ocular Pharmacology-CERFO, University of Catania, Catania, Italy
[c] Angiogenesis LAB, Institute of Genetics and Biophysics "Adriano Buzzati-Traverso", CNR, Naples, Italy
[d] ANBITION s.r.l, Naples, Italy
[e] Department of Surgical Sciences, University of Torino, Torino, Italy
[f] Department of Ophthalmology, University of Lausanne, Hôpital Ophtalmique Jules-Gonin, Lausanne, Switzerland

ARTICLE INFO

Keywords:
Aflibercept
Diabetic retinopathy
VEGF
PlGF
TNF-α

ABSTRACT

Diabetic retinopathy (DR) is a secondary complication of diabetes. DR can cause irreversible blindness, and its pathogenesis is considered multifactorial. DR can progress from non-proliferative DR to proliferative DR, characterized by retinal neovascularization. The main cause of vision loss in diabetic patients is diabetic macular edema, caused by vessel leakage and blood retinal barrier breakdown. Currently, aflibercept is an anti-VEGF approved for diabetic macular edema. Aflibercept can bind several members of vascular permeability factors, namely VEGF-A, B, and PlGF. We analyzed the aflibercept-PlGF complex at molecular level, through an *in silico* approach. In order to explore the role of PlGF in DR, we treated primary human retinal endothelial cells (HRECs) and mouse retinal epithelial cells (RPEs) with aflibercept and an anti-PlGF antibody. We explored the hypothesis that aflibercept has anti-inflammatory action through blocking of PlGF signaling and the ERK axis in an *in vitro* and *in vivo* model of DR. Both aflibercept and the anti-PlGF antibody exerted protective effects on retinal cells, by inhibition of the ERK pathway. Moreover, aflibercept significantly decreased ($p < 0.05$) the expression of TNF-α in an *in vitro* and *in vivo* model of DR. Therefore, our data suggest that inhibition of PlGF signaling, or a selective blocking, may be useful in the management of early phases of DR when the inflammatory process is largely involved.

1. Introduction

Diabetic retinopathy (DR) is one of the main causes of vision loss in developed countries [1]. DR is a chronic progressive disease affecting retinal microvasculature, mainly due to uncontrolled hyperglycemia and other conditions such as hypertension [2,3]. The pathogenesis of diabetic retinopathy is still not completely clear, because hyperglycemia can activate multiple biochemical pathways [4,5]. DR can progress from non-proliferative diabetic retinopathy (NPDR), characterized by retinal microvascular damage, to proliferative diabetic retinopathy (PDR), characterized by retinal neovascularization. One complication of PDR is the diabetic macular edema, which is currently treated with steroids and anti-VEGF agents, injected into the vitreous cavity [6].

The hyperglycemia-induced pathogenesis of diabetic retinopathy has been related to several biochemical alterations, such as increased advanced glycation end-product (AGE) formation and activation of protein kinase C (PKC) isoforms [7–9]. These activated pathways were linked to increased oxidative stress, inflammation, and vascular dysfunction, which can either affect the inner blood retinal barrier (pericytes and endothelial cells) or the external blood retinal barrier (RPE, retinal pigment epithelium), thus leading to vascular occlusion, retinal ischemia and blood retinal barrier (BRB) breakdown. Furthermore, these events cause the upregulation of proangiogenic and inflammatory factors such as the vascular endothelial growth factor (VEGF) [10].

Vascular endothelial growth factor A (VEGF-A) is the main angiogenetic factor that contributes to the development of choroidal

* Corresponding author at: Department of Biomedical and Biotechnological Sciences, School of Medicine, University of Catania, Catania, Italy.
E-mail addresses: claudio.bucolo@unict.it, bucocla@unict.it (C. Bucolo).
[1] These authors equally contributed to the work.

https://doi.org/10.1016/j.bcp.2019.07.021
Received 10 May 2019; Accepted 23 July 2019
Available online 25 July 2019

neovascularization [11,12]. VEGF-A belongs to the VEGF family, which includes other members: VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF) [13,14]. VEGFs exert their biological effects by binding VEGF receptors (VEGFR-1, VEGFR-2 and VEGFR-3) [15]. VEGF receptors are expressed in various cell types, e.g. vascular endothelial cells, epithelial cells, pericytes, monocytes and macrophages [16]. The VEGF-A isoform is upregulated in response to ischemia, hypoxia, inflammation, and trauma. Therefore, the anti-angiogenic therapies have been shown to be effective for the treatment of several eye diseases (neovascular age-related macular degeneration, diabetic macular edema, neovascular glaucoma), via the pharmacological inhibition of VEGF-A signaling [17–21].

Aflibercept is a soluble VEGF decoy receptor (fusion protein) generated with Trap technology and it binds several members of the VEGF family: VEGF-A, VEGF-B and PlGF, which activate the VEGFR1 [12,22]. Therefore, aflibercept can further inhibit VEGFR1 signaling by trapping the PlGF ligand. The signaling of VEGFR1 has been reported to modulate vascular development, angiogenesis, cell survival, inflammation and chemotaxis of inflammatory cells [23]. Moreover, PlGF is a multifunctional peptide associated with angiogenesis-dependent pathologies in several ocular diseases. In fact, vitreous levels of PlGF positively correlated with retinal ischemia in DR patients [24,25]. Furthermore, it has been demonstrated that a PlGF antibody reduces inflammation and angiogenesis in a mouse model of DR [26].

Regarding biochemical pathways, VEGF signaling is a complex pathway that includes various receptor/growth factor interactions and subsequent activation of kinases. Particularly, VEGF-A and PlGF binding to VEGFR1 leads to phosphorylation and activation of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) [27], and also VEGFR2 signaling has been linked to ERK activation, e.g. for mesenchymal stem cell differentiation to endothelial cells [28]. Furthermore, expression of inflammatory cytokines might be mediated by activation of mitogen-activated protein kinases (MAPKs) [29]. The MAPKs, as well as ERKs, are kinases that regulate several cellular processes, such as stress response, inflammation, proliferation, differentiation, apoptosis, and survival [30]. MAPKs in turn can influence VEGF levels, thus leading to increased vascular permeability and angiogenesis [31]. Moreover, it has been reported that the ERK pathway can influence NF-κB activation, by the regulation of NF-κB-dependent genes expression, e.g. inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and tumor necrosis factor-alpha (TNF-α) [32].

Firstly, we assessed at the atomistic level (molecular dynamics simulations and prediction of binding free energy of complexes) the interaction of aflibercept with PlGF and compared this complex with the all-atom model of aflibercept bound to VEGFA. After the *in silico* analysis, to study the mechanism of high glucose (HG)-induced inflammation in human retinal endothelial cells (HRECs) and retinal pigmented epithelial (RPE) cells, we hypothesized that the effects of HG on the expression of inflammatory cytokines might be mediated by the ERK pathway. In order to evaluate and discriminate the effects of aflibercept and anti-PlGF antibody on the ERK pathway, we evaluated the expression levels of VEGF-A, PlGF and TNF-α in two *in vitro* models. Moreover, we analyzed TNF-α expression in retinas of diabetic rats.

2. Material and methods

2.1. In silico studies

Molecular modeling of the binding domain of aflibercept (VEGFR1d2_R2d3) was carried out as reported previously [33]. Structures of VEGF-A and PlGF were retrieved from the Protein Data Bank repository, PDB:1CZ8 and PDB:1RV6, respectively. In the 1CZ8 structure VEGF-A is in complex with Fab of bevacizumab, whereas in 1RV6 PlGF is in complex with domain 2 of VEGFR1. VEGFR1d2_R2d3, VEGFA and PlGF structures were optimized with an energy minimization protocol, before protein-protein docking. Protein-protein docking was carried out by accessing to the web server PyDock, as previously described [33]. VEGFR1d2_R2d3/VEGFA and VEGFR1d2_R2d3/PlGF complexes were subjected to all atom molecular dynamics simulations (MD) in water environment with GROMACS 4.6 [34]. Three equilibration steps were carried out before MD simulation (20 ns per each independent replica, 3 replicas per simulated complex). The simulation protocol was previously described [33]. We herein analyzed the root mean square deviation (RMSD), root mean square fluctuation (RMSF) and number of contacts (within 3.5 Å) at the protein-protein interface. Low and stable RMSD is commonly considered as an index of structural stability of protein during simulation. However, RMSF is more descriptive of conformational stability of proteins, than RMSD. Molecular Mechanics-Poisson Boltzmann Surface Area calculation (MM-PBSA) was carried out on three independent replicas of MD simulations of VEGFR1d2_R2d3/VEGFA and VEGFR1d2_R2d3/PLGF complexes. MMPBSA calculations are time-consuming and generally aimed at the evaluation of each energetic contribution (electrostatic, Van der Waals, polar and apolar free energy) to overall binding free energy [33].

2.2. Materials

Rabbit phospho-p44/42 MAPK (phospho-Erk1/2, catalog n. 9101), rabbit p44/42 MAPK (Erk1/2, catalog n. 9102), rabbit anti-TNF-α (catalog n. 11948) and rabbit anti-vinculin (catalog n. 13901) were purchased from Cell Signaling Technology (Danvers, MA); mouse anti-GAPDH (catalog n. 2118) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary goat anti-rabbit IRDye 800CW conjugated antibody (#926-32211) and secondary goat anti-mouse IRDye 680LT, (#926-68020) were purchased from LI-COR (Lincoln, NE). Aflibercept (Eylea ®) was purchased from Bayer Pharma (Berlin, Germany). The anti-PlGF was purchased from Thrombogenics (Leuven, Belgium).

2.3. In vitro studies

Human retinal endothelial cells (HRECs) were purchased from Innoprot® (Derio – Bizkaia, Spain). Cells were cultured at 37 °C, in humidified atmosphere (5% CO_2), in Endothelial cell medium (ECM) supplemented with 5% fetal bovine serum (FBS), 1% ECGS (Endothelial Cell Growth Supplement) and 100 U/ml penicillin 100 µg/ml streptomycin. After reaching confluence (approximately 70%), cells were starved for 16 h; then the medium was shifted to an ECM supplemented with 2.5% FBS. Cells growth in medium containing 5 mM glucose (physiological glucose concentration) served as control group. HRECs were also exposed to medium containing 40 mM glucose (high glucose, HG) [35]. HRECs, exposed to HG, were treated with aflibercept (1, 10, 40 and 100 µg/ml) or anti-PlGF (1, 10, 25 and 50 µg/ml) for 48 h. Primary mouse RPE cells (pmRPE) were isolated from 6 to 8 weeks-old C57Bl6/J. Male mice C57Bl6/J, weighing 20–25 g, were purchased from Charles River (Calco, Italy). Retinas sampling from mice was carried out in accordance with European directives no. 2010/63/UE and Italian directives D.L. 26/2014. Experimental protocols were approved by the Italian Ministry of Health (authorization no. 695/2015-PR of July 17, 2015). Mouse sacrifice was carried out through cervical dislocation. Enucleated eyes from sacrificed mice were cleaned of fat and extra tissues and incubated in a 1:1 mixture of 0.8 mg/ml collagenase and 4% dispase. Then, the cornea and anterior segments were removed and the 'eye cup' was flattened with four incisions, rinsed in chelating agent (Versene; Invitrogen, Life Technologies, Carlsbad, CA), then incubated with 0.48 mM EDTA and incubated for 10 min in a moisture box with 2% dispase. Eye cups were washed with PBS, therefore, retinas were gently removed and the RPE were digested with 2% dispase for 10 min at 37 °C. The digested eye cup was then washed with PBS in order to remove the dispase solution; 20 µL of DMEM 10% FBS was added and RPE were then gently scraped from the "eye cup" in a 12 well plate (1 eye/well), by rubbing the cup against the bottom of

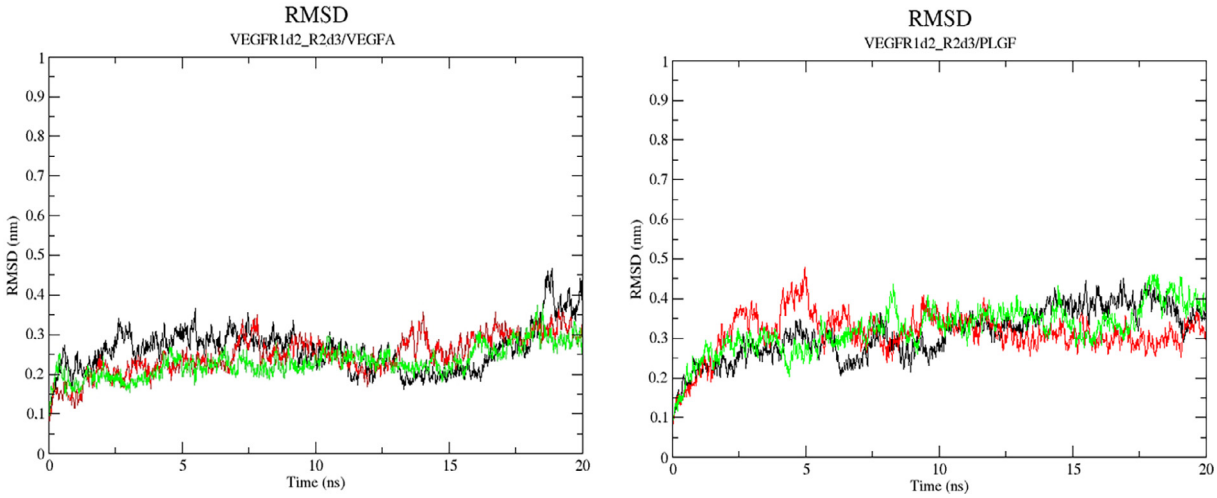

Fig. 1. RMSD of VEGFR1d2_R2d3/VEGFA and VEGFR1d2_R2d3/PlGF complexes (3 independent replicas of 20 ns simulations). RMSD profiles of VEGFR1d2_R2d3/VEGFA and VEGFR1d2_R2d3/PlGF complexes. Black lines represent replica 1, red lines represent replica 2 and green lines represent replica 3 of each simulated system. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

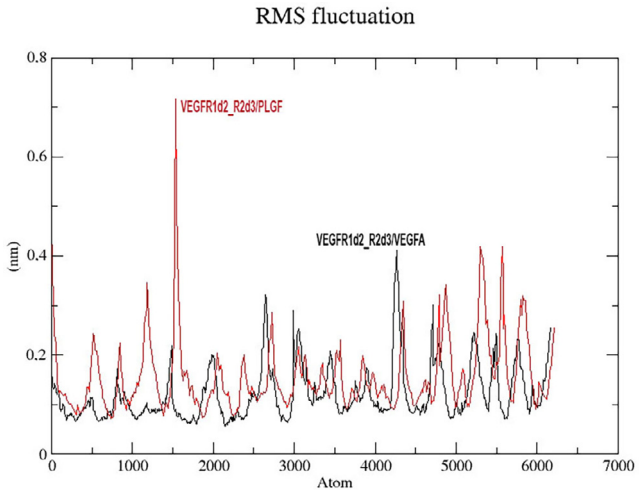

Fig. 2. Mean RMSF of complexes during 20 ns of simulations. Red line represents RMSF of VEGFR1d2_R2d3/PlGF and black line represents RMSF of VEGFR1d2_R2d3/VEGFA. The RMSF profile is the mean of 3 replicas of each simulated complex during 20 ns of MD simulations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

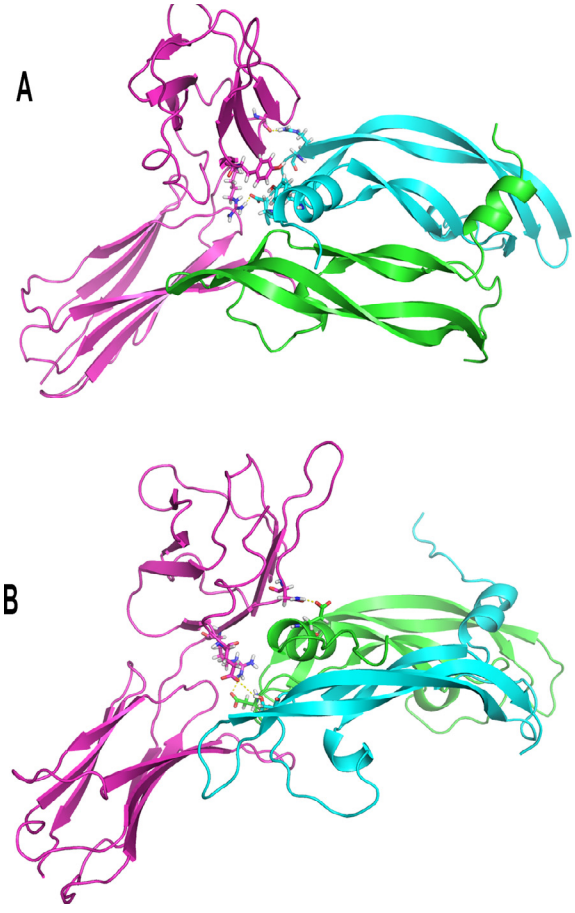

Fig. 3. Main contacts at VEGFR1d2_R2d3 and growth factor interface. Panel A. VEGFR1d2_R2d3/VEGFA; panel B VEGFR1d2_R2d3/PlGF. Magenta cartoon represents the VEGFR1d2_R2d3 bound to dimer of growth factors (green and cyan cartoons). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the well with a pipette tip. Finally, 20% FBS DMEM with antibiotics was added in the plate wells and cells were grown until confluence. RPE cells were pooled and growth in DMEM (Dulbecco's Minimal Essential Medium, Euroclone, Milan Italy) containing 20% FBS and standard antibiotics at 37 °C in a humidified environment containing 5% CO_2. RPE cells (passage 5) were plated at 70–80% of confluence in DMEM + 20% FBS. After 24 h, medium was replaced with DMEM + 1% FBS containing normal glucose (5 mM) or high glucose (25 mM). The RPE cells were treated with equimolar amounts of aflibercept (1, 10 and 50 μg/mL) or anti-PlGF (1.3, 13 and 65 μg/mL) for 24 h.

2.3.1. Cell viability

HRECs viability was evaluated with quantification of LDH levels in cell media. Cells were seeded in 96-well plates at a density of $2x10^4$ cells/well, to obtain optimal cell density. LDH release was assessed with a commercial kit (Roche Diagnostics, Basel, Switzerland) and reported as LDH (% control): $(abs_x \div abs_{ctrl+}) \times 100$. In the equation abs_x is absorbance in the x well, and abs_{ctrl+} is the average absorbance of positive control cells (retinal endothelial cells lysed accordingly to manufacturer's protocol); absorbance values were corrected by subtracting blank. The absorbance values were measured at 450 nm using a

Table 1
Interactions between aflibercept binding domain and growth factors. Residue X of aflibercept directly interacts with residue Y of growth factors.

Complex	X (aflibercept)-Y (growth factor) interacting residues
VEGFR1d2_R2d3/VEGFA	Asn91-Glu11 Hbond, Glu16-Ser40 Hbond, Arg96-Asp65 Hbond
VEGFR1d2_R2d3/PlGF	Glu73-Arg103 Hbond, Arg96-Asp63 Hbond, Tyr 92-Tyr 21 π-π stacking

microplate reader (VarioSkan, Thermo Fisher Scientific, Waltham, MA).

2.3.2. Western Blot

HRECs were cultured in 60 mm Petri dishes ($2x10^5$). Proteins of whole cell lysates were extracted with RIPA Buffer, including protease and phosphatase inhibitors cocktail (Sigma-Aldrich, St. Louis, MO). Total protein content, in each cell lysate sample, was determined by means of the BCA Assay Kit (Pierce™ BCA Protein Assay Kit, Invitrogen, Life Technologies, Carlsbad, USA). Extracted proteins (30 μg) were loaded on 4–12% tris–glycine gel. After electrophoresis, proteins were transferred into a nitrocellulose membrane (Invitrogen, Life Technologies, Carlsbad, CA). Immunoblot was preceded by 30 minutes incubation with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) to membranes. Membranes were incubated overnight (4 °C) with appropriate primary phospho-p44/42 MAPK (1:500 dilution), p44/42 MAPK (1:500 dilution) and anti-GAPDH (1:500 dilution) antibodies. After overnight incubation, the membranes were then incubated with secondary fluorescent antibodies (1: 20,000 dilution) for 1 h at room temperature. Immunoblot was detected through Odyssey imaging system (LI-COR Biosciences, Lincoln, NE). Densitometry analyses of blots were performed at non-saturating exposures and analyzed using the ImageJ software (NIH, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Values were normalized to GAPDH, which was also used as loading control.

Total protein extracts from pmRPE cells were obtained by cell lysis in Tris-HCl 20 mM pH 8, NaCl 150 mM, Triton X-100 1%, EDTA 10 mM, Glycerol 10% and ZnAc 1 mM supplemented with protease and phosphatase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). The protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Milan, Italy). 100 μg of total protein extracts were separated in a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline-Tween 0.1% (TBS-T) and then probed with antibodies against: p-ERK (1:1000), ERK (1:1000), TNF-α (1:1000) and Vinculin (1:10,000), purchased from Santa Cruz Biotechnology, Heidelberg, Germany. The secondary antibodies were purchased from DAKO Agilent Santa Clara, CA (1:10,000). The signals were visualized by chemiluminescence using ECL substrate (Advansta, San Jose, CA). Densitometric analyses were performed using ImageJ software (NIH, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

2.3.3. Extraction of total RNA and cDNA synthesis

Extraction of total RNA, from HRECs, was performed with TRIzol Reagent (Invitrogen, Life Technologies, Carlsbad, CA). The A_{260}/A_{280} ratio of the optical density of RNA samples in agarose gel electrophoresis blots (measured with Multimode Reader Flash, VarioSkan™) was 1.95–2.01, assessing the adequate RNA purity. cDNA was synthesized from 2 μg RNA with a reverse transcription kit (SuperScript™ II Reverse Transcriptase, Invitrogen, ThermoFisher Scientific, Carlsbad, CA).

2.3.4. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Real-time RT-PCR was carried out with the LightCycler ® 2.0 (Real-Time PCR System Roche Life Science, Roche Diagnostics GmbH, Mannheim, Germany). The amplification reaction mix included iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Milan, Italy) and 1 μL (100 ng) of cDNA. Forty-five amplification cycles were carried out for each sample. Results were analyzed with the $2^{-\Delta\Delta}$Ct method. VEGF and PlGF mRNAs expression (*in vitro* experiments) were normalized to β-actin mRNA levels. On the contrary, TNF-α mRNA (*in vivo* experiments) expression was normalized to 18S mRNA. Primers were purchased from Eurofin Genomics (Milan, Italy). Quantitative PCR experiments followed the MIQE guidelines. Forward and reverse primer sequences (for human and rat analyzed genes) are herein listed: i. human β-actin (Forward 5′-TCCACCTTCCAGCAGATGTG-3′, Reverse 5′-GCATTTGCGGTGGACGAT-3′); ii. human PlGF (Forward 5′-ATGTTC AGCCCATCCTGTGT-3′, Reverse 5′-CTTCATCTTCTCCCGCAGAG-3′); iii. human VEGF-A (Forward 5′-GAGGTTTGATCCGCATAATCTG-3′, Reverse 5′-ATCTTCAAGCCATCCTGTGTGC- 3′); iv. rat TNF-α (Forward 5′-ACCACGCTCTTCTGTCTACTG-3′, Reverse 5′CTTGGTGGTTTGCTAC GAC-3′); v. rat 18S (Forward 5′-CATTCGAACGTCTGCCCTAT-3′, Reverse 5′GTTTCTCAGGCTCCCTCTCC-3′).

2.3.5. Enzyme-linked immunosorbent assay (ELISA)

HRECs were cultured in 60 mm Petri dishes (2×10^5). VEGF-A and TNF-α levels were quantified in 200 μL cell media. Commercial ELISA kits (ENZ-KIT156-0001 and ADI-900-099, Enzo Life Science, Farmingdale, NY) were used for VEGFA and TNF-α detection, respectively. Total protein content in cell medium was analyzed prior to the ELISA assay, accordingly to the manufacturer's instructions.

2.4. In vivo studies

Sprague-Dawley rats (200–250 g) were purchased from Envigo (San Pietro a Nadisone, Udine, Italy). Animals were housed under standard conditions, with free access to standard chow and water, in a light-controlled (12-h light/12-h dark; lights on at 6 am) room with standard temperature and humidity conditions (21 ± 3 °C and 54 ± 4% humidity). Animals were randomly assigned to four experimental groups: 1) negative control group (Ctrl) non-diabetic rats (n = 4) injected (i.p.) with citrate buffer, and after 15 days injected intravitreally with vehicle; 2) non-diabetic rats (n = 4) treated with intravitreal injection of aflibercept (160 μg) (Ctrl + afl group); 3) diabetic rats (STZ group; glycemia > 250 mg/dl) injected (i.p.) with streptozotocin (STZ) and after 15 days injected intravitreally with vehicle (n = 4); 4) diabetic rats (STZ + afl group; glycemia > 250 mg/dl) injected (i.p.) with streptozotocin (STZ) and after 15 days injected intravitreally with aflibercept (n = 4). Animals were sacrificed 48 h after intravitreal injection (vehicle or 160 μg aflibercept).

Rats were treated accordingly to the Association for Research in

Table 2
Energetic contribution to binding free energy.

Complex	Binding Parameters			MM-PBSA Energy terms (KJ/mol)				
	$K_{on}/10^5$ (M^{-1} s^{-1})	$K_{off}/10^{-5}$ (s^{-1})	K_D (pM)	$\Delta E_{binding}$	ΔE_{VdW}	$\Delta E_{electrostatic}$	ΔG_{Polar}	ΔG_{Apolar}
VEGFR1d2_R2d3/VEGFA	410	2.01	0.49	−1440 ± 90	−307 ± 50	−1433 ± 100	1050 ± 100	−750 ± 40
VEGFR1d2_R2d3/PLGF	17.5	6.81	38.9	−1270 ± 10	−280 ± 40	−1170 ± 40	840 ± 70	−660 ± 30

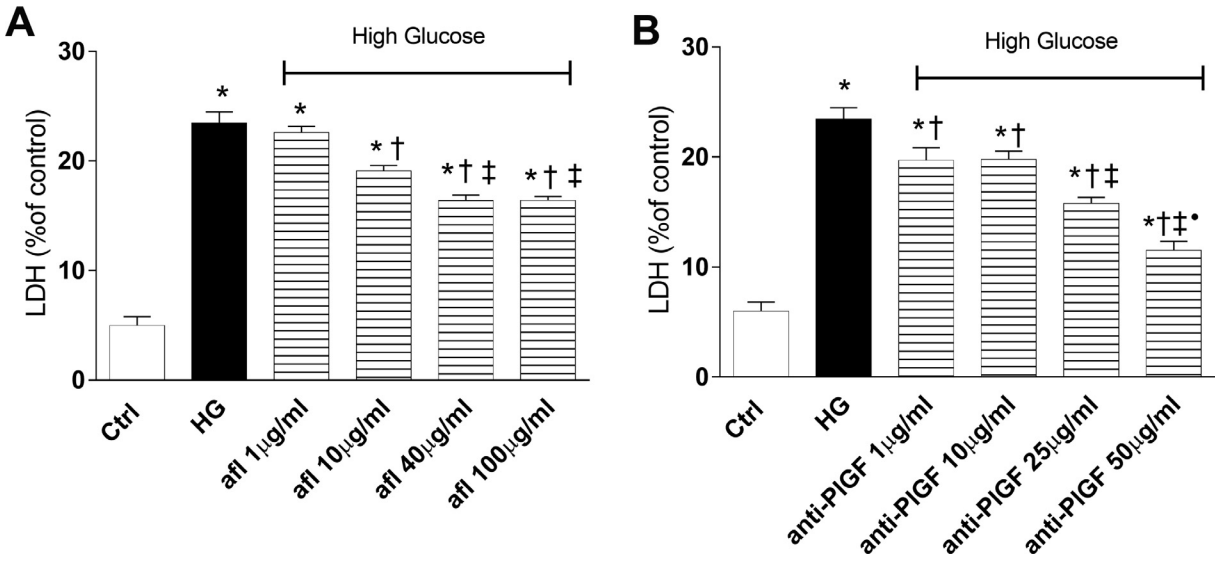

Fig. 4. LDH assay. Aflibercept (1–100 μg/mL) (A) and anti-PlGF (1–50 μg/mL) (B) protect human retinal endothelial cells from high glucose-induced LDH release, 48 h after incubation. Data are shown as % of control LDH release. Ctrl stands for cells grown in medium containing 5 mM glucose concentration. HG stands for cells grown in medium containing 40 mM glucose concentration Values are reported as mean ± SD (n = 8; each run in triplicate). *p < 0.05 *vs.* control; † p < 0.05 *vs.* HG, ‡ p < 0.05 *vs* 10 μg/mL aflibercept or anti-PlGF, • p < 0.05 compared to 25 μg/mL anti-PlGF treatment.

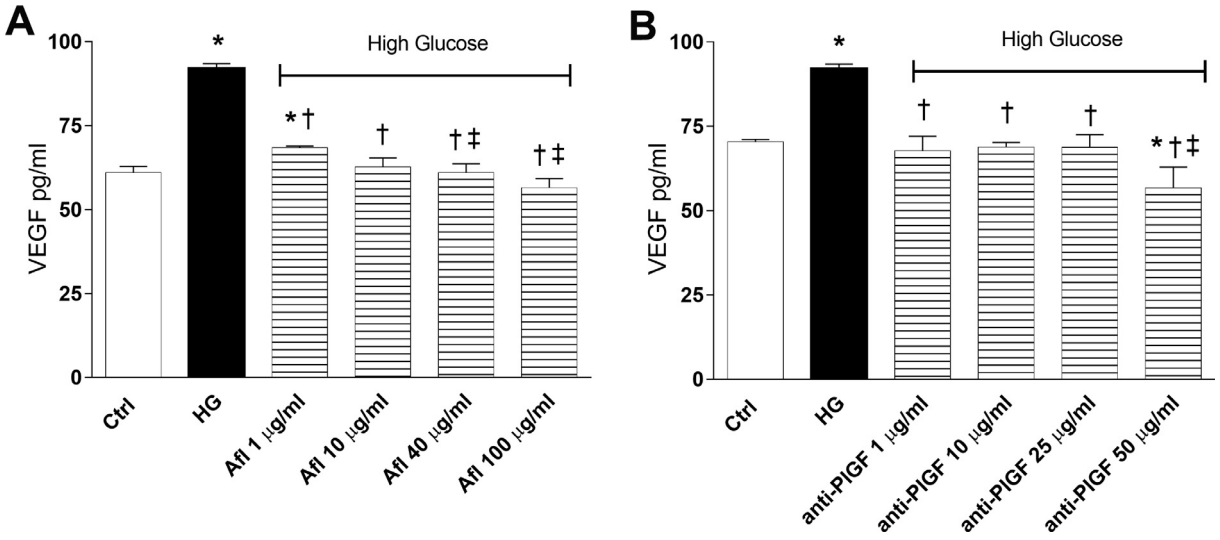

Fig. 5. VEGF-A ELISA. Aflibercept (1–100 μg/mL) (A) and anti-PlGF (1–50 μg/mL) (B) decreased VEGF-A release induced by high glucose levels, 48 h after incubation. Values are reported as mean ± SD (n = 8; each run in triplicate). * p < 0.05 *vs.* control; † p < 0.05 *vs.* HG. ‡ p < 0.05 *vs* 10 μg/mL aflibercept or 25 μg/mL anti-PlGF.

Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Protocols were approved by the Italian Ministry of Health (authorization no. 1172/2016-PR of November 13, 2016). Diabetes was induced with a single injection (i.p.) of streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO) as previously described [36,37]. After 12 h fasting, two groups of animals (STZ and STZ + afl groups) received a single injection of STZ (60 mg/kg body weight) in 10 mM sodium citrate buffer, pH 4.5 (1 mL/kg dose/volume). The control group of animals received a (4 μL) injection of citrate buffer. Twenty-four hours post STZ injection blood glucose levels were measured. Rats with blood glucose levels > 250 mg/dL were considered diabetic. Glycaemia was monitored with a blood glucose meter (Accu–CheckActive; Roche Diagnostic, Milan, Italy), along with rat body weight. Two weeks after STZ administration, diabetic rats received by intravitreal administration, 160 μg of aflibercept dissolved in PBS solution (final volume = 4 μL). This dose, converted to human equivalent dose (HED) 1.3 mg/ml (160 μg/20 μL of rat vitreous = 8 mg/ml × 0.162 [38]), is comparable with the three monthly intravitreal doses of aflibercept (2 mg/4.4 mL of human vitreous × 3 = 1.35 mg/ml aflibercept dose). Before intravitreal injection, animals were anesthetized by intravenous injection of 5 mg/kg Zoletil® (tiletamine HCl and zolazepam HCl, Virbac, Milan, Italy), and 1 drop in each treated eye of local anesthetic 0.4% oxybuprocaine (Novesina®, Novartis, Origgio, Italy). Vehicle treated animals were subjected to intravitreal injection of 4 μL of PBS solution (pH 7.4). Aflibercept treated animals received 4 μL intravitreal injection of aflibercept (40 μg/μL) solution. Forty-eight hours after treatment, rats were killed by CO_2 inhalation, the eye enucleated, the retina collected and treated with TRIzol reagent in order to extract total RNA (methods 2.3.3 and 2.3.4) or RIPA buffer (ThermoFisher Scientific, Carlsbad, CA), including protease and phosphatase inhibitors cocktail (Sigma-Aldrich, St. Louis, MO) for ELISA quantification of retinal TNFα (methods 2.3.5). TNFα quantification in the rat retina was carried out following manufacturer's instructions of ELISA kit, and RIPA buffer was used as blank within

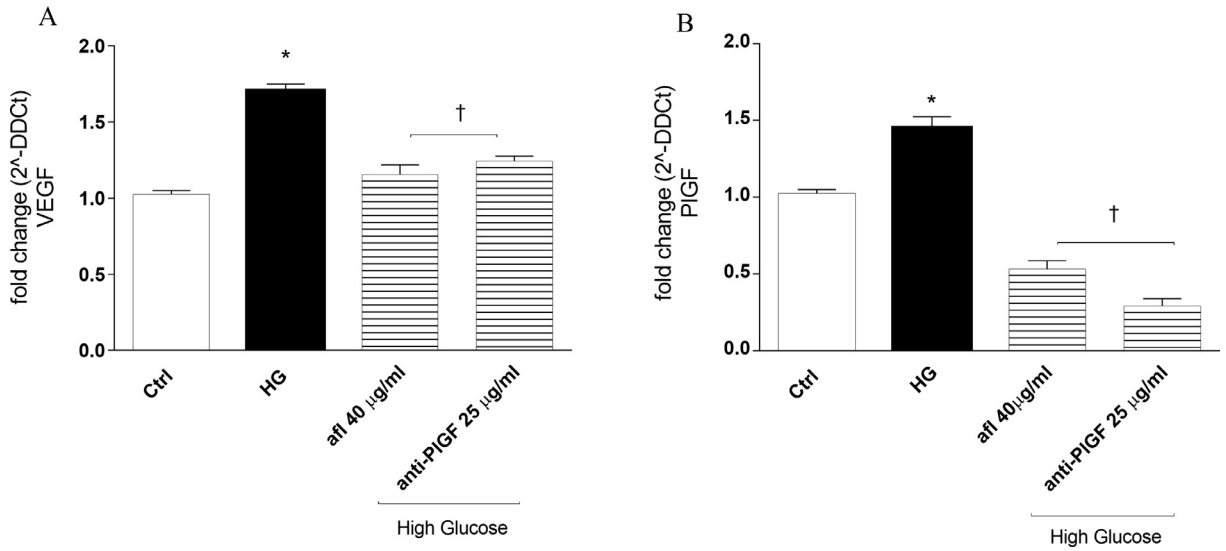

Fig. 6. Real-time PCR. Aflibercept and anti-PlGF reduced VEGF-A (A) and PlGF (B) mRNA expression. HRECs were treated for 48 h with high glucose concentrations (40 mM), aflibercept (40 µg/ml), and anti-PlGF (25 µg/ml). The mRNA levels were evaluated by qPCR. Each bar represents the mean value ± SD (n = 4; each run in triplicate). *p < 0.05 *vs.* control; †p < 0.05 *vs.* HG.

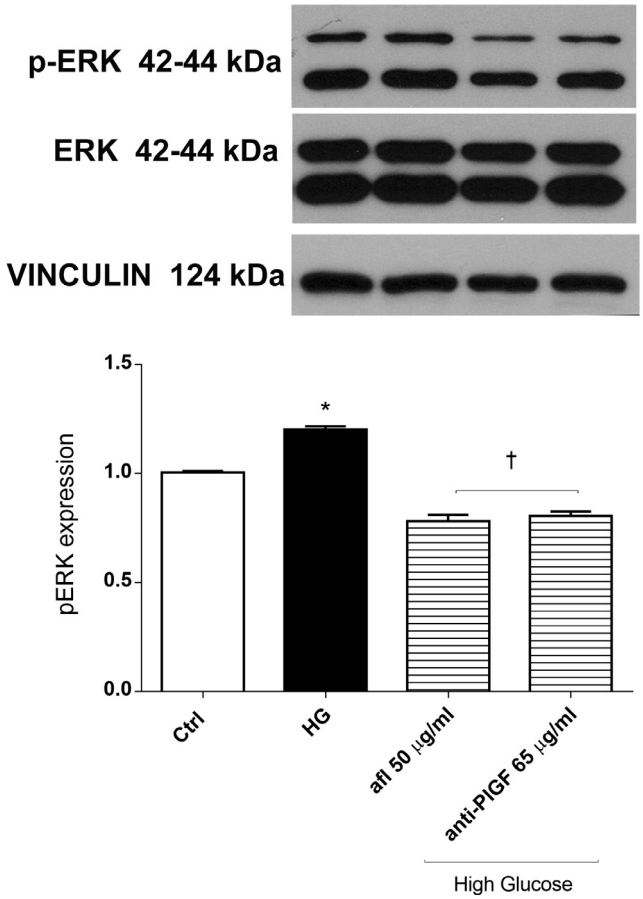

Fig. 7. Western Blot pERK with aflibercept and anti-PlGF in pmRPE cells. Aflibercept and anti-PlGF effect on the inflammatory pathway activated by high glucose (HG) in pmRPE cells. Immunoblot analysis of ERK1/2 phosphorylation in lysates from pmRPE treated for 24 h with HG, aflibercept (50 µg/mL) and anti-PlGF (65 µg/mL). Bar graphs show the densitometry analysis of each band, carried out with the Image J program. Each bar represents the means ± SD (n = 4; each run in triplicate). * p < 0.05 *vs.* control; † p < 0.05 *vs.* HG.

plate preparation procedures. We loaded retinal lysate samples containing 40 µg proteins, determined as described in methods Section 2.3.2. TNFα levels were normalized to total retinal protein content.

2.5. Statistical analysis

All results are reported as mean ± SD, experiments were carried out at least 4 times, each run in triplicate. The results were analyzed using one-way ANOVA followed by Tukey-Kramer post-hoc multiple comparisons test. Differences between groups were considered significant for p-value < 0.05. Graph design and statistical analysis were carried out using GraphPad Prism 5 software (GraphPad Inc., San Diego, CA).

3. Results

3.1. PlGF interacts preferentially with the VEGFR1 domain 2 of aflibercept

The output of PyDock is a score used for rough prediction of binding energy of protein complexes. Furthermore, the PyDock score provided some hints about the weight of each component to the binding free energy ($\Delta G_{binding}$) of VEGFA- and PlGF-aflibercept complexes. VEGFA binding to aflibercept, as predicted by PyDock, was characterized by a better desolvation energy (-12 Kcal/mol), if compared with PlGF-aflibercept complex (-2 Kcal/mol). Moreover, PyDock predicted for the PlGF/aflibercept complex a slightly better electrostatic energy (-18 Kcal/mol), compared to the VEGFA/aflibercept complex (-11 Kcal/mol). Van der Waals (VdW) component of PyDock score was similar for PlGF and VEGF bound to the aflibercept binding domain, −38 and −34 Kcal/mol, respectively. In order to carry out binding energy prediction, with less approximations as applied by PyDock, 20 ns molecular dynamics simulations of the aflibercept binding domain (VEGFR1d2_R2d3) in complex with VEGFA and PlGF were carried (3 independent replicas for each system).

Aflibercept binding domain stabilized more VEGFA (average RMSD 0.32 ± 0.01 nm, whole complex, over 2000 steps of three replicas) than PlGF (average RMSD 0.36 ± 0.01 nm, whole complex over 2000 steps of three replicas) (Fig. 1). RMSD was also calculated for each interactors of complexes: VEGFR1d2_R2d3, VEGFA or PLGF. VEGFA bound to the aflibercept binding domain was more stabilized (RMSD 0.20 ± 0.01 nm, over 2000 steps of three replicas), in comparison with PLGF bound to aflibercept (RMSD 0.30 ± 0.02 nm, over 2000 steps of

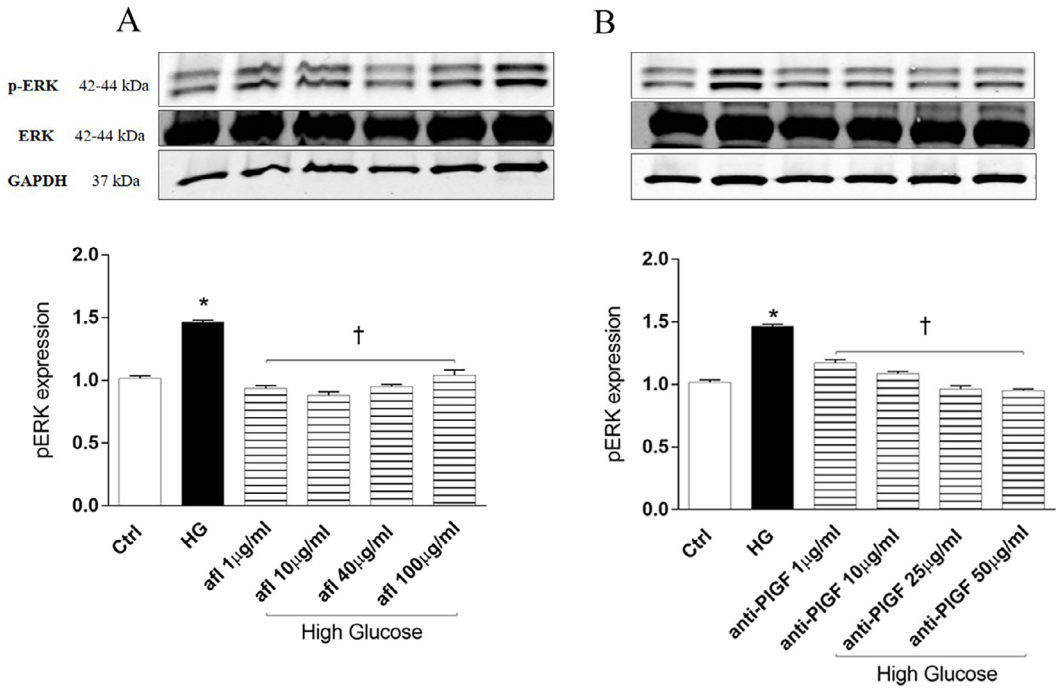

Fig. 8. Western Blot pERK with aflibercept and anti-PlGF. Aflibercept (A) and anti-PlGF (B) effect on the inflammatory pathway activated by high glucose (HG) in HRECs. Immunoblot analysis of ERK1/2 phosphorylation in lysates from HRECs treated for 48 h with HG, aflibercept (1–100 µg/mL) and anti-PlGF (1–50 µg/mL). Bar graphs show the densitometry analysis of each band, carried out with the Image J program. Each bar represents the means ± SD (n = 4; each run in triplicate). * $p < 0.05$ *vs.* control; † $p < 0.05$ *vs.* HG.

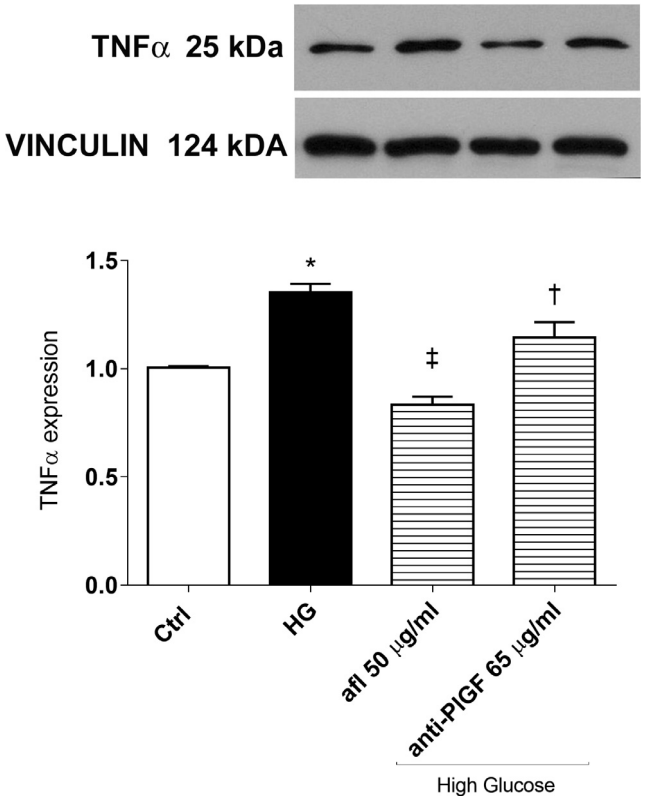

Fig. 9. TNF-α levels in RPE. Immunoblot analysis of TNF-α in lysates from pmRPE treated for 24 h with high glucose (HG), aflibercept (50 µg/mL) and anti-PlGF (65 µg/mL). Bar graphs show the densitometry analysis of each band, carried out with the Image J program. Each bar represents the means ± SD (n = 4; each run in triplicate). *$p < 0.05$ *vs* control; †$p <$ *vs* HG, ‡$p < 0.05$ aflibercept *vs* anti-PlGF treatment.

three replicas).

Furthermore, the analysis of root mean square fluctuation (RMSF) of α-carbons revealed that the VEGFR1d2_R2d3/VEGFA complex was more stable than VEGFR1d2_R2d3/PLGF (Fig. 2). We have calculated the number of contacts at protein–protein interface, applying a 0.35 nm cut-off, which takes into account coulombic and Van der Waals (VdW) interactions. The complex VEGFR1d2_R2d3/VEGFA formed during simulation 290 ± 2 contacts (over 2000 steps of 3 replicas); while the VEGFR1d2_R2d3/PlGF complex formed 260 ± 20 (over 2000 steps of 3 replicas) at protein–protein interface. Additionally, we have identified the main residue-residue interactions at the interface of each VEGFR1d2_R2d3/growth factor complex (Fig. 3, Table 1). Table 1 shows the interactions also shown in Fig. 3; these interactions mainly involved the domain VEGFR1_d2 of aflibercept and the loop 1 of growth factors. Moreover, VEGFA formed through Asp34 a stable salt bridge with the N atom in the amide bond of Glu159, which is in VEGFR2 domain 3 of aflibercept (not shown in Fig. 3). Furthermore, MM-PBSA calculations were carried on three replicas of MD of complexes. MM-PBSA results compared with experimental binding affinity data [39] are shown in Table 2. The predicted binding energy of VEGFR1d2_R2d3/PlGF complex was less negative (less favorable) than predicted binding energy of VEGFR1d2_R2d3/VEGFA, accordingly to experimental binding affinity results [39].

3.2. Aflibercept and anti-PlGF treatment protect human retinal endothelial cells from high glucose insult

We assessed the protective effect of aflibercept and anti-PlGF treatment on HRECs damaged by high-glucose levels, by LDH assay. After 48 h, high glucose levels induced a significant increase of LDH release (Fig. 4), compared to control cells grown in medium with physiological glucose levels (*$p < 0.05$ *vs.* Ctrl). LDH release was significantly reduced, in a concentration-dependent manner, after aflibercept (Fig. 4A) and anti-PlGF treatment (Fig. 4B), compared to cells grown in high glucose medium (HG group) (†$p < 0.05$ *vs.* HG).

Modulation of VEGF-A cell release by aflibercept or anti-PlGF

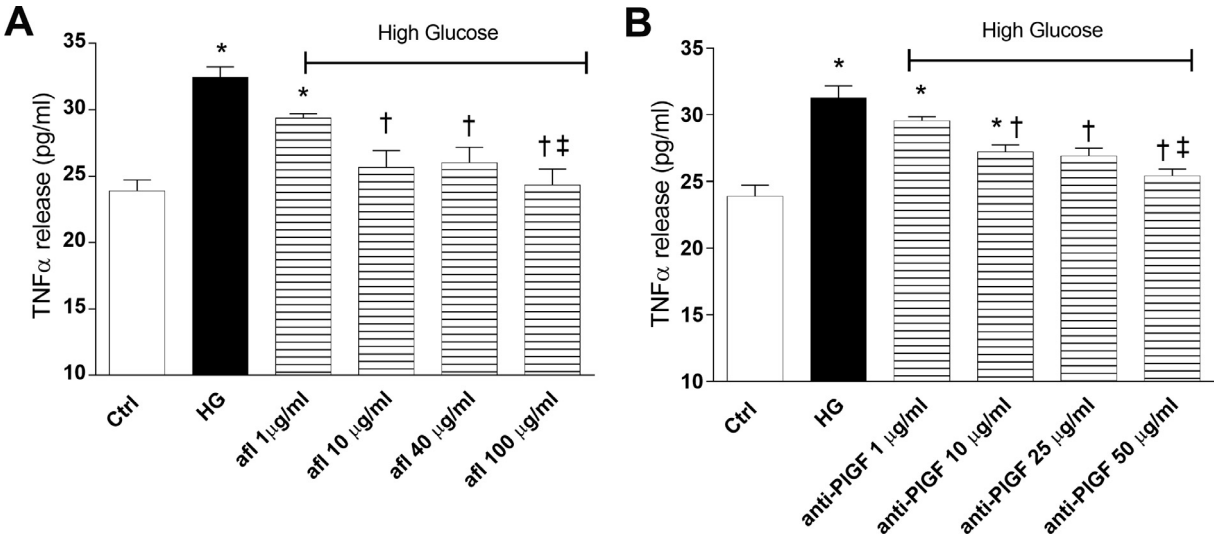

Fig. 10. TNF-α levels in HREC (ELISA). Aflibercept (1–100 μg/mL) (A) and anti-PlGF (1–50 μg/mL) (B) treatment decreased TNF-α release induced by 48 h exposure to high glucose (HG) levels. Ctrl stands for control cells, growth in medium with physiological glucose concentrations (5 mM). HG stands for cells grown in medium with high glucose (40 mM) levels. Bars represent mean values ± SD (n = 8; each run in triplicate). *p < 0.05 *vs.* control; †p < 0.05 *vs.* HG, ‡ p < 0.05 *vs* 40 μg/mL aflibercept or 25 μg/mL anti-PlGF.

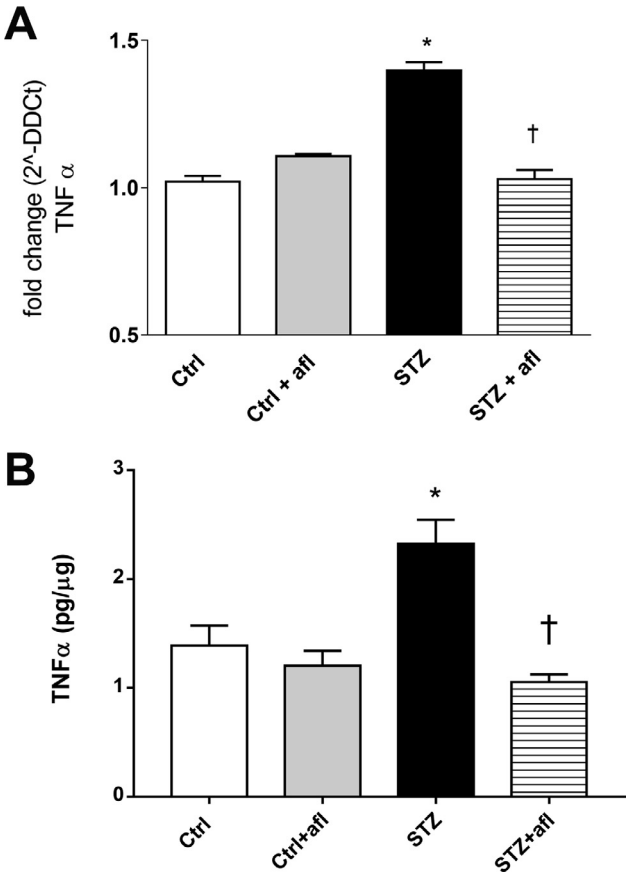

Fig. 11. Aflibercept reduces TNF-α mRNA expression (A) and protein levels (B) in the retina of streptozotocin-induced diabetic rats (STZ + afl group). Ctrl stands for non-diabetic rats. Ctrl + afl stands for non-diabetic rats treated with an intravitreal injection of aflibercept. STZ stands for diabetic rats treated with vehicle. STZ + afl are diabetic rats that were treated with aflibercept (160 μg; intravitreally). Each bar represents the mean value ± SD (n = 4; each run in triplicate). *p < 0.05 *vs.* control; †p < 0.05 *vs.* STZ.

treatments was evaluated in HRECs, challenged with high glucose levels. After 48 h of high glucose concentration exposure, VEGF-A protein levels were significantly increased in HRECs, compared to control cells. The aflibercept (Fig. 5A) and anti-PlGF (Fig. 5B) treatments were able to reduce significantly ($p < 0.05$) VEGF-A protein levels.

Moreover, we evaluated the expression of VEGF-A (Fig. 6A) and PlGF (Fig. 6B) by quantitative PCR measurements. The VEGF-A and PlGF mRNA levels were significantly increased in HRECs challenged with high glucose levels, compared to control cells. The results from the ELISA assay were confirmed by quantitative PCR and both aflibercept and anti-PlGF treatments reduced significantly ($p < 0.05$) the VEGF-A mRNA levels in HRECs exposed to hyperglycemia insult (Fig. 6A). Furthermore, similarly to the effects on VEGFA expression, the treatment with aflibercept and the anti-PlGF antibody decreased PlGF mRNA levels, in HRECs challenged with high glucose levels (Fig. 6B).

3.3. ERK/TNF-α inflammatory pathway is modulated by aflibercept and anti-PlGF treatment on retinal pigmented epithelial cells and endothelial cells

After 24 h of high-glucose exposure, the ERK phosphorylation levels were significantly increased in retinal pmRPE cells, compared with control cells (Fig. 7). While, after 48 h, the ERK pathway was significantly activated in HRECs exposed to high glucose levels, compared with control cells.

Treatment with aflibercept and anti-PlGF significantly reduced ERK levels in a concentration-dependent manner both in retinal pigmented primary mouse cells (Fig. 7) and in human retinal endothelial cells (Fig. 8 A, B).

We also tested the effect of aflibercept and anti-PlGF treatment on the expression of the inflammatory cytokine TNF-α, which is downstream to the ERK pathway. The aflibercept and anti-PlGF antibody treatment significantly ($p < 0.05$) reduced TNF-α levels in pmRPE cells, compared with non-treated cells, 24 h after exposure to HG levels (Fig. 9). The anti-inflammatory action of aflibercept and anti-PlGF antibody in terms of TNF-α levels was also demonstrated in HRECs exposed to high glucose levels for 48 h (Fig. 10).

3.4. Retinal TNF-α levels in diabetic rats are attenuated by aflibercept

We tested the anti-inflammatory effect of aflibercept in an *in vivo* model of diabetic retinopathy. Diabetes was induced in rats by STZ

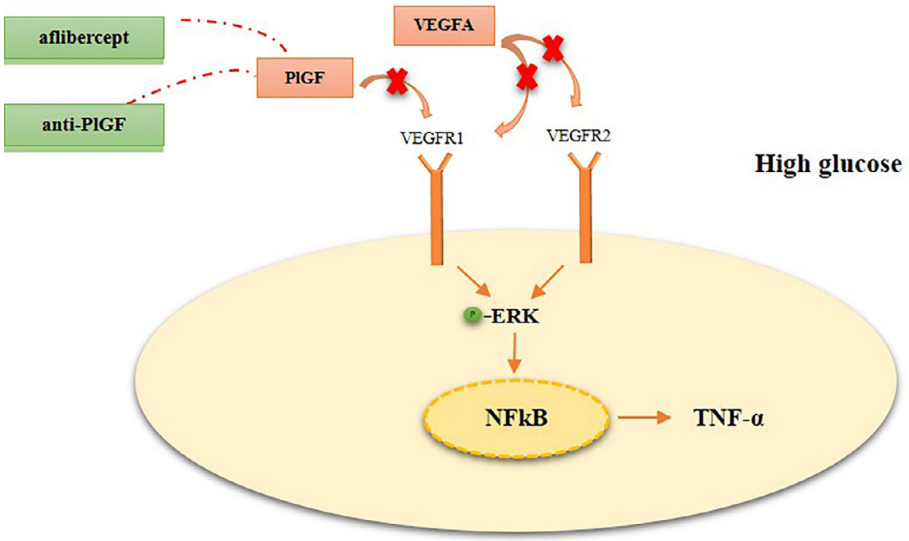

Fig. 12. High glucose-induced damage in retinal endothelial cells. Proposed anti-inflammatory activity of aflibercept and anti-PlGF.

(60 mg/kg), and after 15 days (glycemia > 250 mg/dL) we intravitreally injected aflibercept (160 μg). It should be noted that the dose of aflibercept used in our study (160 μg) was superimposable to the dose used in clinical practice [40].

Rats were sacrificed 48 h after pharmacological treatment. Diabetes induction significantly increased TNF-α expression in the retina of diabetic rats (STZ group), compared to control rats (citrate buffer, Fig. 11A). The intravitreal injection of aflibercept in non-diabetic rats did not affect retinal TNF-α levels compared to control non-diabetic rats. On the contrary, aflibercept significantly ($p < 0.05$) reduced the retinal TNF-α expression in diabetic rats (STZ + AFL, Fig. 11A), compared with vehicle-treated diabetic rats (STZ).

TNF-α protein levels in retina of diabetic rats were measured with ELISA kit, accordingly to qPCR analysis TNF-α retinal levels were increased in diabetic rat, compared to control animals (Fig. 11B). Aflibercept intravitreal injection effectively decreased TNF-α protein levels in the retina of treated diabetic rats, compared to untreated animals (Fig. 11B)

4. Discussion

Aflibercept (VEGF-trap agent) is currently used for treatment of retinal diseases such as age-related macular degeneration (AMD), retinal vein occlusion and diabetic macular edema. It has been reported that aflibercept, through its binding to VEGF-A and PlGF, can effectively inhibit the signaling activated by these ligands [39]. Aflibercept therapeutic action is accounted mainly to the neutralization of VEGF-A activity, therefore preventing the binding and activation of its receptors (VEGFR1 and VEGFR2).

The binding of PlGF to aflibercept was experimentally found to be less favorable (38.9 nM dissociation constant – K_D), compared to VEGF-A/aflibercept complex formation (0.49 nM dissociation constant – K_D). In agreement with experimental data [39] we demonstrated through *in silico* studies (molecular dynamics combined with MM-PBSA calculations), that PlGF forms less stable non-covalent interactions with aflibercept, compared with VEGFA. The binding of PlGF and other growth factors to $VEGFR_{1\text{-}2\text{-}3}$ receptors was previously studied, by molecular dynamics simulations [41]. However, the authors did not carry out specific energetic analysis of growth factors/receptor complex formation, and analyzed RMSD profile of a single replica of each complex [41]. On the contrary, we applied a previous published approach [33,42,43], that combines molecular dynamics, MM-PBSA calculation and statistical analysis to obtain prediction of energetic components of binding free energy of complex formation. Indeed, we described for the first time the binding of aflibercept (fusion protein and decoy receptor) to PlGF and compared its properties to aflibercept/VEGFA complex. Particularly, our *in silico* studies confirmed that PlGF preferentially binds the VEGFR1 domain 2 of aflibercept, as previously reported [44,45], while VEGFA forms a stable salt bridge with a residue of VEGFR2_d3 domain of the aflibercept binding domain. Moreover, we found that the binding of aflibercept to growth factors (VEGA and PlGF) is mainly guided by electrostatic interactions, whereas ranibizumab and bevacizumab interact with VEGFA mainly through VdW interactions [33].

It is known that VEGF-A plays a central role in both physiological and pathological conditions, whereas the role of PlGF seems to be specific to pathological conditions [36]. It has been demonstrated that the loss of PlGF significantly protects mice against retinal neovascularization and hypoxic ischemic retinopathy [37,38]. Indeed, the aflibercept's capability to bind both VEGFA and PlGF could deliver additional beneficial effects in diabetic retinopathy, compared with biologics that are capable of binding only VEGFA. Therefore, on the basis of previous reports, reporting the detrimental role of PlGF in the development and progression of diabetic retinopathy [24,46,47], we tested *in vitro* and *in vivo* the hypothesis that aflibercept, by binding also to PlGF, could exert an anti-inflammatory action in the diabetic retina.

We demonstrated that inhibition of PlGF signaling improved the diabetes-associated inflammation in retinal cells exposed to high glucose levels, through the administration of aflibercept and of an anti-PlGF antibody. The latter binds PlGF selectively, directly influencing the activation of VEGFR1 and indirectly the activation of VEGFR2 (heterodimeric form with VEGF-A), while as stated before aflibercept can bind different isoforms of VEGF, including PlGF. In the current study we used human retinal endothelial cells (HRECs) and mouse primary retinal pigmented epithelial cells (RPE) exposed to high glucose levels, as *in vitro* models of the early insult occurring in diabetic retinopathy and in two different areas of the retina.

We demonstrated that both aflibercept and anti-PlGF antibody protected human RECs and mouse RPE cells from high glucose damage, by blocking the activation of the ERK pathway with the subsequent suppression of TNF-α release. This effect could be related to regulation of NF-kB signaling [32,48]. According to previous findings, we also found that high glucose induces the overexpression of PlGF and VEGF-A [49,50]. We found that HRECs, challenged with high glucose levels, were protected from cell death after treatment with aflibercept and anti-PlGF. Therefore, the inhibition of PlGF signaling may protect the

inner blood retinal barrier. Moreover, aflibercept and anti-PlGF treatment decreased also VEGFA and PlGF release by HRECs challenged with high glucose levels. It has recently been demonstrated [26] that anti-PlGF antibody and aflibercept were able to attenuate the inflammatory response in a choroidal neovascularization mouse model, on the contrary, the selective anti-VEGF antibody had no effect on inflammation.

These findings highlight a possible favorable effect of blocking PlGF, compared to canonic VEGF-inhibitors, for which possible concerns have been reported as regards potential side effects [51]. High glucose can activate several biochemical pathways, including AKT and ERK [52]. Besides AKT and ERK pathways, toll-like receptors [53] and receptors for advanced glycation end-products (RAGE) were found to be activated in several models of diabetic retinopathy; furthermore, their activation was directly linked to NFκB activation and transcription of pro-inflammatory cytokines, such as IL-1β [9,54,55].

Previous data, borrowed from cancer research, showed that PlGF activates ERK1/2 phosphorylation [56]; which is involved in triggering of inflammation [29,30,32]. Similar effects on ERK activation were also observed in conditions where VEGFA was overexpressed. We herein found that block of PlGF signaling, by aflibercept and anti-PlGF treatments, inhibited ERK phosphorylation in HRECs and primary mouse RPE challenged with high glucose levels (Fig. 12). Both aflibercept and PlGF treatments were able to inhibit EKR activation along with TNF-α release, the downstream protein of this pathway. The findings obtained from the *in vivo* model of diabetic retinopathy supported this hypothesis, because rat retinal TNF-α levels were significantly decreased by aflibercept treatment. The present findings show that aflibercept has an anti-inflammatory action through the PlGF/ERK pathway. Furthermore, our results support the development of a new strategy to counteract diabetic retinopathy by selective inhibition of the PlGF signaling pathway. Particularly, the present data suggest that PlGF signaling can modulate the retinal inflammatory response elicited by high glucose through ERK and TNF-α regulation. This approach could avoid the over-activation of VEGFR1 maintaining physiological actions of VEGF, without inducing VEGF inhibitors treatment-related complications. In conclusion, our data suggest that PlGF may be considered a selective target to manage diabetic retinopathy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by: National Grant PRIN 2015JXE7E8 from Ministry of Education, University and Research (MIUR); “Piano Triennale per la Ricerca – Linea Intervento 2, University of Catania, Italy”; Ministry of Economic Development (MISE) PON-Innovative PhD Program; unrestricted grant from Bayer.

References

[1] N. Congdon, Y. Zheng, M. He, The worldwide epidemic of diabetic retinopathy, Ind. J. Ophthalmol. (2012), https://doi.org/10.4103/0301-4738.100542.
[2] G. Giurdanella, C.D. Anfuso, M. Olivieri, G. Lupo, N. Caporarello, C.M. Eandi, F. Drago, C. Bucolo, S. Salomone, Aflibercept, bevacizumab and ranibizumab prevent glucose-induced damage in human retinal pericytes in vitro, through a PLA2/COX-2/VEGF-A pathway, Biochem. Pharmacol. (2015), https://doi.org/10.1016/j.bcp.2015.05.017.
[3] R.B. Caldwell, M. Bartoli, M.A. Behzadian, A.E.B. El-Remessy, M. Al-Shabrawey, D.H. Platt, R.W. Caldwell, Vascular endothelial growth factor and diabetic retinopathy: pathophysiological mechanisms and treatment perspectives, Diabetes/Metabol. Res. Rev. (2003), https://doi.org/10.1002/dmrr.415.
[4] C.B.M. Platania, G.M. Leggio, F. Drago, S. Salomone, C. Bucolo, Computational systems biology approach to identify novel pharmacological targets for diabetic retinopathy, Biochem. Pharmacol. 158 (2018), https://doi.org/10.1016/j.bcp.2018.09.016.
[5] G.B. Stefano, S. Challenger, R.M. Kream, Hyperglycemia-associated alterations in cellular signaling and dysregulated mitochondrial bioenergetics in human metabolic disorders, Eur. J. Nutr. (2016), https://doi.org/10.1007/s00394-016-1212-2.
[6] C.H. Meyer, Current treatment approaches in diabetic macular edema, Ophthalmologica (2007), https://doi.org/10.1159/000098257.
[7] M. Brownlee, The pathobiology of diabetic complications: a unifying mechanism, Diabetes (2005), https://doi.org/10.2337/diabetes.54.6.1615.
[8] C.D. Anfuso, G. Lupo, L. Romeo, G. Giurdanella, C. Motta, A. Pascale, C. Tirolo, B. Marchetti, M. Alberghina, Endothelial cell-pericyte cocultures induce PLA $_2$ protein expression through activation of PKCα and the MAPK/ERK cascade, J. Lipid Res. (2007), https://doi.org/10.1194/jlr.M600489-JLR200.
[9] G. Giurdanella, F. Lazzara, N. Caporarello, G. Lupo, C.D. Anfuso, C.M. Eandi, G.M. Leggio, F. Drago, C. Bucolo, S. Salomone, Sulodexide prevents activation of the PLA2/COX-2/VEGF inflammatory pathway in human retinal endothelial cells by blocking the effect of AGE/RAGE, Biochem. Pharmacol. (2017), https://doi.org/10.1016/j.bcp.2017.06.130.
[10] A. Das, S. Rangasamy, P. McGuire, Diabetic retinopathy and inflammation: novel therapeutic targets, Middle East Afr. J. Ophthalmol. (2012), https://doi.org/10.4103/0974-9233.92116.
[11] T. Agawa, Y. Wakabayashi, Y. Okunuki, M. Juan, K. Umazume, T. Kezuka, M. Takeuchi, Y. Yamauchi, H. Goto, Profile of intraocular immune mediators in patients with age-related macular degeneration and the effect of intravitreal bevacizumab injection, Retina (2014), https://doi.org/10.1097/IAE.0000000000000157.
[12] M.W. Stewart, The expanding role of vascular endothelial growth factor inhibitors in ophthalmology, Mayo Clin. Proc. (2012), https://doi.org/10.1016/j.mayocp.2011.10.001.
[13] D.I.R. Holmes, I. Zachary, The vascular endothelial growth factor (VEGF) family: angiogenic factors in health and disease, Genome Biol. (2005), https://doi.org/10.1186/gb-2005-6-2-209.
[14] M. Shibuya, Vascular endothelial growth factor (VEGF) and its receptor (VEGFR) signaling in angiogenesis: a crucial target for anti- and pro-angiogenic therapies, Genes Cancer. (2011), https://doi.org/10.1177/1947601911423031.
[15] A.K. Olsson, A. Dimberg, J. Kreuger, L. Claesson-Welsh, VEGF receptor signalling – in control of vascular function, Nat. Rev. Mol. Cell Biol. (2006), https://doi.org/10.1038/nrm1911.
[16] A.N. Witmer, G.F.J.M. Vrensen, C.J.F. Van Noorden, R.O. Schlingemann, Vascular endothelial growth factors and angiogenesis in eye disease, Prog. Chem. Org. Nat. Prod. Retinal Eye Res. (2003), https://doi.org/10.1016/S1350-9462(02)00043-5.
[17] C. Arnott, G. Punnia-Moorthy, J. Tan, S. Sadeghipour, C. Bursill, S. Patel, The vascular endothelial growth factor inhibitors ranibizumab and aflibercept markedly increase expression of Atherosclerosis-Associated inflammatory mediators on vascular endothelial cells, PLoS ONE (2016), https://doi.org/10.1371/journal.pone.0150688.
[18] E.W.M. Ng, A.P. Adamis, Targeting angiogenesis, the underlying disorder in neovascular age-related macular degeneration, Can. J. Ophthalmol. (2005), https://doi.org/10.1016/S0008-4182(05)80078-X.
[19] N. Ferrara, L. Damico, N. Shams, H. Lowman, R. Kim, Development of ranibizumab, an anti-vascular endothelial growth factor antigen binding fragment, as therapy for neovascular age-related macular degeneration, Retina (2006), https://doi.org/10.1097/01.iae.0000242842.14624.e7.
[20] Y. Sun, Y. Liang, P. Zhou, H. Wu, X. Hou, Z. Ren, X. Li, M. Zhao, Anti-VEGF treatment is the key strategy for neovascular glaucoma management in the short term, BMC Ophthalmol. (2016), https://doi.org/10.1186/s12886-016-0327-9.
[21] C. Bucolo, G. Campana, R. Di Toro, S. Cacciaguerra, S. Spampinato, σ Recognition sites in rabbit iris-ciliary body: topical σ-1-site agonists lower intraocular pressure, J. Pharmacol. Exp. Therapeutics (1999).
[22] M.W. Stewart, S. Grippon, P. Kirkpatrick, Aflibercept, Nat. Rev. Drug Discov. (2012), https://doi.org/10.1038/nrd3700.
[23] J. He, H. Wang, Y. Liu, W. Li, D. Kim, H. Huang, Blockade of vascular endothelial growth factor receptor 1 prevents inflammation and vascular leakage in diabetic retinopathy, J. Ophthalmol. (2015), https://doi.org/10.1155/2015/605946.
[24] Q.D. Nguyen, S. De Falco, F. Behar-Cohen, W.C. Lam, X. Li, N. Reichhart, F. Ricci, J. Pluim, W.W. Li, Placental growth factor and its potential role in diabetic retinopathy and other ocular neovascular diseases, Acta Ophthalmol. (2018), https://doi.org/10.1111/aos.13325.
[25] L. Kowalczuk, E. Touchard, S. Omri, L. Jonet, C. Klein, F. Valamanes, M. Berdugo, P. Bigey, P. Massin, J.C. Jeanny, F. Behar-Cohen, Placental growth factor contributes to micro-vascular abnormalization and blood-retinal barrier breakdown in diabetic retinopathy, PLoS ONE (2011), https://doi.org/10.1371/journal.pone.0017462.
[26] T. Van Bergen, T.T. Hu, I. Etienne, G.E. Reyns, L. Moons, J.H.M. Feyen, Neutralization of placental growth factor as a novel treatment option in diabetic retinopathy, Exp. Eye Res. (2017), https://doi.org/10.1016/j.exer.2017.09.012.
[27] E. Szabo, H. Schneider, K. Seystahl, E.J. Rushing, F. Herting, K.M. Weidner, M. Weller, Autocrine VEGFR1 and VEGFR2 signaling promotes survival in human glioblastoma models in vitro and in vivo, Neuro-Oncol. (2016), https://doi.org/10.1093/neuonc/now043.
[28] S.G. Almalki, D.K. Agrawal, ERK signaling is required for VEGF-A/VEGFR2-induced differentiation of porcine adipose-derived mesenchymal stem cells into endothelial cells, Stem Cell Res. Ther. 8 (2017) 113, https://doi.org/10.1186/s13287-017-0568-4.
[29] Y. Son, Y.-K. Cheong, N.-H. Kim, H.-T. Chung, D.G. Kang, H.-O. Pae, Mitogen-activated protein kinases and reactive oxygen species: how can ROS activate MAPK pathways? J. Sig. Transduction (2011), https://doi.org/10.1155/2011/792639.

[30] M. Cargnello, P.P. Roux, Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases, Microbiol. Mol. Biol. Rev. (2011), https://doi.org/10.1128/MMBR.00031-10.
[31] W. Lassoued, D. Murphy, J. Tsai, R. Oueslati, G. Thurston, W.M.F. Lee, Effect of VEGF and VEGF Trap on vascular endothelial cell signaling in tumors, Cancer Biol. Ther. (2010), https://doi.org/10.4161/cbt.10.12.14009.
[32] B. Jiang, S. Xu, X. Hou, D.R. Pimentel, P. Brecher, R.A. Cohen, Temporal control of NF-κB activation by ERK differentially regulates interleukin-1β-induced gene expression, J. Biol. Chem. (2004), https://doi.org/10.1074/jbc.M307521200.
[33] C.B.M. Platania, L. Di Paola, G.M. Leggio, G.L. Romano, F. Drago, S. Salomone, C. Bucolo, Molecular features of interaction between VEGFA and anti-angiogenic drugs used in retinal diseases: a computational approach, Front. Pharmacol. 6 (2015) 248, https://doi.org/10.3389/fphar.2015.00248.
[34] S. Pronk, S. Páll, R. Schulz, P. Larsson, P. Bjelkmar, R. Apostolov, M.R. Shirts, J.C. Smith, P.M. Kasson, D. Van Der Spoel, B. Hess, E. Lindahl, GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit, Bioinformatics (2013), https://doi.org/10.1093/bioinformatics/btt055.
[35] D. Huang, C. Zhao, R. Ju, A. Kumar, G. Tian, L. Huang, L. Zheng, X. Li, L. Liu, S. Wang, X. Ren, Z. Ye, W. Chen, L. Xing, Q. Chen, Z. Gao, J. Mi, Z. Tang, B. Wang, S. Zhang, C. Lee, X. Li, VEGF-B inhibits hyperglycemia- and Macugen-induced retinal apoptosis, Sci. Rep. (2016), https://doi.org/10.1038/srep26059.
[36] M. Amadio, C. Bucolo, G.M. Leggio, F. Drago, S. Govoni, A. Pascale, The PKCβ/HuR/VEGF pathway in diabetic retinopathy, Biochem. Pharmacol. (2010), https://doi.org/10.1016/j.bcp.2010.06.033.
[37] C. Bucolo, G.M. Leggio, F. Drago, S. Salomone, Eriodictyol prevents early retinal and plasma abnormalities in streptozotocin-induced diabetic rats, Biochem. Pharmacol. (2012), https://doi.org/10.1016/j.bcp.2012.03.019.
[38] A.B. Nair, S. Jacob, A simple practice guide for dose conversion between animals and human, J. Basic Clin. Pharm. (2016), https://doi.org/10.4103/0976-0105.177703.
[39] N. Papadopoulos, J. Martin, Q. Ruan, A. Rafique, M.P. Rosconi, E. Shi, E.A. Pyles, G.D. Yancopoulos, N. Stahl, S.J. Wiegand, Binding and neutralization of vascular endothelial growth factor (VEGF) and related ligands by VEGF Trap, ranibizumab and bevacizumab, Angiogenesis (2012), https://doi.org/10.1007/s10456-011-9249-6.
[40] C. Ozsaygili, Z. Duru, A. Cicek, D.M. Ulusoy, A.A. Demirtas, N. Duru, The effect of age on aflibercept (eylea) response in diabetic macular edema, Retina (2019), https://doi.org/10.1097/iae.0000000000002504.
[41] B.A.C. Horta, A.C.R. Sodero, R.B. de Alencastro, Investigating the differential activation of vascular endothelial growth factor (VEGF) receptors, J. Mol. Graph. Model. (2009), https://doi.org/10.1016/j.jmgm.2009.08.007.
[42] L. Di Paola, C.B.M. Platania, G. Oliva, R. Setola, F. Pascucci, A. Giuliani, Characterization of protein-protein interfaces through a protein contact network approach, Front. Bioeng. Biotechnol. 3 (2015) 170, https://doi.org/10.3389/fbioe.2015.00170.
[43] D. Corrada, G. Colombo, Energetic and dynamic aspects of the affinity maturation process: characterizing improved variants from the bevacizumab antibody with molecular simulations, J. Chem. Inf. Model. 53 (2013) 2937–2950, https://doi.org/10.1021/ci400416e.
[44] T. Torimura, H. Iwamoto, T. Nakamura, M. Abe, Y. Ikezono, F. Wada, T. Sakaue, H. Masuda, O. Hashimoto, H. Koga, T. Ueno, H. Yano, Antiangiogenic and Antitumor Activities of Aflibercept, a Soluble VEGF Receptor-1 and -2, in a Mouse Model of Hepatocellular Carcinoma, Neoplasia (United States) (2016), https://doi.org/10.1016/j.neo.2016.05.001.
[45] S. Takahashi, Vascular endothelial growth factor (VEGF), VEGF receptors and their inhibitors for antiangiogenic tumor therapy, Society (2011), https://doi.org/10.1248/bpb.34.1785.
[46] I. Apicella, V. Cicatiello, D. Acampora, V. Tarallo, S. De Falco, Full functional knockout of placental growth factor by knockin with an inactive variant able to heterodimerize with VEGF-A, Cell Rep. (2018), https://doi.org/10.1016/j.celrep.2018.05.067.
[47] Y.H. Aldebasi, A.H. Rahmani, A.A. Khan, S. Mesalhy Aly, The effect of vascular endothelial growth factor in the progression of bladder cancer and diabetic retinopathy, Int. J. Clin. Exp. Med. (2013).
[48] A.B. Carter, G.W. Hunninghake, A constitutive active MEK → ERK pathway negatively regulates NF-κB-dependent gene expression by modulating TATA-binding protein phosphorylation, J. Biol. Chem. (2000), https://doi.org/10.1074/jbc.M003599200.
[49] L.E. Benjamin, Glucose, VEGF-A, and diabetic complications, Am. J. Pathol. (2001), https://doi.org/10.1016/S0002-9440(10)64066-7.
[50] X. Chen, J. Li, M. Li, M. Zeng, T. Li, W. Xiao, J. Li, Q. Wu, X. Ke, D. Luo, S. Tang, Y. Luo, KH902 suppresses high glucose-induced migration and sprouting of human retinal endothelial cells by blocking VEGF and PIGF, Diabetes, Obes. Metabol. (2013), https://doi.org/10.1111/dom.12008.
[51] T. Van Bergen, I. Etienne, F. Cunningham, L. Moons, R.O. Schlingemann, J.H.M. Feyen, A.W. Stitt, The role of placental growth factor (PlGF) and its receptor system in retinal vascular diseases, Prog. Chem. Org. Nat. Prod. Retinal Eye Res. (2019), https://doi.org/10.1016/j.preteyeres.2018.10.006.
[52] Q. Huang, N. Sheibani, High glucose promotes retinal endothelial cell migration through activation of Src, PI3K/Akt1/eNOS, and ERKs, AJP: Cell Physiol. (2008), https://doi.org/10.1152/ajpcell.00322.2008.
[53] L. Hu, H. Yang, M. Ai, S. Jiang, Inhibition of TLR4 alleviates the inflammation and apoptosis of retinal ganglion cells in high glucose, Graefe's Arch. Clin. Exp. Ophthalmol. (2017), https://doi.org/10.1007/s00417-017-3772-0.
[54] M.R. Dasu, S. Devaraj, L. Zhao, D.H. Hwang, I. Jialal, High glucose induces toll-like receptor expression in human monocytes Mechanism of activation, Diabetes (2008), https://doi.org/10.2337/db08-0564.
[55] C.B.M. Platania, G. Giurdanella, L. Di Paola, G.M. Leggio, F. Drago, S. Salomone, C. Bucolo, P2X7 receptor antagonism: implications in diabetic retinopathy, Biochem. Pharmacol. (2017), https://doi.org/10.1016/j.bcp.2017.05.001.
[56] A.P. Taylor, E. Leon, D.M. Goldenberg, Placental growth factor (PlGF) enhances breast cancer cell motility by mobilising ERK1/2 phosphorylation and cytoskeletal rearrangement, Br. J. Cancer (2010), https://doi.org/10.1038/sj.bjc.6605746.