

Original Paper

Aflibercept and Ranibizumab Modulate Retinal Pigment Epithelial Cells Function by Acting on Their Cross Talk with Vascular Endothelial Cells

Stefano De Cillà^a Serena Farruggio^{b,c} Grazia Cocomazzi^{b,c} David Mary^{b,c}
Micol Alkabes^a Luca Rossetti^d Stela Vujosevic^a Elena Grossini^{a,c}

^aOphthalmology Unit, Department of Health Sciences, University East Piedmont, Azienda Ospedaliera Universitaria Maggiore della Carità, Novara, Italy, ^bLaboratory of Physiology and Experimental Surgery, Department of Translational Medicine, University East Piedmont, Novara, Italy, ^cAGING Project, Department of Translational Medicine, University of Eastern Piedmont, Novara, Italy, ^dEye Clinic, San Paolo Hospital, University of Milan, Milan, Italy

Key Words

Anti-VEGF • Cell viability • Cross-talk • Mitochondrial function

Abstract

Background/Aims: We performed co-culture experiments between human RPE cells (ARPE-19) and human umbilical vascular endothelial cells (HUVEC) in order to evaluate how anti-VEGF drugs could affect NO release, mitochondrial function, the oxidative status, proliferation and migration of RPE cells through modulation of their cross talk with vascular endothelial cells. **Methods:** The co-culture HUVEC/RPE, was exposed to Ranibizumab/Aflibercept in the absence/presence of the NO synthase (NOS) inhibitor, the phosphatidylinositol 3'-kinase (PI3K), the extracellular-signal-regulated kinases 1/2 (ERK1/2) and the p38 mitogen-activated protein kinase (p38 MAPK) blockers. Specific kits were used for cell viability, mitochondrial membrane potential, NO, ROS and GSH production. Western blot was performed for apoptosis markers, NOS isoforms, and others kinases detection. Cell migration was analyzed by scratch assay, whereas cell proliferation and cell cycle through xCELLigence and flow cytometry. **Results:** In RPE cells co-cultured with HUVEC in physiological conditions, Aflibercept/Ranibizumab increased NO release in a dose and time-dependent way. Opposite results were obtained in peroxidative conditions. Both anti-VEGF agents were able to prevent the fall of cell viability and mitochondrial membrane potential, an effect which was reduced by various inhibitors, and increased cell migration. Aflibercept/Ranibizumab counteracted the changes of apoptosis markers, NOS expression/activation, PI3K and ERK1/2 activation caused by peroxidation.

These results were confirmed by cell cycle analysis. **Conclusion:** This study has shown new mechanisms at the basis of protective effects elicited by Aflibercept/Ranibizumab in RPE cells. HUVEC stimulated with Aflibercept/Ranibizumab, could release some paracrine factors that can modulate the RPE cells response in both physiologic and peroxidative conditions.

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Introduction

The age-related macular degeneration (AMD), is a complex chronic neurodegenerative disease and is the major cause of blindness among old people [1-3]. Although the precise etiopathogenesis of AMD is still unknown, it has been postulated that the reduced choroidal blood flow would result in changes in the retinal pigment epithelial (RPE) function with accumulation of lipoproteins that ultimately lead to RPE cell degeneration and altered permeability of the Bruch's membrane [4-7].

There is strong evidence suggesting that oxidative stress has a major role in the development and progression of AMD. The retina and RPE are extremely susceptible to oxidative stress damage: they both have high metabolic demands and require large amounts of adenosine triphosphate to support their functions [8]. Due to its high metabolic activity, RPE cells are enriched with mitochondria, which act as major source of ROS in RPE cells. Increased mitochondrial damage and generation of ROS are associated with AMD, suggesting that damaged mitochondria and other oxidatively modified components are not efficiently removed by the aged- RPE cells [9].

Although nitric oxide (NO) could play an important role at the onset of AMD by its action on the vascular tone and ocular blood flow, there has been only a few studies to characterize the nitric oxide synthase (NOS) isoforms in the choroid [10-15]. In this context, small amount of eNOS-derived NO could act as a potent vasodilator and play a key role in the physiological regulation of ocular blood flow [10, 16, 17]. Furthermore, in RPE cells, NO would contribute to the function of phagocytosis of rod outer segments and regulation of vascular endothelial growth factor gene expression. Under oxidative stress however, a high concentration of NO would be converted into peroxynitrite (ONOO⁻), which would cause retinal damage [13, 14]. In a previous study performed in RPE cells, we have found that the anti-VEGF drugs, Aflibercept and Ranibizumab, which are widely used for the treatment of exudative AMD [1, 18], were able to increase NO production through the eNOS isoform activation in RPE cells cultured physiological conditions, while they caused opposite effects in RPE cells that underwent peroxidation. Those effects were related to the activation of intracellular signaling downstream Akt and extracellular-signal-regulated kinases 1/2 (ERK1/2) [19]. In spite of this, the cellular mechanisms and signaling pathways conferring the protective effect of the anti- VEGF agents are only partially understood. In particular and up to date, no information is available about the modulation of the cross-talk between RPE and vascular endothelial cells, which appears critical for the maintenance of the outer-retinal structure and function. Thus, in the present study, we have compared the effects of Aflibercept and Ranibizumab on NO release, mitochondria function, oxidative stress and proliferation by human RPE cells (ARPE-19 cell line) in co-culture with human umbilical vascular endothelial cells (HUVEC). The use of NOS inhibitor, phosphatidylinositol 3'-kinase (PI3K), ERK1/2 and p38 mitogen-activated protein kinase (p38 MAPK) blockers furthermore allowed us to examine the involvement of those intracellular pathways

Materials and Methods

Culture of RPE and HUVEC

Human retinal pigment epithelium (RPE) cell line, was obtained from the American Type Culture Collection (ATCC; Rockville, Maryland, USA; catalog. no. CRL-2302TM; ARPE-19), and was maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, Milan, Italy) supplemented with 10% fetal bovine

serum (FBS; Euroclone, Pero, Milan, Italy), and 2 mM L-glutamine (Euroclone), 1% penicillin-streptomycin (P/S; Euroclone), at 37°C with 5% CO₂ in incubator. Human umbilical vein endothelial cell line (HUVEC), was purchased from ATCC (catalog. no. CRL-1730™), and was maintained in Kaighn's Modification of Ham's F-12 Medium (F-12K Medium; ATCC), containing 2 mM L-glutamine, 1500 mg/L sodium bicarbonate, and supplemented with 0.1 mg/ml heparin (Sigma), 100 µg/ml endothelial cell growth supplement (ECGS; Sigma), 1% P/S, and 10% FBS.

Experimental protocol

For co-culture experiments, HUVEC were plated in the apical compartment of the insert, while RPE cells were plated in the basal compartment. Transwell inserts were used by initially adding medium to the multi-well plate, which was followed by addition of the Transwell inserts, and lastly of the medium and cells to the inside compartment. After an initial equilibration period useful to improve cell attachment, the plate was then incubated for at least one hour at the same temperature used to grow the cells. The cells were then added in fresh medium to the Transwell inserts and returned to the incubator over-night. Next day, HUVEC were stimulated with Aflibercept (0.025 mg/ml and 0.5 mg/ml, for 30 min) and Ranibizumab (0.025 mg/ml and 0.5 mg/ml, for 30 min). In addition, in some experiments, Aflibercept (0.5 mg/ml) and Ranibizumab (0.5 mg/ml) were also given after pre-treatment with the NOS inhibitor, L-NAME (4 µM, for 15 min; Sigma), the p38 MAPK inhibitor, SB203580 (4 µM, for 30 min; Sigma), the phosphatidylinositol 3'-kinase (PI3K) inhibitor, wortmannin (4 µM, for 15 min; Sigma), or the MAPK/ERK inhibitor, UO126 (4 µM, for 15 min; Sigma). After the treatment, the medium was changed and newly completed fresh medium was added for 24 h, in order to give the HUVEC time enough to induce any responses from the RPE cells. Next day, all Transwell inserts were removed by using sterile forceps, and some samples of RPE cells were also treated with 200 µM H₂O₂ for 30 min, in order to induce oxidative stress. At the end of stimulations, various assays were performed. All experiments were conducted in triplicate and repeated at least five times.

NO release

NO production was measured in RPE cells culture supernatants by using the Griess method (Promega, Milan, Italy), as previously performed in the same or similar cellular models [19-24]. For the experiments, 7500 cells for well/insert were plated in 96-Transwell plates. HUVEC were stimulated with Aflibercept (0.025 mg/ml and 0.5 mg/ml, for 30 min) and Ranibizumab (0.025 mg/ml and 0.5 mg/ml, for 30 min). Another pool of cells was treated with Aflibercept (0.5 mg/ml, for 30 min) and Ranibizumab (0.5 mg/ml, for 30 min), after pre-treatment with various inhibitors, as described above. Next day, some RPE cell samples were treated with 200 µM H₂O₂ for 30 min. At the end of the stimulations, NO production in the sample's supernatants was examined by adding an equal volume of Griess reagent following the manufacturer's instruction. At the end of incubation, the absorbance at 570 nm was measured by a spectrometer (VICTOR™ X Multilabel Plate Reader; PerkinElmer; Waltham, Massachusetts, USA) and the NO production. The value of each sample was quantified in respect to nitrite standard curve and expressed as nitrite production (µM).

Cell viability

Cell viability was examined in RPE cells by using the 1% 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT; Life Technologies Italia, Monza, Italy) dye, as previously described [19-25]. For the experiments, 10000 cells for well/insert were plated in 96-Transwell plates and treated as described for Griess assay. After each treatment, the medium was removed, and fresh culture medium without red phenol and FBS and with 0, 5 mg/ml MTT dye was added to the 96-well plate containing the cells that were kept for 2 h at 37°C in an incubator. Thereafter, the medium was removed, and an MTT solubilization solution (dimethyl sulfoxide; DMSO; Sigma) in equal volume to the original culture medium was added and mixed in a gyratory shaker until the complete dissolution of formazan crystals. Cell viability was determined by measuring the absorbance through a spectrometer (VICTOR™ X Multilabel Plate Reader; PerkinElmer) with a wavelength of 570 nm and cell viability was calculated by setting control cells as 100%.

Mitochondrial membrane potential

Mitochondrial membrane potential measurement in RPE cells was performed with JC-1 assay, as previously described [19, 21, 23-25] and following the experimental protocol used for MTT assay. After stimulations, the medium of cells plated in starvation medium was removed and the cells were incubated

with 5, 51, 6,61-tetrachloro-1, 11, 3,31 tetraethylbenzimidazolyl carbocyanine iodide (JC-1) 1X diluted in Assay Buffer 1X for 15 min at 37°C in an incubator, following the manufacturer's instruction (Cayman Chemical, Ann Arbor, MI, USA). After incubation, the cells were washed twice with Assay Buffer 1X and then the mitochondrial membrane potential was determined by measuring the red (excitation 550 nm/emission 600 nm) and green (excitation 485 nm/emission 535 nm) fluorescence through a spectrometer (VICTOR™ X Multilabel Plate Reader; PerkinElmer). To identify cells undergoing apoptosis, the ratio of fluorescent intensity of J-aggregates to fluorescent intensity of monomers was used as an indicator of cell health. The data were normalized versus control cells.

Wound-healing migration

Cell migration was measured in RPE cells, as previously described [24]. For the experiments, 25000 cells for well/insert were plated in 24-Transwell plate. HUVEC were stimulated with Aflibercept (0.025 mg/ml and 0.5 mg/ml, for 30 min) and Ranibizumab (0.025 mg/ml and 0.5 mg/ml, for 30 min). Images of cell monolayers were taken using an optical microscope (Leica ICC50HD) with a digital camera to evaluate wound closure. Migration was quantified by calculating the area of wound at time points T0 (time of wound), T24 (24 h after wound) and T48 (48 h after wound) by using ImageJ software (National Institutes of Health, Bethesda, MA, USA). For each condition, the percentage of wound closure at several time points throughout the course of the assay, was obtained through the formula:

% wound closure: $[(WA0-WA)/WA0]*100$, where WA= wound area and WA0=original size of the wound area.

ROS release

The ROS generation was performed with a specific kit (Abcam, Cambridge, United Kingdom) [19, 21-24]. For the experiments, 25000 cells for well/insert were plated in 96-Transwell plate and the same protocol used for Griess, MTT and JC-1 methods was followed, but in the absence of inhibitors. Briefly, after treatments, the reactions were stopped by removing the medium and washing the cells with PBS which was followed by staining with 10 μ M H2DCFDA for 20 min at 37°C. The fluorescence intensity of DCF was measured at an excitation and emission wavelength of 485 nm and 530 nm, respectively, by using a spectrophotometer (VICTOR™ X Multilabel Plate Reader; PerkinElmer). The amount of intracellular ROS was proportional to the intensity of DCF fluorescence, and the fluorescence intensity was recorded directly to indicate the relative amount of ROS. Results were expressed as DCF fluorescence intensity, which was proportional to the amount of intracellular ROS.

Glutathione (GSH) quantification

GSH measurement was performed with a specific kit (Cayman Chemical) as previously described [19, 21, 24, 25]. For the experiments, 400000 cells for well/insert were plated in 6-Transwell plate. HUVEC were treated as described for ROS quantification. Briefly, after treatments, cells were lysed by using the 50 mM 2-(N-morpholino) ethanesulphonic acid (GSH MES Buffer) and a rubber policeman, and were centrifuged at 10000 g for 15 min at 4°C. After centrifugation, the supernatant was treated with an equal volume of metaphosphoric acid (Sigma) for 5 min and centrifuged at 2000 g for at least two min. The supernatant was collected and supplemented with 50 μ l per ml of 4 M solution of triethanolamine (Sigma). Fifty μ l of the samples was transferred to a 96-well plate where GSH was detected following the manufacturer's instructions through a spectrometer (VICTOR™ X Multilabel Plate Reader; PerkinElmer) at excitation/emission wavelengths of 405–414 nm. Glutathione was expressed as GSH production (μ M).

Cell proliferation

The RPE cell proliferation was performed with xCELLigence™ MP Instrument (Roche, Basel, Switzerland) [19]. For the experiments, 3000 cells for well/ insert were plated in 16-well E-plate. HUVEC were stimulated with Aflibercept (0.5 mg/ml, for 30 min) and Ranibizumab (0.5 mg/ml, for 30 min). After stimulations, the medium of HUVEC was changed and new F-12K medium was added. At the same time and in order to induce peroxidation, some RPE cells were treated with 200 μ M H₂O₂, which was left to act all the time of the analysis. The proliferation rate was determined by the Cell Index, and analyzed after 12 h, 24 h and 48 h.

Cell-sensor impedance was expressed as an arbitrary unit called the Cell Index. The Cell Index at each time point is defined as $(Rn-Rb)/15$, where Rn is the cell-electrode impedance of the well when it

contains cells and Rb is the background impedance of the well with the media alone. The system measures the electrical impedance across interdigitated microelectrodes integrated at the bottom of E-plates. The data were analyzed with real-time cell analysis (RTCA) software, which includes real-time data display and analysis functions.

Cell cycle analysis

Flow cytometry was used for cell cycle analysis, as previously described [24]. For the experiments, 400000 cells for well/insert were plated in 6-Transwell plates. HUVEC were stimulated with Aflibercept (0.5 mg/ml, for 30 min) and Ranibizumab (0.5 mg/ml, for 30 min). Next day, some RPE cell samples were treated with 200 μ M H₂O₂ for 30 min, in order to induce oxidative stress. At the end of each stimulations, the culture medium was collected from each well and transferred into a 15 ml tube in order to collect the cells that were eventually detached, through trypsin-EDTA. Thereafter, an appropriate volume of culture medium was added, and cell suspension was transferred into a tube and centrifuged at 900 g for 5 min at room temperature. The supernatant was discarded, and cells were fixed in 1 ml 70% ethanol for 1 h at -20°C. After 1 h, the cells were centrifuged at 900 g for 5 min, and ethanol, as well as, the supernatants were discarded. Cells were washed with PBS and centrifuged again 900 g for 5 min. Each pellet of cells was resuspended in 200 μ l propidium iodide buffer (3.4 mM trisodium citrate, 9.65 mM sodium chloride, 0.003% tergitol), 25 μ l RNasi A (10 ng/ml; Cabru), and 10 μ l propidium iodide (1 mg/ml; Cabru).

Then, 50 μ l of each sample was transferred in a 96-well plate in triplicate, and after 15 min at 37°C in the dark, the analysis was performed by using Attune NxT (Life Technologies).

Cell lysates

For protein expression/activation, HUVEC were stimulated as described for cell cycle analysis. For the experiments, 400000 cells for well/insert were plated in 6-Transwell plates. At the end of stimulation, RPE cells were lysed in iced Ripa buffer, as described previously [19, 21-25].

Western blot analysis

Cell lysates (30 μ g of each protein sample) were loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis and, thereafter, they were transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories), which were incubated with specific primary antibodies: anti phospho-eNOS (1:1000; Ser1177, Cell Signaling Technologies), anti eNOS (1:1000; Cell Signaling Technologies), anti iNOS (1:500; Santa Cruz Biotechnology), anti phospho-Akt (1:1000; Ser 473, Santa Cruz Biotechnology), anti Akt (1:1000; Santa Cruz Biotechnology) anti phospho-ERK 1/2 (1:1000; Thr 202/Tyr 204, Santa Cruz Biotechnology), anti ERK 1/2 (1:1000; Santa Cruz Biotechnology), anti Cleaved Caspase-9 (1:1000; Abcam, Cambridge, UK) and anti Cytochrome C (1:1000, Santa Cruz Biotechnology). The membranes were washed and incubated with horseradish peroxidase-coupled goat anti-rabbit IgG (Sigma), peroxidase-coupled rabbit anti-goat IgG and horseradish peroxidase-coupled goat anti-mouse IgG (Sigma) for 45 min and were developed through Western Lightning Chemiluminescence (PerkinElmer Life and Analytical Sciences). Protein expression was calculated as a ratio towards specific total protein expression or β -actin (1:5000; Santa Cruz Biotechnology) detection.

Statistical analysis

All data were recorded using the Institution's database. Statistical analysis was performed by using STATVIEW version 5.0.1 for Microsoft Windows (SAS Institute Inc., Cary NC, USA). Data were checked for normality before statistical analysis. All the results obtained were examined through one-way ANOVA followed by Bonferroni *post hoc* tests. All data are presented as means \pm standard deviation (SD) of five independent experiments for each experimental protocol. A value of P <0.05 was considered statistically significant.

Results

Effects of Aflibercept and Ranibizumab administrated to HUVEC/RPE cells co-culture on cell viability, mitochondrial membrane potential and NO release in RPE cells

As illustrated in Fig. 1A and C, in physiological conditions the HUVEC treatment induced a dose-dependent increase of NO release in RPE cells, while reducing the NO release caused by peroxidation (Fig. 1B and D). It is notable that the pre-treatment with inhibitors counteracted the effects of Aflibercept and Ranibizumab on NO release, in both physiological conditions (Fig. 1A and C), and peroxidation (Fig. 1B and D).

In physiological conditions the administration of Aflibercept and Ranibizumab to HUVEC/RPE cells co-culture caused a dose-response increase in RPE cells viability (Fig. 2A and C). Similar effects were observed as regarding mitochondrial membrane potential (Fig. 3A and C); however, in this case, while both doses Aflibercept were able to induce an increase, the same effect was observed with 0.5 mg/ml Ranibizumab, only. In peroxidative conditions, both anti VEGF agents prevented the reduction of cell viability (Fig. 2B and D) and the collapse of mitochondrial membrane potential (Fig. 3B and D) caused by H₂O₂ in RPE cells. Of note, in physiological and peroxidative conditions, the protective effects elicited by HUVEC treated with Aflibercept and Ranibizumab on RPE cells, were reduced or abolished by UO126, wortmannin, SB203580 and L-NAME (Fig. 2 and 3).

Changes of activation/expression of eNOS/iNOS, Akt, ERK1/2, Cleaved Caspase 9 and Cytochrome C in RPE cells in co-culture with HUVEC treated with Aflibercept and Ranibizumab

As shown in Fig. 4, the effects of co-culture HUVEC/RPE cells on NO release by RPE cells, were accompanied by changes in eNOS/iNOS activation/expression. In physiological conditions, eNOS phosphorylation was increased (Fig. 4A), while iNOS expression was reduced (Fig. 4B). In RPE cells subjected to peroxidation, both kinases activation/expression were inhibited.

As shown in Fig. 5A and B, the treatment of HUVEC with Aflibercept and Ranibizumab increased p-Akt and p-ERK1/2 in RPE cells cultured in physiological conditions and prevented their inhibition caused by hydrogen peroxide.

In addition, the anti VEGF drugs were able to counteract the effects of hydrogen peroxide on apoptosis in RPE cells (Fig. 5C and D).

Effects of Aflibercept and Ranibizumab administration to HUVEC/RPE cells co-culture on ROS and GSH production by RPE cells

The stimulation of HUVEC with the two anti-VEGF drugs, reduced ROS production by RPE cells in a dose-dependent manner with a maximum effect at 0.5 mg/ml for Aflibercept and 0.2 mg/ml for Ranibizumab (Fig. 6A). Moreover, as shown in Fig. 6B, an increase in GSH production was observed with both anti-VEGF drugs.

Effects of Aflibercept and Ranibizumab administration to HUVEC/RPE cells co-culture on RPE cells migration and proliferation

As shown in Fig. 7A and B, Aflibercept and Ranibizumab increased RPE cells migration with a maximum effect at the major dose until 24 h from the wound. Furthermore, in physiological conditions, HUVEC stimulated with Aflibercept and Ranibizumab, were able to increase RPE cells proliferation (Fig. 7C) only up to 12 h of stimulation, while, after 24 h and 48 h, cell proliferation was reduced in a time-dependent way. Both agents counteracted the effects of hydrogen peroxide (Fig. 7D) from 12 h to 48 h of stimulation, too.

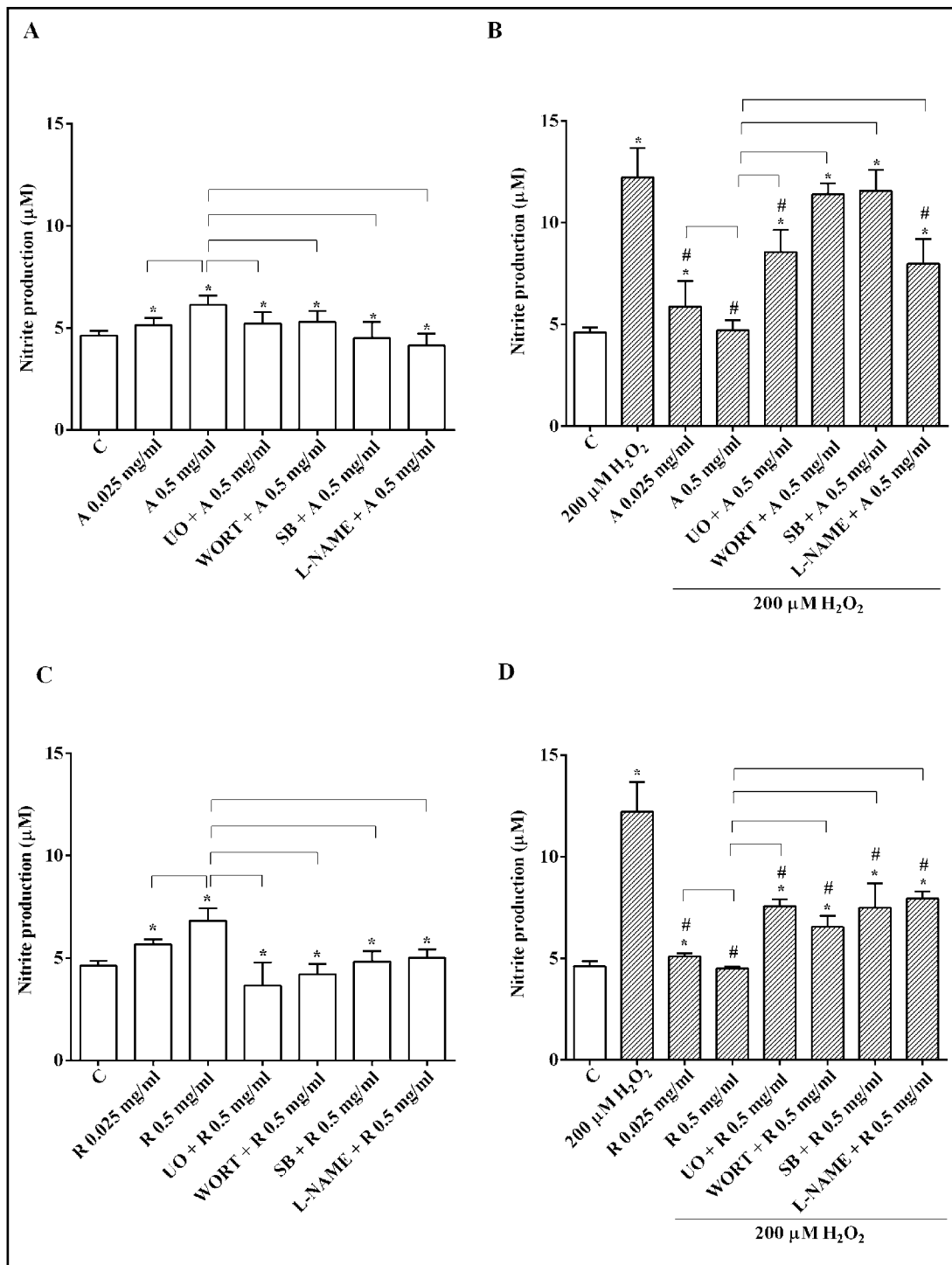


Fig. 1. Effects of HUVEC/RPE cells co-culture on NO release in RPE cells, in physiological (A and C) or peroxidative (B and D) conditions. In A and B, the effects of Aflibercept (A), and in C and D, the effects of Ranibizumab (R), are shown. The values obtained correspond to the nitrite (μM) produced after each stimulation. C=control (non- treated cells). UO (UO126 $4 \mu\text{M}$ for 15 min); WORT (wortmannin $4 \mu\text{M}$ for 15 min); SB (SB203580 $4 \mu\text{M}$ for 15 min); L-NAME ($4 \mu\text{M}$ for 15 min). Reported data are means \pm SD of five independent experiments for each experimental protocol. Significance between groups: * $P < 0.05$ vs C; # $P < 0.05$ vs $200 \mu\text{M H}_2\text{O}_2$. Short square brackets indicate significance between groups ($P < 0.05$).

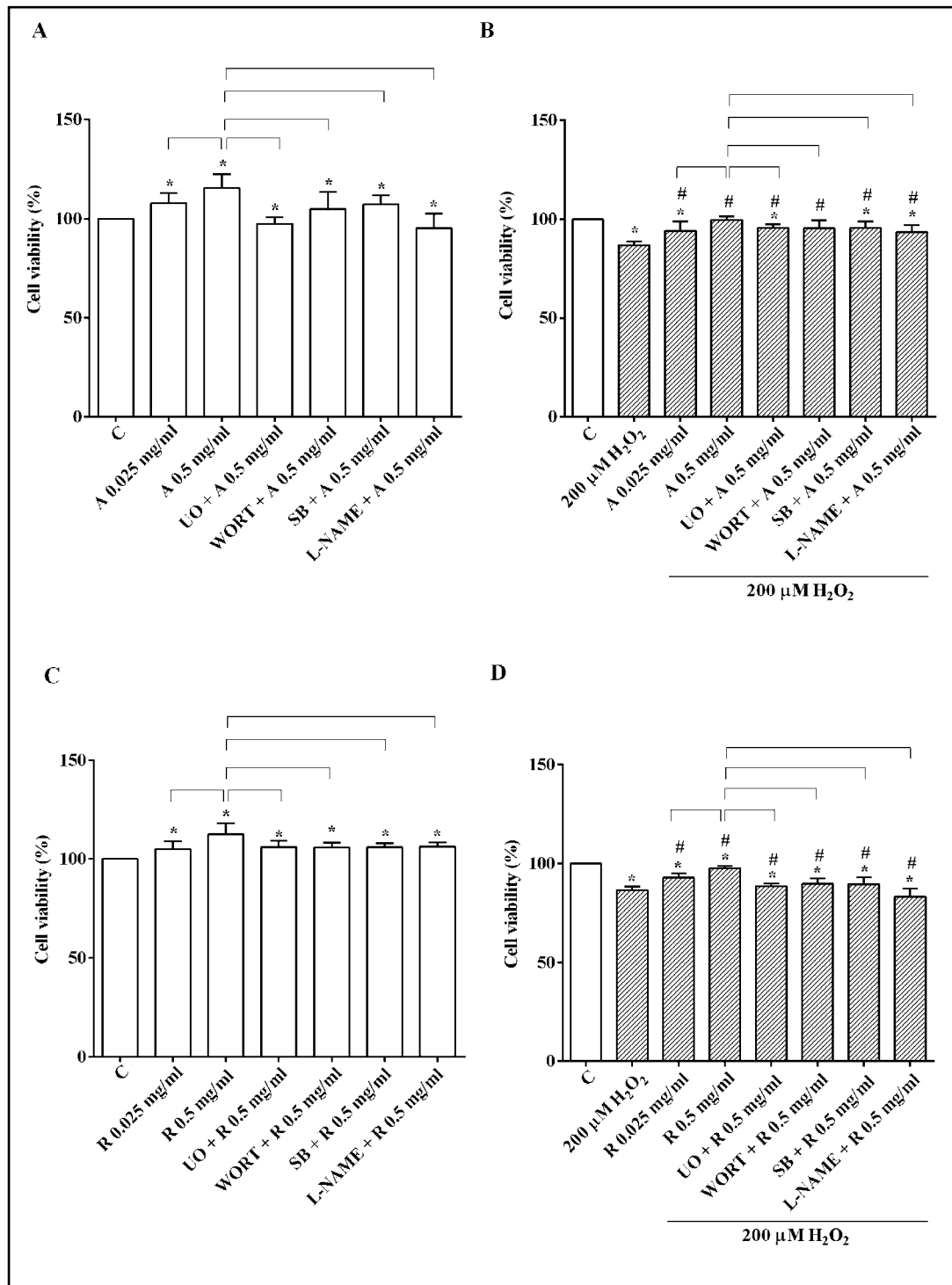


Fig. 2. Effects of HUVEC/RPE cells co-culture on RPE cell viability in physiological (A and C) and peroxidative (B and D) conditions. In A and B, the effects of Aflibercept, and in C and D, the effects of Ranibizumab, are shown. Abbreviations are as in the previous Fig.. The data were normalized versus control value. Reported data are means ± SD of five independent experiments for each experimental protocol. Significance between groups: *P<0.05 vs C; #P<0.05 vs 200 μM H₂O₂. Short square brackets indicate significance between groups (P<0.05).

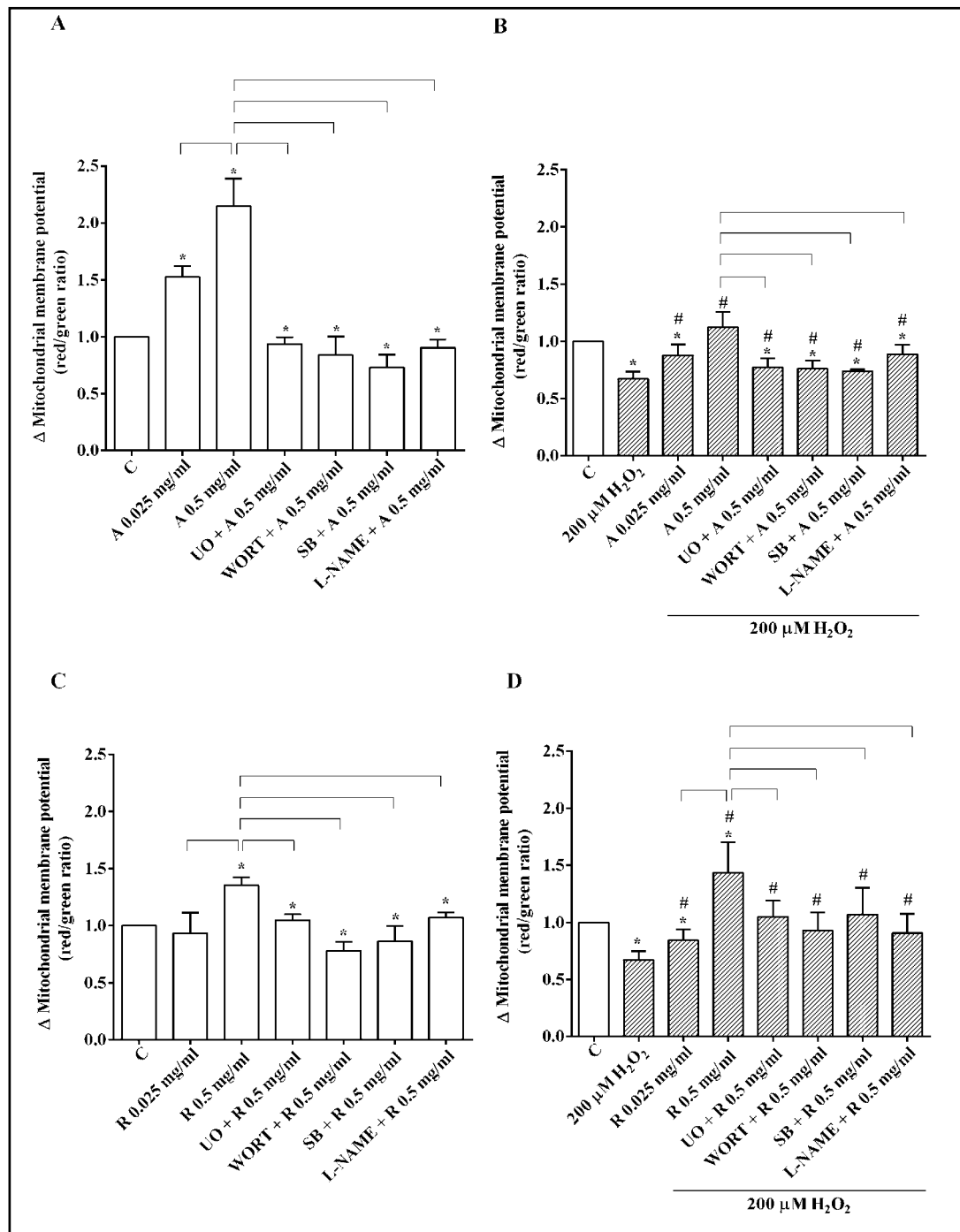
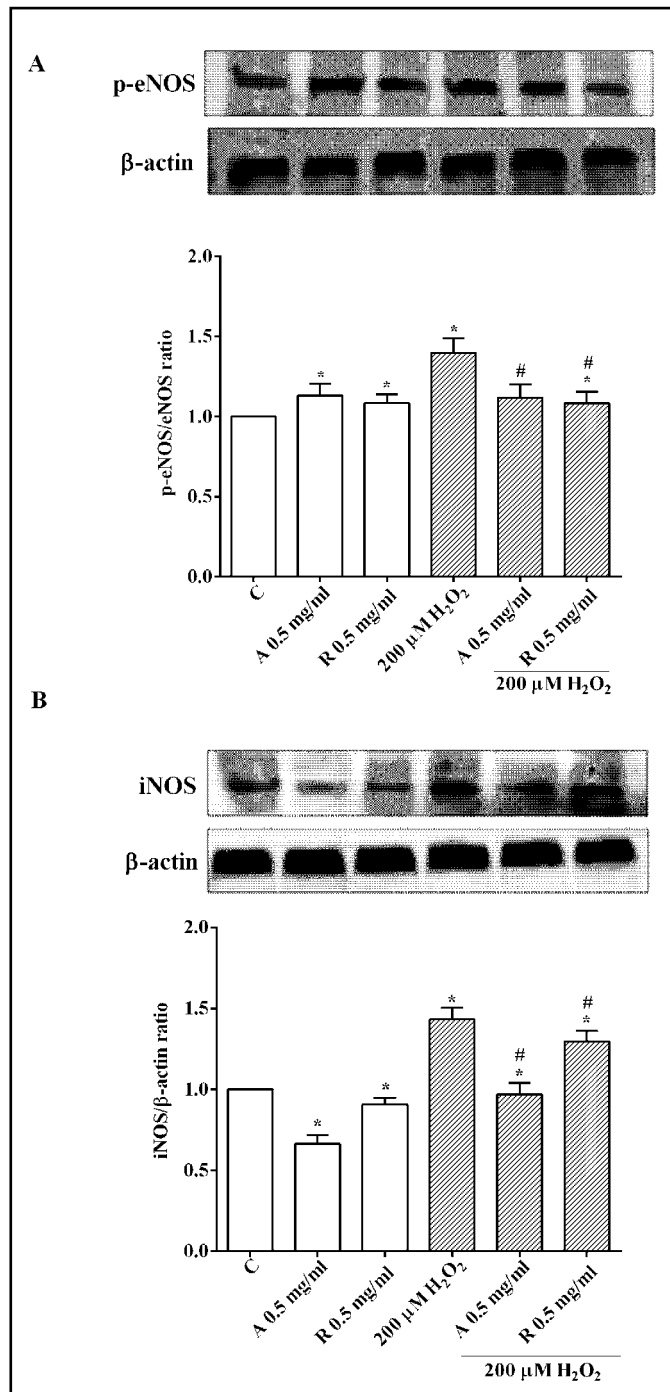


Fig. 3. Effects of HUVEC/RPE cells co-culture on mitochondrial membrane potential in RPE cells, in physiological (A and C) and peroxidative (B and D) conditions. In A and B, the effects of Aflibercept, and in C and D, the effects of Ranibizumab, are shown. Abbreviations are as in previous Fig.s. The data were normalized versus control value. Reported data are means ± SD of five independent experiments for each experimental protocol. Significance between groups: *P<0.05 vs C; #P<0.05 vs 200 μM H₂O₂. Short square brackets indicate significance between groups (P<0.05).

Fig. 4. Variation in eNOS phosphorylation and iNOS expression measured in RPE cells by Western blot and densitometric analysis. In A and B, densitometric analysis and an example of Western blot of p-eNOS and iNOS are respectively shown. Abbreviations are as in previous Fig.s. The data were normalized versus control cells. Reported data are means \pm SD of five independent experiments for each experimental protocol. Significance between groups: * $P < 0.05$ vs C; # $P < 0.05$ vs $200 \mu\text{M H}_2\text{O}_2$.



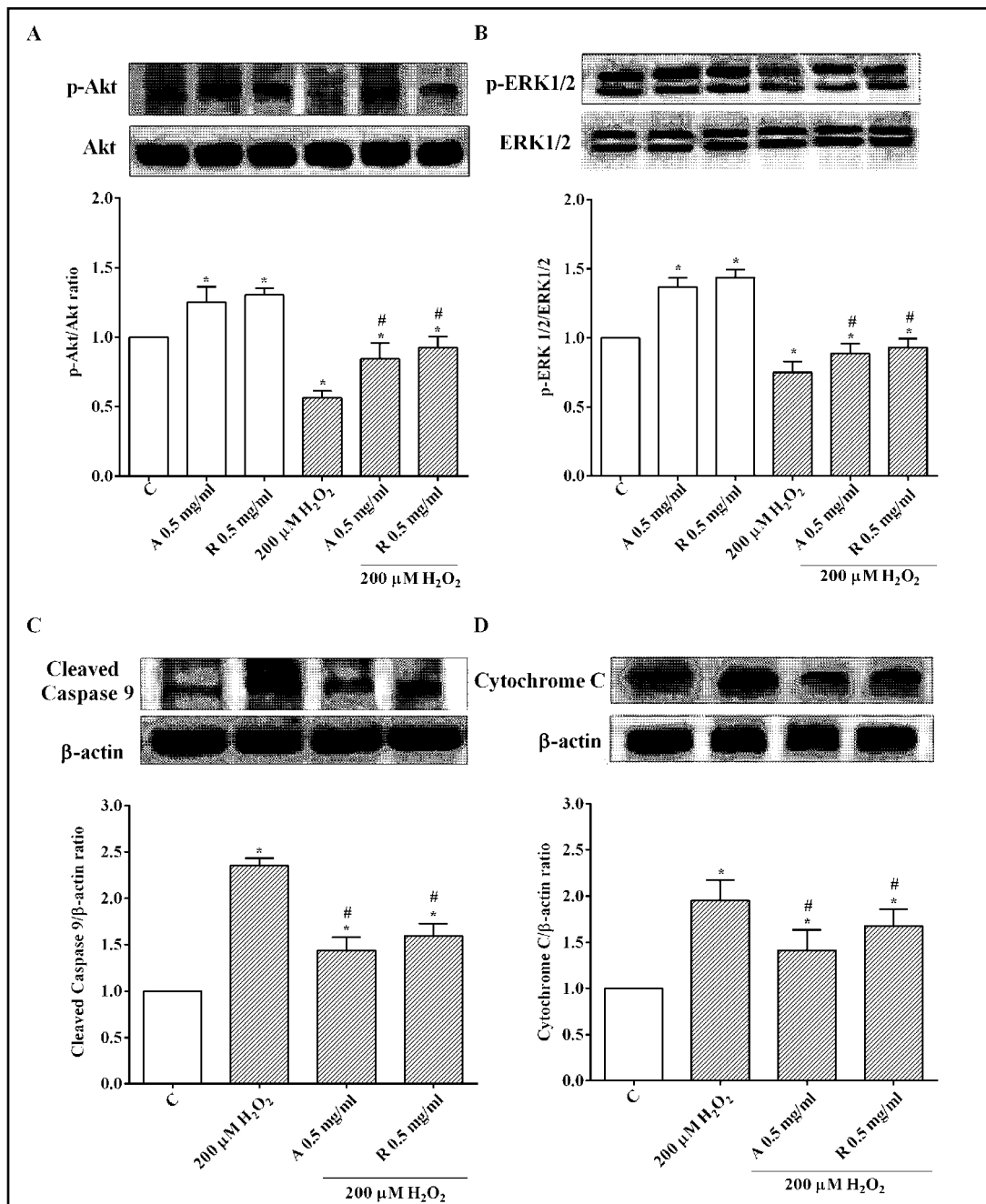
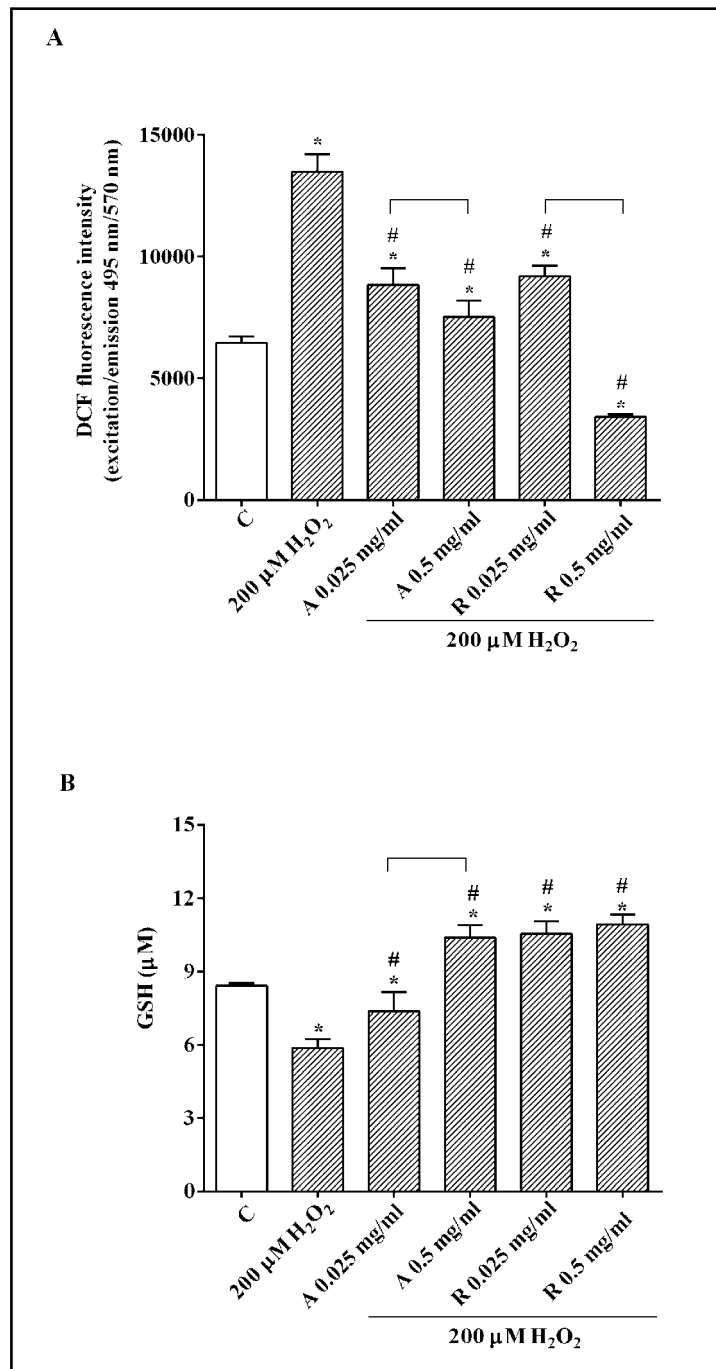


Fig. 5. Variation in Akt and ERK 1/2 phosphorylation (A and B) and in Cleaved Caspase 9 and Cytochrome C expression (C and D) in RPE cells, measured by Western blot and densitometric analysis. Densitometric analysis and an example of Western blot taken from 5 different experiments are shown in Figs A and B for p-Akt and p-ERK1/2, respectively, and in C and D for Cleaved Caspase 9 and Cytochrome C, respectively. Abbreviations are as in previous Fig.s. The data were normalized versus control value. Reported data are means \pm SD of five independent experiments for each experimental protocol. Significance between groups: * $P < 0.05$ vs C; # $P < 0.05$ vs 200 μ M H₂O₂.

Fig. 6. Effects of HUVEC/RPE cells co-culture on ROS (A) and GSH (B) production in RPE cells. Abbreviations are as in previous Figs. In A, results are expressed as DCF fluorescence intensity, which is proportional to the amount of intracellular ROS. In B, results are expressed as GSH (μM) produced after each stimulation. Reported data are means \pm SD of five independent experiments for each experimental protocol. Significance between groups: * $P < 0.05$ vs C; # $P < 0.05$ vs 200 μM H_2O_2 . Short square brackets indicate significance between groups ($P < 0.05$).



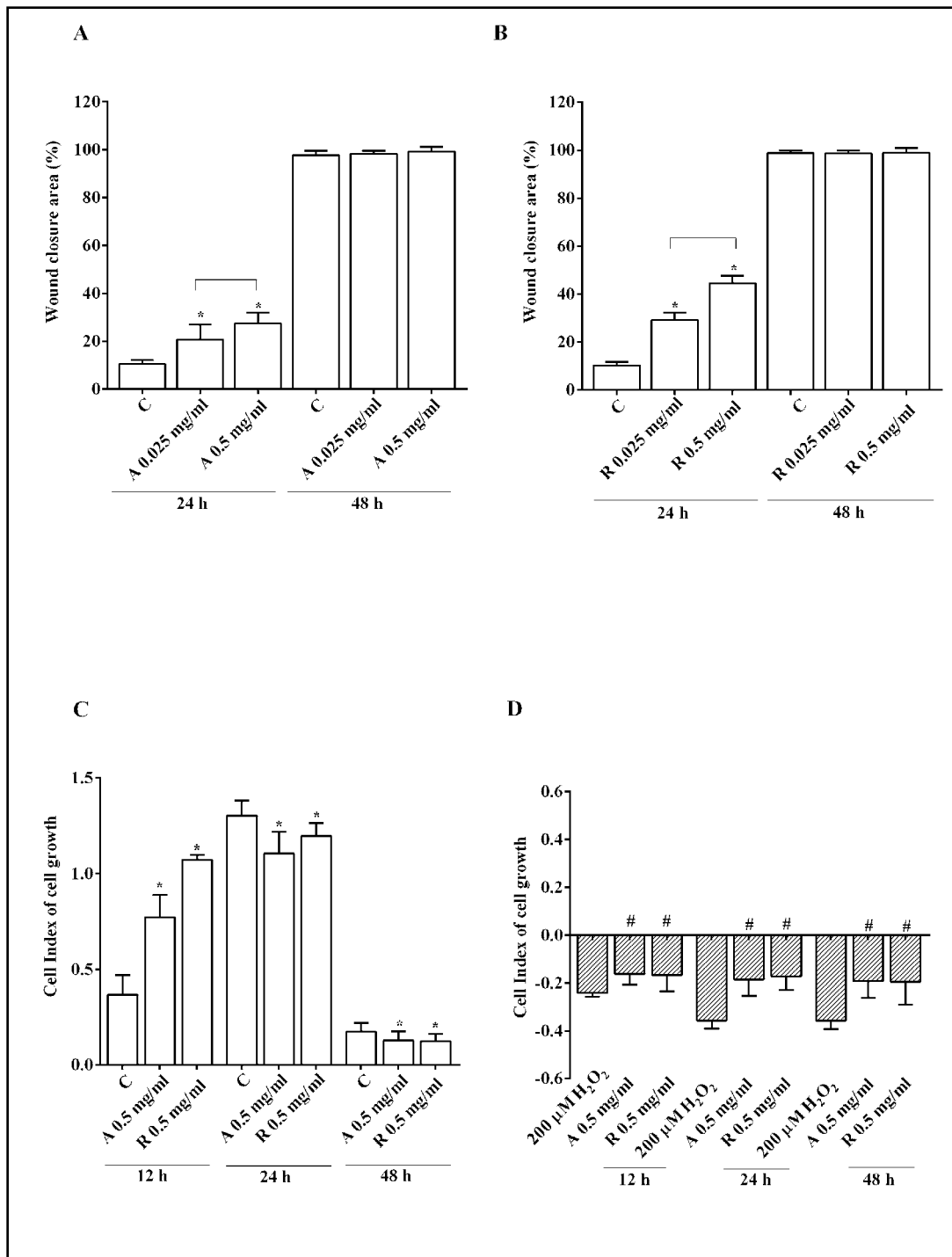
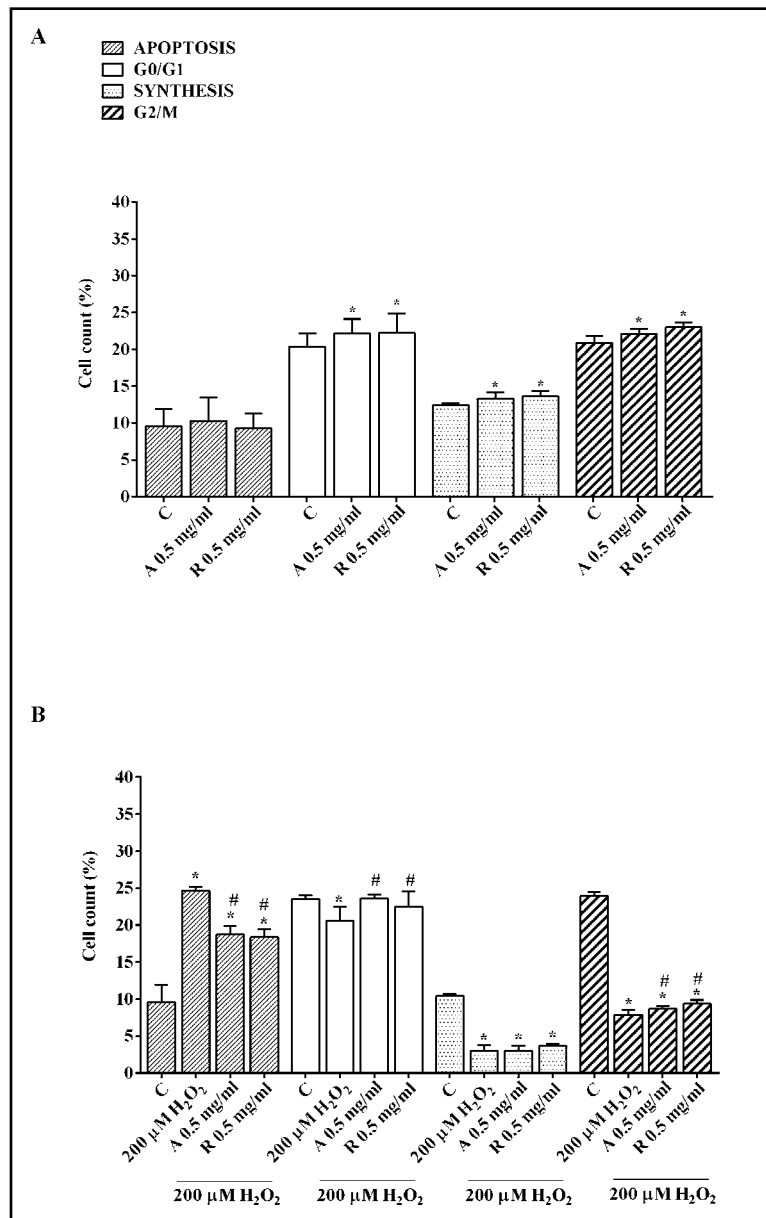


Fig. 7. Effects of HUVEC/RPE cells co-culture on RPE proliferation (A and B) and RPE migration (C and D). Abbreviations are as in previous Figs. In A (Aflibercept) and B (Ranibizumab), quantified data for the wound-healing assay are shown. The wound area and the percentage of healing were calculated for each time point. In C (physiological condition) and D (peroxidative condition), Cell index obtained from the xCELLigence system is shown. Abbreviations are as in previous Figs. Reported data are means \pm SD of five independent experiments for each experimental protocol. Significance between groups: * $P < 0.05$ vs C; # $P < 0.05$ vs 200 μM H₂O₂. Short square brackets indicate significance between groups ($P < 0.05$).

Fig. 8. Effects of HUVEC/RPE cells co-culture on RPE cell cycle progression, in physiological (A) and peroxidative (B) conditions. Cell cycle distribution was measured by flow cytometry using propidium iodide stain and quantitative analysis of apoptosis, G0/G1, synthesis, and G2/M phase is shown in a bar graph form. Abbreviations are as in previous Figs. Reported data are means \pm SD of five independent experiments for each experimental protocol. Significance between groups: * $P < 0.05$ vs C; # $P < 0.05$ vs $200 \mu\text{M H}_2\text{O}_2$. Short square brackets indicate significance between groups ($P < 0.05$).



Effects of Aflibercept and Ranibizumab administration to HUVEC/RPE cells co-culture on RPE cell cycle

The effects observed previously, were partly confirmed by the cell cycle analysis. Thus, in physiological conditions, HUVEC stimulated with Aflibercept and Ranibizumab, increased the percentage of RPE cells in G0/G1, S and G2/M phase (Fig. 8A). In peroxidative conditions, HUVEC stimulated with both anti-VEGF drugs, reduced the percentage of RPE cells in apoptosis. Moreover, in comparison with hydrogen peroxide, Aflibercept and Ranibizumab were able to increase the percentage of RPE cells in G0/G1 AND G2/M (Fig. 8B).

Discussion

This study has shown for the first time that anti-VEGF agents, Aflibercept and Ranibizumab, play an important role in the modulation of cell viability, mitochondrial membrane potential, proliferation and migration of RPE cells co-cultured with HUVEC, in either physiological or pathological conditions, by mechanisms related to NO release and cell survival-related pathways.

In a previous study, Aflibercept and Ranibizumab were able to increase the NO release by RPE cells cultured in physiological conditions and to cause opposite results in peroxidation. Moreover, beneficial effects on cell viability and mitochondrial function were observed [19]. However, since the normal interaction between RPE cells and retinal vascular endothelial cells appears critical for the maintenance of the outer-retinal structure and function [26, 27] and for the onset of vision-threatening pathological conditions such as AMD [28] a better characterization of the homeostatic RPE cells-vascular endothelial cells relationships would therefore be of particular interest [27].

Thus, in the present study we focused on the effects of the anti-VEGF drugs on the cross talk between RPE cells and HUVEC, which were directly treated with the two anti-VEGF drugs, at doses similar to the ones achievable in humans after intravitreal injections, and previously used in RPE cells [19, 29].

Moreover, since oxidative stress to RPE cells over time is theorized to produce tissue dysfunction that contributes to the development of AMD [19, 30] H_2O_2 at a concentration which is widely adopted to induce oxidative stress in various cell lines, among which RPE [19, 31-33] was used in some experiments.

The results obtained confirmed our previous observations about NO release, cell viability and mitochondria function [19]. Hence, Aflibercept and Ranibizumab increased the NO release by RPE cells co-cultured in physiological conditions with HUVEC. Opposite results were obtained in peroxidation, when both Aflibercept and Ranibizumab reduced the NO release caused by hydrogen peroxide. These effects on NO release were accompanied by changes in eNOS/iNOS activation/expression.

Those findings would confirm the fact that differences of NO concentration and of activity of the constitutive or inducible NOS could account for the beneficial or harmful effects played by NO in RPE [10, 13, 14].

It is also of note that the administration of the NOS blocker, L-NAME, reduced the effects of the anti VEGF drugs on cell viability and mitochondrial membrane potential of RPE cells co-cultured with HUVEC, which would highlight the role of NO in the determining these responses.

The findings we have obtained about the protective effects of anti-VEGF agents on mitochondrial membrane potential could be of significant clinical relevance. Mitochondrial membrane potential has been considered as a good indicator of the energetic status of the mitochondria and, above all, of cellular homeostasis. Interestingly, changes in mitochondrial membrane potential have been reported to be correlated with cell survival or death through apoptosis [34-37]. Previous data have shown a clear association between RPE cell health and compromised mitochondrial function [33, 38, 39]. In particular, mitochondrial depolarization has been reported to precede RPE cell death caused by peroxidation through the reduction of energy production, as well as, the increase in Cytochrome C release and ROS [40-42].

In our study both anti-VEGF agents, in addition to preventing the fall of cell viability and mitochondrial membrane potential, were also able to increase cell proliferation and migration, and to counteract the effects of H_2O_2 . These results were confirmed by cell cycle analysis, too.

The above findings could also be of particular relevance in clinical conditions. Hence, there are emerging indications that anti-VEGF treatment can potentially increase development of RPE cell atrophy leading to geographic atrophy (GA) [17, 42-45]. Our data showing protective effects elicited by anti-VEGF agents in RPE cell proliferation and migration are in contrast with findings reported from *in vivo* studies [46]. It could, however, be argued that

the increased cell proliferation was observed in RPE cells with Aflibercept and Ranibizumab treatments up to 12 h, only. Findings taken with longer exposure would be in agreement with previous data. Changes in ocular microenvironment or effects of the anti-VEGF agents on retinal cells other than RPE cells could contribute to those discrepancies. Although the exact mechanisms of anti-VEGF effect on macular atrophy has not been fully elucidated, data from the present study may indicate that the keeping of mitochondria function could be hypothesized to play a key role.

The protective effect of Aflibercept and Ranibizumab on RPE cells response to oxidative stress, has been confirmed by the analysis of ROS release and GSH content.

Oxidative stress is one of the leading factors triggering RPE cells apoptosis. Furthermore, while the accumulation of ROS in mitochondria can lead to apoptotic cell death and ROS, it may also have direct effects on cellular structure and function. In our study Aflibercept and Ranibizumab, were able to reduce ROS production induced by H₂O₂, to increase GSH content and to decrease apoptosis, as shown by Western blot. The keeping GSH content could be hypothesized to be involved in the modulation of eNOS function and ROS release. Hence, eNOS itself has been reported to be a redox “hub”, being regulated by GSH-dependent pathways. Also, changes of GSH have been reported to cause eNOS “uncoupling”, which would trigger ROS production from the oxygenase domain [47].

In our study, short-term exposure of RPE cells to H₂O₂ reduced Akt activation, as previously shown in the same cellular model [19, 48]. This effect was hindered in RPE cells co-cultured with HUVEC treated with either Aflibercept or Ranibizumab. Thus, although not directly examined, the activation of intracellular signaling downstream Akt and ERK1/2 could be hypothesized to be involved in the protective effects elicited by the anti-VEGF agents against AMD. The signaling pathways downstream Akt and ERK1/2 activation are known to be involved in the regulation of cellular proliferation, differentiation, and survival processes in many cell lines, amongst which are RPE cells. In this respect, it is notable that the effects of the two anti-VEGF agents on cell viability and mitochondrial membrane potential were reduced in the presence of PI3K, ERK1/2, and p38MAPK inhibitors. In particular, the modulation of PI3K/Akt pathway could represent a promising therapeutic tool for the prevention of the RPE degeneration, and theoretically, for the treatment of eye disorders, such as AMD [49, 50].

Conclusion

In conclusion, the co-culture experiments would confirm the importance of the cross talk between HUVEC and RPE cells in the prevention of oxidative stress damage and RPE cell apoptosis, and in eliciting the protective effects of Aflibercept and Ranibizumab.

Our results could also indicate that the progression of atrophy in patients with neovascular AMD treated with Ranibizumab or Aflibercept are not due to the treatment itself, but to additional local factors. Specific subtypes of neovascular AMD have shown different natural evolution, thus, with major probability to progress to the atrophy stage. Therefore, further clinical and experimental studies are needed to better understand the interaction between anti-VEGF treatment and the progression of atrophy in patients with AMD.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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