

Basal and apical regulation of VEGF-A and placenta growth factor in the RPE/choroid and primary RPE

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Purpose: Members of the vascular endothelial growth factor (VEGF) family are strongly involved in pathological processes in the retina, such as age-related macular degeneration and diabetic retinopathy. Cells of the retinal pigment epithelium (RPE) constitutively secrete VEGF-A, and the secretion of placental growth factor (PIGF) has also been described. RPE cells are strongly polarized cells with different secretome at the apical and basal side. In this study, we evaluated the basal and apical regulation of VEGF-A and PIGF secretion in RPE/choroid explants and primary RPE cells.

Methods: RPE/choroid tissue explants were prepared from porcine eyes and cultivated in modified Ussing chambers, separating apical (RPE) and basal (choroid) supernatant. Primary RPE cells were also prepared from porcine eyes and cultivated on Transwell plates. Explants and cells were treated with inhibitors for VEGFR-2 (SU1498), p38 (SB203580), and the transcription factors nuclear factor-kappa B (NF- κ B) and SP-1 (mithramycin), respectively. VEGF-A and PIGF content was evaluated with enzyme-linked immunosorbent assay (ELISA). In addition, western blots were performed.

Results: In the RPE/choroid, VEGF-A can initially be found on the apical and basal sides with significantly more pronounced secretion on the basal side. VEGF-A secretion is differentially regulated on the apical and basal sides, with the inhibition of SP-1 and NF- κ B showing strong effects apically and basally after 24 h and 48 h, the inhibition of p38 displaying its effect mainly on the basal side with some effect apically after 48 h, and the inhibition of VEGFR-2 reducing the secretion of VEGF only on the apical side at 24 h and 48 h. In the RPE cell culture, similar effects were found, with inhibition of NF- κ B or SP-1 displaying a strong decrease in VEGF-A on both sides, and p38 inhibition displaying only an inhibitory effect on the basal side. In contrast, an apical effect of VEGFR-2 inhibition was not found. However, the western blot experiments exhibited a significant decrease in the VEGF-A protein under SU1498 treatment. In the RPE/choroid organ cultures, PIGF was initially found mainly on the basal site with only minute amounts of PIGF found apically. NF- κ B and SP-1 were strongly involved in PIGF regulation apically and basally, while VEGFR2 and to a lesser degree p38 displayed some regulation at the basal site. In the primary RPE cell culture, PIGF was not found on the apical or basal side.

Conclusions: VEGF-A and PIGF were constitutively secreted and regulated by the RPE/choroid complex, with PIGF secreted mainly by the choroid. Although the transcription factors NF- κ B and SP-1 were involved in apical and basal regulation of both growth factors, VEGFR-2 displayed a strong polarity, with regulation of apical VEGF-A and basal PIGF secretion.

The vascular endothelial growth factor (VEGF) family consists of various members (VEGF-A, -B, -C, -D, -E, -F, and placental growth factor), of which VEGF-A is most important for angiogenesis. In the developing organism, the loss of a single allele of VEGF-A is lethal [1,2]. VEGF-A is vital for the development of the retinal and choroidal vasculature as well as the development of the neuroretina [3]. In the adult, VEGF-A is intimately involved in the pathogenesis of exudative age-related macular degeneration and diabetic macular edema, mediating neovascularization and barrier disruption [4,5], and is the major target for the treatment of these diseases [6]. However, in addition to contributing to pathological angiogenesis and edema, VEGF-A exerts various physiologic

functions in the adult, such as protecting the neuroretina, the retinal pigment epithelium (RPE), and the endothelium and upholding the fenestration of the choriocapillaris. VEGF-A is produced by various cells in the retina, such as ganglion cells and Müller cells, but the main source in the retina is the RPE [3]. VEGF-A expression and secretion are regulated in a tight, differentiated manner and may be induced by various factors such as hypoxia, oxidative stress, cytokines, hyperthermia, and others [7-10], but is also strongly constitutively secreted by the RPE and the RPE/choroid [11,12]. Regulation of constitutive VEGF-A expression in the RPE or RPE/choroid has not completely been elucidated, but we have recently shown that it differs from induced VEGF-A regulation and is mediated via nuclear factor-kappa B (NF- κ B), SP-1, p38 mitogen activated protein kinase (MAPK), and autocrine VEGFR-2 regulation [9,12,13].

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In contrast to VEGF-A, the loss of both alleles of placenta growth factor (PlGF) is of no consequence for the development of the embryo [14]. However, PlGF is involved in ischemia-induced and tumor angiogenesis. Furthermore, PlGF has been shown to strongly enhance the effect of VEGF-A on endothelial cells, potentiating its proangiogenic effects [15,16]. Moreover, PlGF is found in choroidal neovascularization (CNV) membranes, enhances angiogenesis CNV models [17], and has recently been introduced as an additional target for anti-VEGF treatment [6]. PlGF has been shown to be expressed in the healthy RPE/choroid [17].

The RPE is a monolayer situated between the choroid and the photoreceptor and is part of a functional complex with Bruch's membrane and the choroid, in which the members tightly interact to protect and maintain the photoreceptors [18]. They are highly polarized cells that exert different functions on their apical and basal sides [19-21]. In this study, we investigated the differential regulation of constitutive VEGF-A and PlGF secretion on the apical and basal sides of the RPE/choroid, showing differential apical and basal regulation of VEGF-A secretion and primarily choroidal secretion of PlGF.

METHODS

RPE/choroid organ culture: RPE/choroid organ cultures were prepared as previously described with modifications [22]. The usage of the eyes for experimental purposes was conducted in agreement with the animal welfare officer of the University of Kiel. According to the German welfare act (TierSchG), it is not considered to be animal research, but an alternative to the use of animals in research. Briefly, porcine eyes were obtained from a local abattoir, and preparation was initiated within 4 h of death. The globes were briefly immersed in antiseptic solution (betaisodona, Mundipharma, Limburg, Germany). The anterior segment, vitreous, and retina were removed, and the RPE/choroid layer gently separated from the sclera using forceps and scissors. The RPE/choroid explant was fixed between the two parts of a fixation ring. Then the rings were transferred to multiwell culture plates (Corning Costar, Lowell, MA) and allowed to equilibrate in culture medium. The culture medium was a mixture of equal amounts of Dulbecco's modified Eagle's medium (DMEM; PAA, Cölbe, Germany) and Ham-F12 medium (PAA), which was supplemented with penicillin/streptomycin (1%), l-glutamine, HEPES (10 mM), sodium pyruvate (110 mg/ml) and porcine serum (10%, PAA), taurine (100 µM), and calcium (2 mM). The cultures then were brought into a modified Ussing chamber with an exposed tissue diameter of 7 mm. This chamber allowed the separation of the apical

(RPE-facing) and basal (choroid-facing) compartments. To evaluate the viability of the cultures, a calcein stain was conducted as previously described after 48 h [12]. Only the supernatants of viable cultures were further analyzed.

Experimental treatment of organ cultures: From one porcine eye, two cultures were prepared. One served as the control, and one was treated with the respective inhibitor (VEGFR-2, 10 µM SU1498 (Calbiochem, Darmstadt, Germany); p38, 10 µM SB203580 (Calbiochem); SP-1, 1 µM mithramycin (Sigma-Aldrich, Munich, Germany), and NF-κB, 1 µM NF-κB inhibitor (Calbiochem)). None of the tested inhibitors has been described as to exert any toxicity in organ or cell culture [12]. Cultures were prepared, and after 24 h, medium was collected, and inhibitors were added. Medium was collected at 24 h and 48 h after the initial application; the inhibitor was readded to the medium after each exchange.

Primary RPE isolation and culture: Porcine RPE cells were isolated as previously described [13]. Briefly, porcine eyes obtained from the local abattoir were cleaned of adjacent tissue and immersed briefly in antiseptic solution (betaisodona, Mundipharma). The anterior part of the eye was removed, as well as the lens, vitreous, and retina. In each eyecup, trypsin was added, and incubated for 10 min at 37 °C. The trypsin solution was removed and substituted with trypsin-EDTA for 45 min at 37 °C. RPE cells were gently pipetted off the choroid, collected in media, and washed. Cells were cultivated in DMEM supplemented with penicillin/streptomycin (1%), HEPES (25 mM), sodium-pyruvate (110 mg/ml), 10% fetal calf serum (Linaris GmbH, Wertheim-Bettingen, Germany), and 1% non-essential amino acids. Cells of the second passage were cultivated on Transwell plates (volume of medium: 200 µg apical, 600 µl basal; Costar Corning), and the transepithelial resistance (TER) was measured with an epithelial voltmeter (EVOM; World Precision Instruments, Sarasota, FL) as previously described [23]. For western blot experiments, RPE cells were cultured on Nunc dishes (Thermo Scientific, Schwerte, Germany).

Experimental treatment of cell culture: In the Transwell plates, the cells were treated after a minimum of 4 weeks of cultivation and a stable TER ($225.2 \pm 54.7 \Omega/\text{cm}^2$). Medium was collected after 24 h of incubation without any inhibitor at 0 h, and fresh medium with the respective inhibitors (see above) was added. The medium was collected again after 24 h, fresh medium supplied with the respective inhibitor, and after 48 h the medium was collected again. The supernatant was centrifuged for 5 min at 4 °C and $16,200 \times g$ and stored at -20 ° until further evaluation. For the western blot experiments, the medium of the confluent cell cultures cultivated on the Nunc dish was replaced with fresh medium containing the

inhibitor (SU1498), and the cells were collected as described below after 48 h.

Evaluation of VEGF-A and PlGF content: The VEGF-A content was measured with a VEGF-A enzyme-linked immunosorbent assay (ELISA; R&D Systems, Wiesbaden, Germany), and the PlGF content was measured using a Human PlGF DuoSet (R&D Systems). To evaluate the Transwell plates, VEGF-A content was normalized for the 600 μ l medium. All ELISA kits were used according to the manufacturer's instructions.

Whole cell lyses: Cell lysis was conducted as previously described [13]. Briefly, the cells were scraped off in PBS (Serva, Heidelberg, Germany; composition 8.0 g/l sodium chloride, 0.2 g/l potassium chloride, 1.15 g/l di-sodium hydrogenphosphate, 0.2 potassium dihydrogenphosphate), centrifuged, and the pellet was resuspended in sodium dodecyl sulfate (SDS)-lysis buffer-buffer (Tris pH 7.4 10 mM, 1% SDS, protease inhibitor, phosphatase inhibitor). Samples were heated at 95° for 5 min, sonicated with ultrasound, and centrifuged. The protein concentration of the supernatant was determined with the BioRad (Munich, Germany) protein assay with bovine serum albumin (BSA) as the standard.

Western blot: Western blot was conducted as previously described with modifications [24]. The cell lysate was separated under reducing conditions on 12% SDS-polyacrylamide gel and transferred onto polyvinylidene fluoride (PVDF) membranes. After blotting the gel, the PVDF membrane (Carl Roth GmbH, Karlsruhe, Germany) was blocked with 4% skim milk in Tris-buffered saline with 0.1% Tween for 1 h at room temperature. The blot was treated with the first antibodies, against beta-actin (Cell Signaling Technology, Leiden, Netherlands) or VEGF-A (A 20; Santa Cruz Biotechnology, Heidelberg, Germany), overnight at 4 °C in 2% skim milk in Tris buffered saline with 0.1% Tween. After washing, the blot was incubated with anti-rabbit immunoglobulin G (IgG), horseradish peroxide-linked antibody (Cell Signaling Technologies) in 2% skim milk in Tris-buffered saline with 0.1% Tween. Following the final washing, the blot was incubated with Immobilon chemiluminescence reagent (Millipore, Schwalbach, Germany), and the signal was detected with MF-ChemiBis 1.6 (Biostep, Jahnsdorf, Germany). The density of the bands was evaluated using Total laboratory software (Biostep), and the signal was normalized for β -actin.

Statistics: Each experiment was independently conducted three to six times. Bars in the figures depict the mean and the standard deviation. Significance was evaluated with the paired Student *t* test using Excel software 2010 (Microsoft, Redmond, WA).

RESULTS

Constitutive secretion of VEGF-A and PlGF: We have previously shown that RPE/choroid cultures constitutively secrete VEGF-A in a stable manner [12,25]. In contrast to the previously published models, the RPE/choroid organ explants in this study were cultivated in modified Ussing chambers that allow differentiation between the apical and basal compartments [22]. Here, instead of the steady-state flow achieved with the perfusion chamber, the medium was collected after 24 h for all experiments, in which the growth factors accumulated in the supernatant.

We found that VEGF-A was secreted at high levels at the apical and basal sides, with more pronounced and significantly higher secretion at the basal side (apical: 231.6 \pm 106.1 pg/ml versus basal: 436.3 \pm 266.2 pg/ml, p <0.001; Figure 1A). In primary cell culture, a similar distribution was found (apical: 225.5 \pm 74.9 pg/ml, basal: 670.3 \pm 200.5 pg/ml, p <0.001; Figure 1C). The secretion of VEGF-A in cell culture was stable over time on the apical (0 h: 206.6 \pm 61.8 pg/ml; 24 h: 206.4 \pm 88.1 pg/ml; 48 h: 200.2 \pm 58.8 pg/ml) and basal sides (0 h: 542.5 \pm 206.7 pg/ml; 24 h: 492.7 \pm 219.5 pg/ml; 48 h: 489.7 \pm 197.8 pg/ml; Figure 1E).

PlGF, however, was also secreted by the explants but was found mainly on the basal side with initially only minute amounts found apically (basal: 190.7 \pm 116.3 versus apical: 14.0 \pm 14.9 pg/ml, p <0.001; Figure 1B). In the RPE cell culture, no PlGF was measured in ELISA, neither at 0 h (Figure 1D) nor at later time points (data not shown). Thus, no further experiments concerning PlGF secretion by RPE cells were conducted.

Regulation of apical and basal VEGF-A secretion: We have previously shown that the transcription factors NF- κ B and SP-1 are involved in the regulation of constitutive VEGF-A secretion in the RPE/choroid. Moreover, autocrine VEGFR-2 regulation was found, in addition to the involvement of p38 that was shown to be independent of NF- κ B [9,12]. We investigated the effect of the inhibition of the respective factor on the apical and basal secretion of VEGF-A. The inhibitors were previously shown to not exert toxicity on RPE cells or RPE organ explants in a perfusion culture [12]. Correspondingly, none of the tested inhibitors showed toxicity on the organ cultures cultivated in the Ussing chambers (Figure 2).

NF- κ B: In the RPE/choroid organ culture, a significant effect of the inhibition of NF- κ B was found on the apical side at 24 h (control: 267.8 \pm 88.1; inhibitor: 180.6 \pm 57.9 pg/ml, p <0.01) as well as at 48 h (control: 307.0 \pm 98.7 pg/ml, inhibitor: 166.2 \pm 91.9 pg/ml, p <0.001) of incubation (Figure 3A). Similar effects were found at the basal side at 24 h

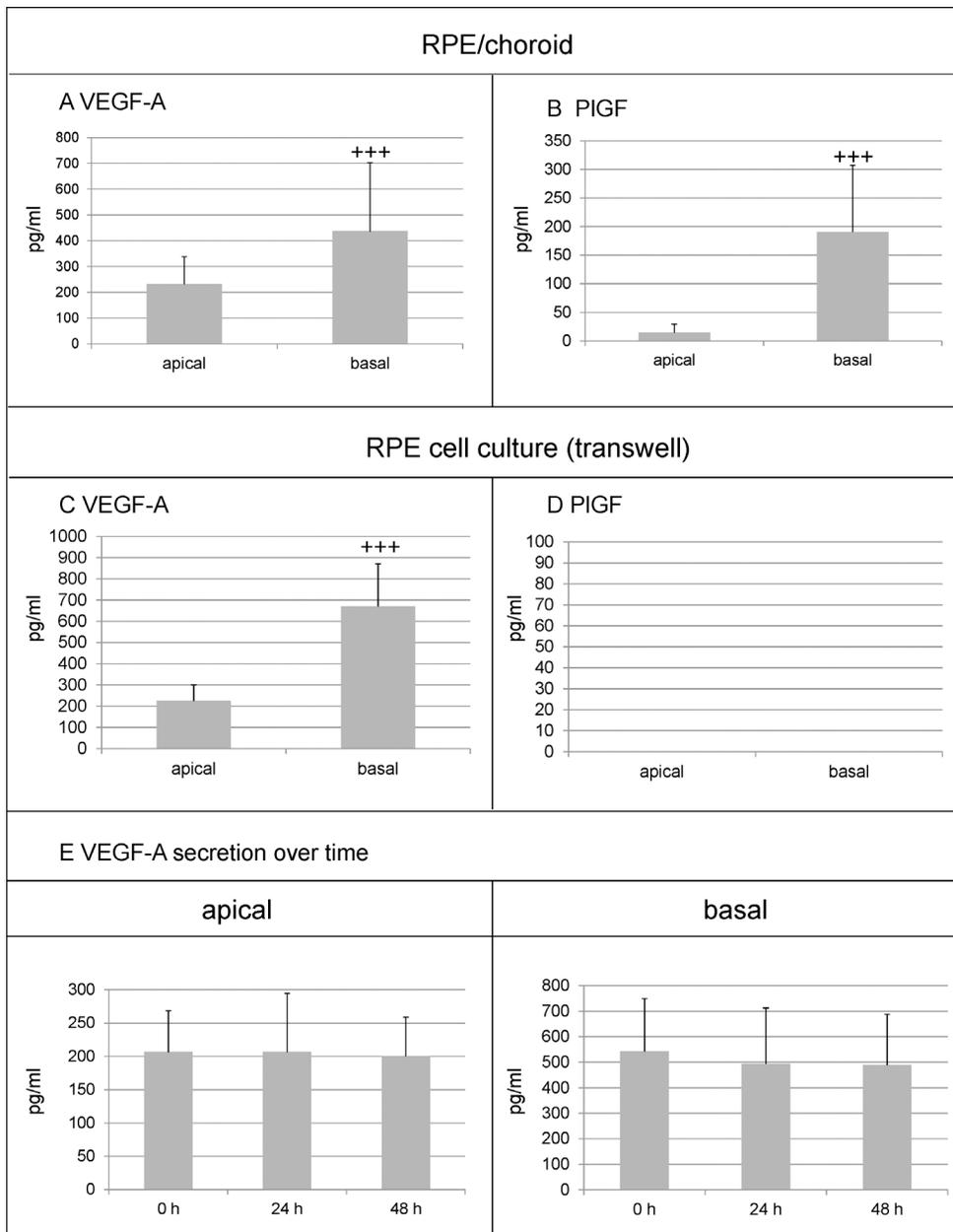


Figure 1. Basal VEGF-A and PIGF secretion. **A:** In the RPE/choroid, vascular endothelial growth factor (VEGF)-A is constitutively secreted with significantly higher secretion on the basal side. **B:** Placental growth factor (PIGF) is also constitutively secreted with only minor secretion on the apical and significantly stronger secretion on the basal side. **C:** In the RPE cell culture, VEGF is basally and apically secreted, with a stronger secretion on the basal side. **D:** In RPE cell culture, no PIGF was found. **E:** The secretion of VEGF in the RPE cell culture is stable over time. Supernatants were collected for 24 h and were analyzed in enzyme-linked immunosorbent assay (ELISA). **E:** Supernatant was collected for 24 h, and the medium was changed and recollected after another 24 h. Significance was determined with the Student *t* test; ⁺⁺⁺ $p < 0.001$. $n \geq 30$ (A–C), $n = 7$ (D), $n = 10$ (E, apical), $n = 6$ (E, basal). Bars depict the mean and the standard deviation.

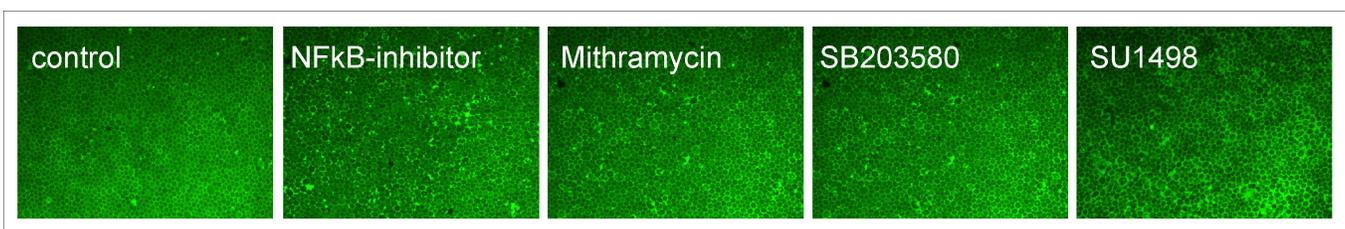


Figure 2. Calcein stains of organ cultures after treatment with the indicated inhibitor.

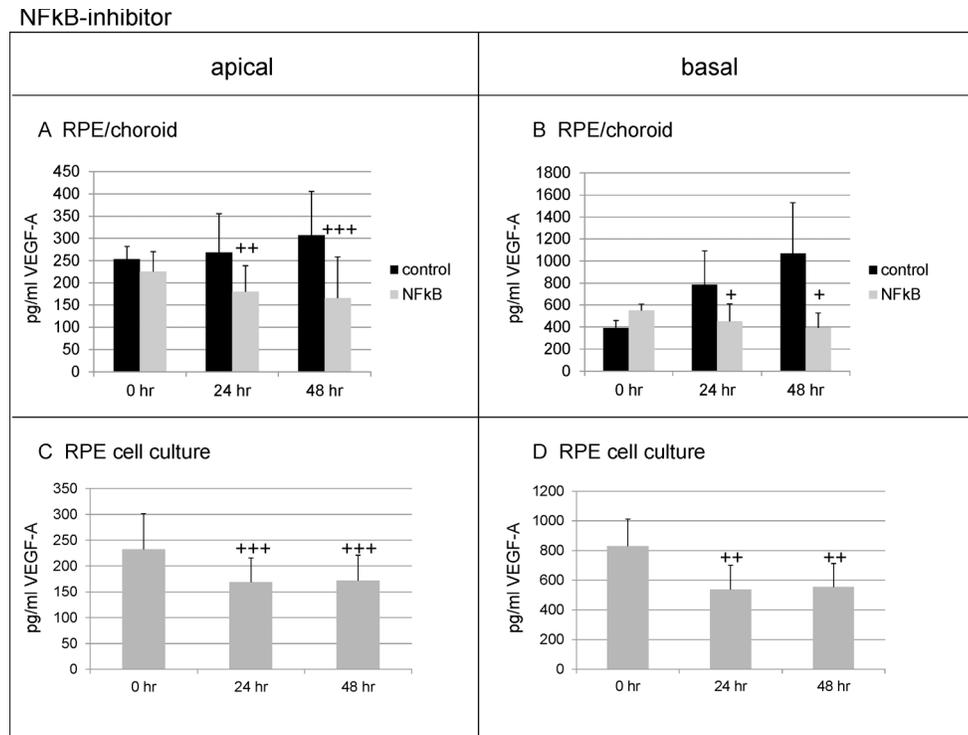


Figure 3. Influence of NF- κ B on apical and basal VEGF-A secretion. In the RPE/choroid, the inhibition of nuclear factor-kappa B (NF- κ B) significantly reduces vascular endothelial growth factor (VEGF)-A at 24 h and 48 h both on the apical (A) and basal sides (B). Similar results are obtained in primary RPE cell culture, with a significant reduction of VEGF-A secretion on the apical (C) and basal (D) sides, at 24 h and 48 h. Supernatants were collected for 24 h and were analyzed in enzyme-linked immunosorbent assay (ELISA). Significance was determined with the Student *t* test; + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$. $n = 4$ (A, B), $n = 6$ (C, D). NF- κ B = NF- κ B inhibitor. Bars depict the mean and the standard deviation.

(control: 786.0 ± 305.6 pg/ml, inhibitor: 451.7 ± 158.1 pg/ml, $p < 0.05$) and 48 h of stimulation (control: 1068.5 ± 460.9 pg/ml, inhibitor: 394.2 ± 132.6 pg/ml, $p < 0.05$; Figure 3B). In the cell culture, the inhibition of NF- κ B significantly reduced the secretion of VEGF-A on the apical side at 24 h compared to the 0 h control (0 h: 232.5 ± 69.0 pg/ml, 24 h: 169.0 ± 46.4 pg/ml, $p < 0.001$), and at 48 h (48 h: 171.8 ± 48.9 pg/ml, $p = 0.001$; Figure 3C). Basally, the inhibition of NF- κ B reduced the secretion of VEGF-A significantly at 24 h (0 h: 829.4 ± 184.4 pg/ml, 24 h: 538.5 ± 162.6 , $p < 0.01$) and at 48 h (554.8 ± 157.6 pg/ml, $p < 0.01$; Figure 3D).

SP-1: In the RPE/choroid organ cultures, a strong effect of the inhibition of SP-1 on VEGF-A secretion was found at the apical side at 24 h (control: 206.0 ± 54.8 pg/ml, inhibitor: 81.0 ± 35.6 pg/ml, $p < 0.01$) and at 48 h of incubation (control: 190.0 ± 64.8 pg/ml, inhibitor: 31.4 ± 36.0 pg/ml, $p < 0.01$; Figure 4A). Similarly, the inhibition of SP-1 reduced VEGF-A secretion on the basal side at 24 h (control: 372.1 ± 63.6 pg/ml, inhibitor: 176.8 ± 83.2 pg/ml, $p < 0.01$) and at 48 h (control: 327.7 ± 86.9 pg/ml, inhibitor: 40.0 ± 21.6 pg/ml, $p < 0.001$; Figure 4B). In the RPE cell culture, a similar effect was found with a highly significant decrease in VEGF-A secretion at the apical side at 24 h (0 h: 246.1 ± 75.0 pg/ml, 24 h: 90.0 ± 29.6 pg/ml, $p < 0.001$) and at 48 h (48 h: 17.9 ± 8.8 pg/ml, $p < 0.001$; Figure 4C). On the basal side, a decrease was also observed at 24 h

(0 h: 655.7 ± 144.2 pg/ml, 24 h: 357.8 ± 118.4 pg/ml, $p < 0.01$) and at 48 h (48 h: 157.4 ± 38.6 pg/ml, $p < 0.001$; Figure 4D).

p38: In the RPE/choroid organ culture, the inhibition of p38 displayed a less pronounced effect on the apical side, with no significant effect found at 24 h (control: 466.7 ± 337.5 pg/ml, inhibitor: 213.3 ± 144.5 pg/ml), and a significant decrease in VEGF-A secretion was observed at 48 h (control: 283.5 ± 100.0 pg/ml, inhibitor: 158.5 ± 90.1 pg/ml, $p < 0.05$; Figure 5A). At the basal side, an effect was observed, with VEGF-A secretion significantly reduced at 24 h (control: 654.6 ± 252.5 pg/ml, inhibitor: 283.4 ± 213.3 pg/ml, $p < 0.05$) and at 48 h (control: 632.4 ± 228.1 pg/ml, inhibitor: 238.1 ± 158.2 pg/ml, $p < 0.01$; Figure 5B). In the cell culture, no significance influence of the inhibition of p38 on VEGF-A secretion was found on the apical side (0 h: 221.4 ± 103.0 pg/ml; 24 h: 184.2 ± 56.6 pg/ml, 48 h: 191.3 ± 56.0 pg/ml; Figure 5C). In contrast, similar to the findings in the RPE/choroid, the inhibition of p38 significantly reduced the secretion of p38 on the basal side at 24 h (0 h: 680.1 ± 175.8 pg/ml, 24 h: 534.2 ± 164.8 pg/ml, $p < 0.001$) and at 48 h (48 h: 518.4 ± 176.9 pg/ml, $p < 0.001$; Figure 5D).

VEGFR-2: In the RPE/choroid organ culture, the inhibition of VEGFR-2 significantly reduced VEGF-A secretion at the apical side at 24 h (control 454.2 ± 225.5 pg/ml, inhibitor: 210.8 ± 106.7 pg/ml, $p < 0.05$) and at 48 h (control: 372.8 ± 105.0 pg/ml, inhibitor: 223.6 ± 96.1 pg/ml, $p < 0.01$; Figure 6A). In contrast, VEGFR-2 inhibition displayed no effect on the basal

SP-1-inhibitor (Mithramycin)

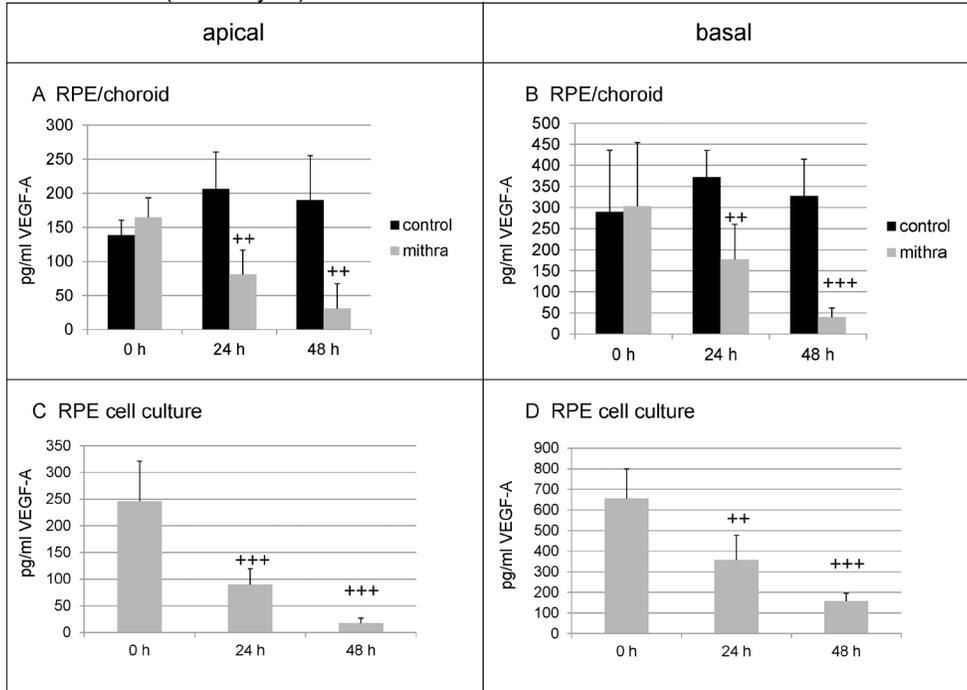


Figure 4. Influence of SP-1 on apical and basal VEGF-A secretion. In the RPE/choroid, the inhibition of SP-1 significantly reduces vascular endothelial growth factor (VEGF)-A at 24 h and 48 h on the apical (A) and basal sides (B). Similar results are obtained in primary RPE cell culture, with a significant decrease in VEGF-A secretion on the apical (C) and basal (D) sides, at 24 h and 48 h. Supernatants were collected for 24 h. Significance was determined with the Student *t* test; ++ $p < 0.01$ +++= $p < 0.001$. $n = 6$. mithra=mithramycin. Bars depict the mean and the standard deviation.

p38-inhibitor (SU203580)

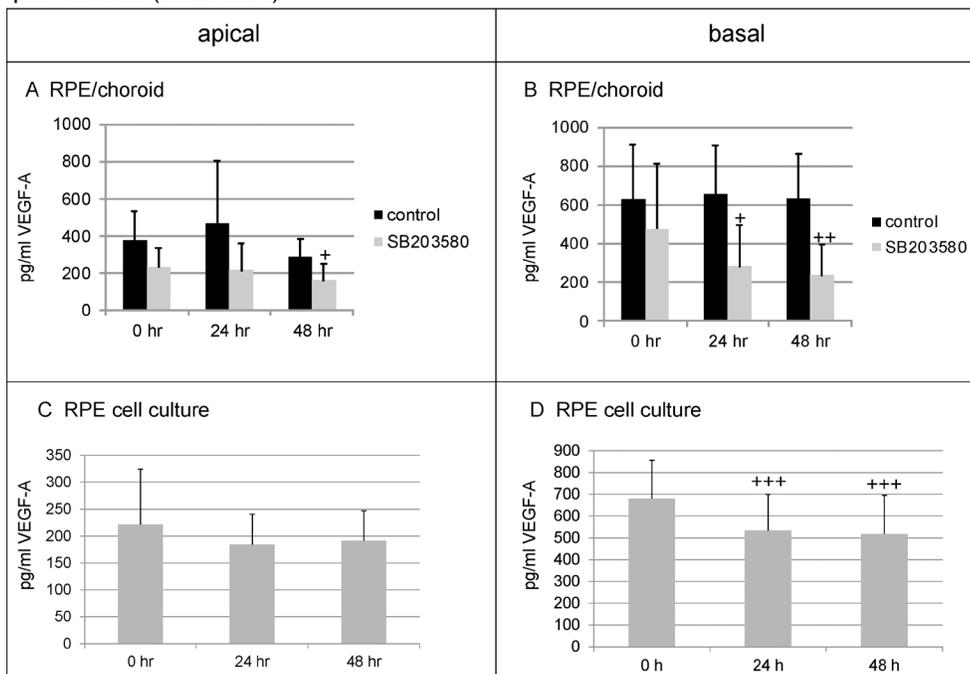


Figure 5. Influence of p38 on apical and basal VEGF-A secretion. A: In the RPE/choroid, inhibition of p38 significantly reduces the secretion of vascular endothelial growth factor (VEGF)-A on the apical side at 48 h. B: On the basal side, the inhibition of p38 reduces VEGF-A secretion at 24 h and at 48 h. In primary RPE cell culture, no influence of p38 on the secretion was found on the apical side (C), while p38 inhibition reduces VEGF-A secretion at the basal side significantly at 24 h and at 48 h (D). Supernatants were collected for 24 h and were analyzed in enzyme-linked immunosorbent assay (ELISA). Significance was

determined with the Student *t* test; + $p < 0.01$, ++ $p < 0.01$ +++= $p < 0.001$. $n = 4$ (A, B), $n = 6$ (C, D). Bars depict the mean and the standard deviation.

secretion of VEGF-A, neither at 24 h (control: 578.8±366.3 pg/ml, inhibitor: 425.6±246.4 pg/ml) nor at 48 h (control: 646.0±237.9 pg/ml, inhibitor: 537.0±274.9 pg/ml; Figure 6B). In the RPE cell culture, the apical inhibition of VEGFR-2 showed no significant effect after 24 h (0 h: 233.7±88.0 pg/ml, 24 h: 233.7±88.0 pg/ml) or 48 h (223.2±88.3; Figure 6C). On the basal side, the inhibition of VEGFR-2 did not show any reduction in VEGF-A secretion, neither at 24 h (0 h: 729.9±151.5 pg/ml, 24 h: 653.5±121.0 pg/ml) nor at 48 h (48 h: 652.5±147.2 pg/ml; Figure 6D). For VEGFR-2, we also conducted western blot experiments to further assess the possible involvement of VEGFR-2 in VEGF-A regulation. We found that the inhibition of VEGFR-2 significantly reduced

the VEGF-A protein levels in RPE cells, to 44.6±35.3% of the control (Figure 6E).

Regulation of apical and basal PIGF secretion: In the RPE/choroid organ culture, we generally found that PIGF secretion increases during the cultivation over time. As described, PIGF was not found in RPE cell culture, neither apically nor basally, and no increase in secretion was detected over time.

NF-κB: At the apical side, NF-κB inhibition significantly reduced PIGF secretion at 24 h (control: 114.5±40.2 pg/ml, inhibitor: 15.3±10.2 pg/ml, $p < 0.05$) and at 48 h (control: 143.6±44.6 pg/ml, inhibitor: 27.0±19.4 pg/ml, $p < 0.05$) compared to the untreated control (Figure 7A). At the basal side, NF-κB inhibition significantly inhibited PIGF secretion

VEGFR-2-inhibitor (SU1498)

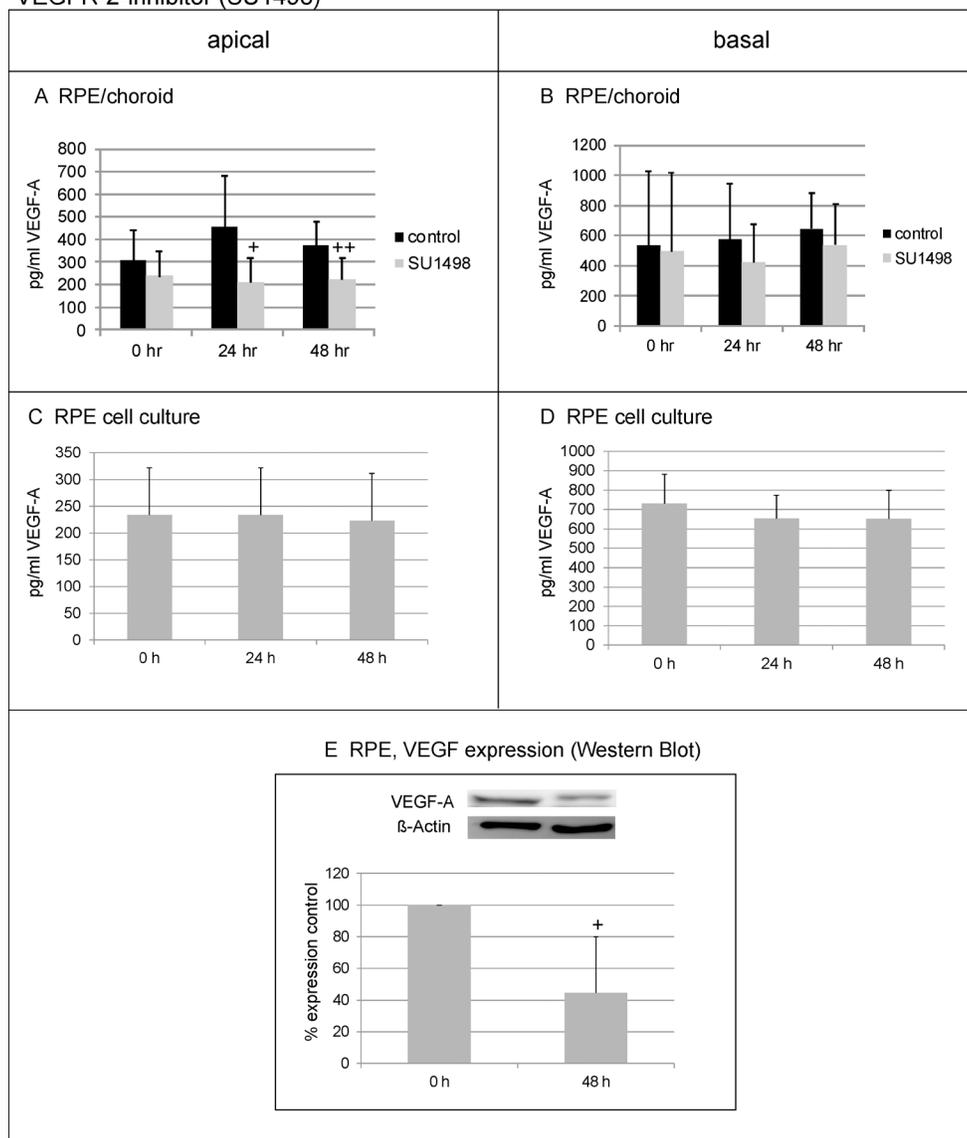


Figure 6. Influence of VEGFR-2 inhibition on apical and basal VEGF-A secretion. In the RPE/choroid, the inhibition of vascular endothelial growth factor (VEGF)-2 reduces VEGF-A secretion on the apical side (A) but not on the basal side (B). In contrast, in primary RPE cell culture, the inhibition of VEGFR-2 did not influence the secretion of VEGFR-2, at the apical side (C) or at the basal side (D). Supernatants were collected for 24 h and were analyzed in enzyme-linked immunosorbent assay (ELISA). In the whole cell lysis of the primary RPE cells, analyzed with western blot, a significant reduction was found in VEGF-A expression after 48 h of VEGFR-2 inhibition. Representative blots of VEGF-A and β-actin control are shown, as well as a densitometric evaluation of VEGF-A normalized for β-actin (E). Significance was determined with the Student *t* test; + $p < 0.01$, ++ $p < 0.01$. $n = 4$ (A), $n = 3$ (B), $n = 6$ (C-E). Bars depict the mean and the standard deviation.

PIGF secretion in RPE/choroid

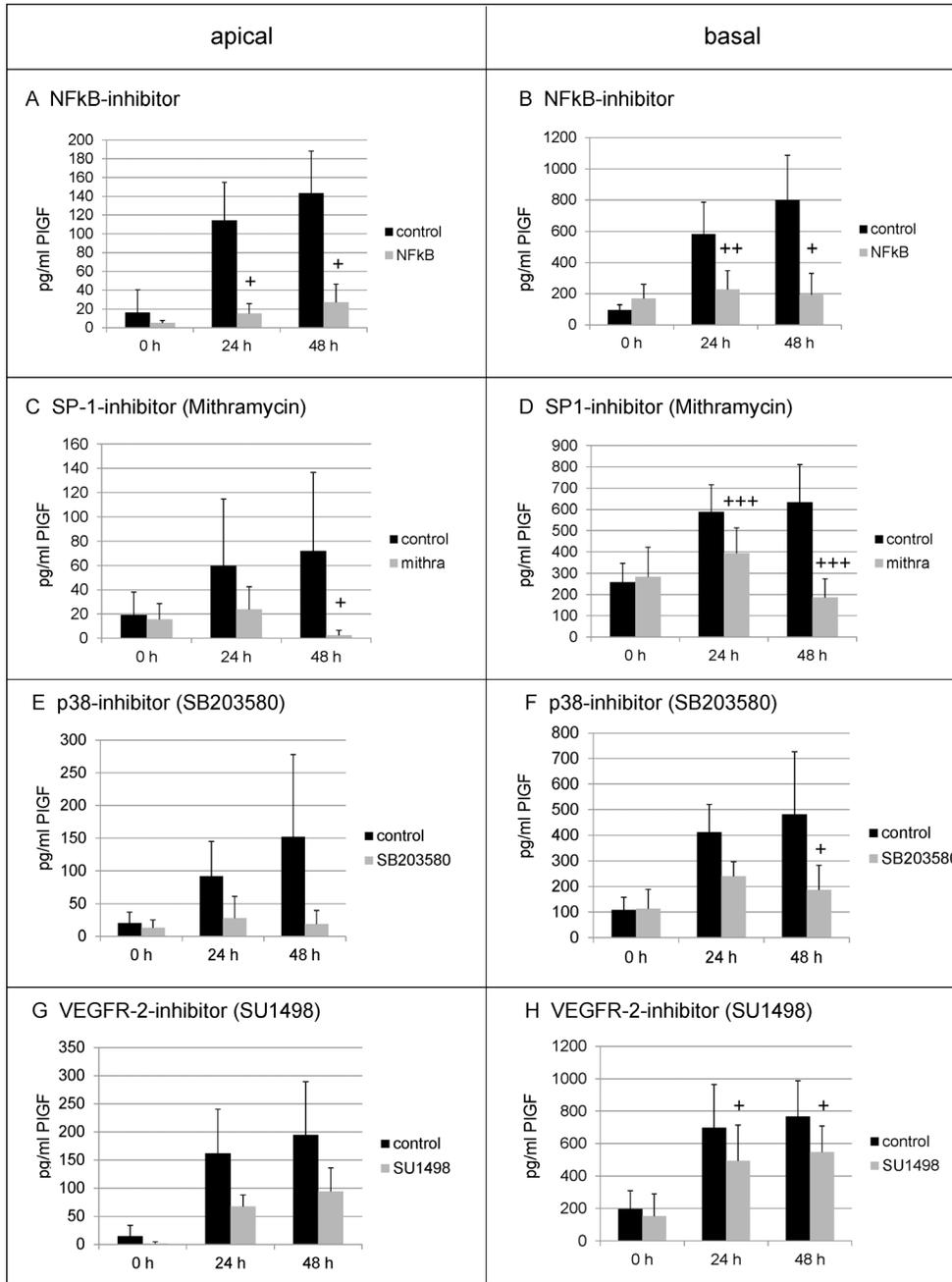


Figure 7. Regulation of PIGF secretion in the RPE/choroid. In general, placental growth factor (PIGF) secretion increased during cultivation. The inhibition of nuclear factor-kappa B (NF-κB) displayed a significant influence on PIGF secretion on the apical side (A) and on the basal side (B) at 24 h and at 48 h. The inhibition of SP-1 significantly reduced vascular endothelial growth factor (VEGF)-A secretion on the apical side after 48 h (C) and on the basal side at 24 h and at 48 h (D). The reduction of PIGF after p38 inhibition did not reach significance on the apical side (E) but did on the basal side after 48 h (F). The inhibition of VEGFR-2 did not significantly reduce PIGF at the apical side (G) but displayed significant effects on the basal side after 24 h and 48 h (H). Supernatants were collected for 24 h and were analyzed in enzyme-linked immunosorbent assay (ELISA). Significance was determined with the Student *t* test; + *p*<0.01, ++ *p*<0.01, +++=*p*<0.001. *n*=3 (A, B, E, F, G), *n*=6 (C, D), *n*=4 (H). Bars depict the mean and the standard deviation.

at 24 h (control: 580.7±205.4 pg/ml, inhibitor: 227.6±118.6 pg/ml, *p*<0.01) and at 48 h (control: 798.6±286.7 pg/ml, inhibitor: 193.0±137.0, *p*<0.05; Figure 7B).

SP-1: In the RPE/choroid organ culture, the inhibition of SP-1 reduced the secretion of PIGF on the apical side, displaying a trend at 24 h (control: 59.9±54.9 pg/ml, inhibitor: 23.7±18.7 pg/ml, *p*=0.057) and reaching significance at 48 h (control: 72.0±64.6 pg/ml, inhibitor 2.4 pg/ml ± 4.2 pg/ml, *p*<0.05;

Figure 7C). At the basal side, the inhibition of SP-1 reduced PIGF secretion significantly at 24 h (control: 588.3±128.1 pg/ml, inhibitor: 394.5±118.8 pg/ml, *p*<0.001) and at 48 h (control: 633.1±177.4 pg/ml, inhibitor 186.5±87.5 pg/ml, *p*<0.001; Figure 7D).

p38: In the RPE/choroid organ culture, the inhibition of p38 displayed no significant effect on apical PIGF secretion, neither at 24 h (control: 92.2±52.5 pg/ml, inhibitor: 27.8±33.1

pg/ml) nor at 48 h (control: 152.0±125.8 pg/ml, inhibitor: 18.6±21.1 pg/ml; Figure 7E). Basally, the inhibition of p38 showed a decrease in PIGF secretion, which displayed a trend at 24 h (control: 411.9±108.6 pg/ml, inhibitor: 240.9±56.2 pg/ml, $p=0.059$) and reached significance at 48 h (control: 481.8±244.5 pg/ml, inhibitor: 186.4±96.2 pg/ml, $p<0.05$; Figure 7F).

VEGFR-2: In the RPE/choroid organ culture, the inhibition of VEGFR-2 displayed no effect on the secretion of PIGF on the apical side, neither at 24 h (control: 161.8±78.5 pg/ml, inhibitor: 67.1±21.0) nor at 48 h (control: 194.6±94.9, inhibitor: 94.1±42.0 pg/ml; Figure 7G). In contrast, the inhibition of VEGFR-2 reduced the secretion of PIGF significantly on the basal side at 24 h (control: 695.8±268.4 pg/ml, inhibitor: 493.7±218.8 pg/ml, $p<0.05$) and at 48 h (control: 766.6±221.5 pg/ml, inhibitor: 546.1±160.8 pg/ml, $p<0.05$; Figure 7H).

DISCUSSION

In this study, we investigated the regulation of VEGF-A and PIGF secretion, using two model systems, an RPE/choroid organ culture and primary RPE cells. The RPE/choroid organ culture is an excellent *ex vivo* model for investigation secreted proteins, especially as it allows the interaction between the RPE, Bruch's membrane, and choroid, and, moreover, consists of highly differentiated tissue that does not undergo the dedifferentiation seen in cell culture passaging. We have previously shown that this system is well suited for investigating and distinguishing between apical and basal compartments [22]. In addition, we used a primary RPE cell culture cultivated on Transwell plates until stable TER was reached, which was in accordance with the TER previously published for polarized primary porcine cells [26].

In both systems, stronger secretion of VEGF-A on the basal than on the apical side was found, which is in concordance with previously published data [21,27] and is a strong indication of cell polarization [26]. VEGF-A has different functions on the apical and basal sides, with the apically secreted VEGF-A exerting primarily neuroprotective functions and the basally secreted maintaining the choriocapillaris [3,28,29]. Accordingly, substantial VEGF-A is found on both sides.

In contrast, PIGF was mostly found on the basal side of RPE/choroidal explants with only minute amounts secreted to the apical side during the first 24 h of cultivation. Interestingly, no PIGF was found secreted in the primary RPE cells, neither on the apical nor on the basal side. This was in contrast to previous studies [30], in which primary RPE cells were shown to secrete and respond to PIGF. However, the secreted PIGF quantities in that study were minute (5 pg/

ml), comparable what we see in our RPE/choroid cultures. Possibly, the RPE cells in our porcine system need the interaction with the choroid to produce these minute amounts of PIGF on the apical site. Generally, however, our data strongly suggest that PIGF is mostly secreted from choroidal cells, not from the RPE itself. We found a substantial increase in PIGF during the time of cultivation of the RPE/choroid tissue. PIGF has little function in quiescent, healthy tissue but is involved in ischemia-induced and pathological angiogenesis, possibly via crosstalk with VEGF-A [15]. The upregulation of PIGF in our system could imply an ischemic situation in culture; however, we did not find a similar increase in VEGF-A, which would have been a strong indicator of ischemic/hypoxic signaling. Possibly, PIGF is upregulated as a response to the trauma of explant preparation, as PIGF has been described as important in wound healing [14].

The role of physiologic PIGF has not been satisfactorily elucidated thus far. PIGF is generally of little importance for physiologic angiogenesis [14]; however, strong secretion in the choroid might indicate a relevant, yet not defined, purpose in the choroid. PIGF has been indicated to increase the efficacy of VEGF-A; thus, PIGF may be involved in the maintenance of the choroid, together with VEGF-A.

The main topic of this study was the apical and basal regulation of VEGF-A and PIGF secretion in the RPE/choroid. We have previously shown that several factors are involved in the constitutive secretion of VEGF-A, namely, the transcription factors NF- κ B and SP-1, as well as the MAPK p38, in contrast to Erk1/2, which participates in oxidative stress-induced VEGF-A upregulation [9,12,31]. Furthermore, we previously described autocrine VEGFR-2-mediated regulation that has been shown to be mediated via phosphatidylinositol 3 (PI3) kinase signaling [12].

Biochemical inhibitors were used, since siRNA or specific antibodies are not feasible and/or affordable in this system. These biochemical inhibitors have been well described in the literature [32-40]. However, the specificity of some biochemical inhibitors is under debate [40-43], which is a limitation of this study.

Within the limitation of the model system, we confirmed the involvement of the transcription factors NF- κ B and SP-1 in the regulation of constitutive VEGF-A secretion in this study. Both transcription factors do not differentiate between apical and basal regulation, displaying a strong effect on the secretion on either site. The effect of SP-1 inhibition on VEGF-A secretion is particularly strong, especially after long-term (48 h) stimulation, indicating a profound influence of SP-1 on constitutive VEGF-A regulation, similarly to what is seen in lung tumor cells [44]. Interestingly, cytokine-induced

VEGF-A secretion in RPE cells is not mediated by SP-1, indicating that SP-1 is part of the constitutive pathway [45].

We also confirmed the previously shown involvement of p38 [9,12]. In contrast to the transcription factors, the influence of p38 was stronger on the basal side, both in RPE cell culture, where no influence on the apical side was found, and on the RPE/choroid, with a significant influence on the basal side found after 48 h. These data indicate that p38, via the induction of basal VEGF-A, is important for the maintenance of the choroid. p38 is not only involved in constitutive VEGF-A secretion in the RPE; we have recently shown that hyperthermia induces VEGF-A via p38. Interestingly, p38 is not involved in the induction of VEGF-A after oxidative stress insult [9,31]. This could indicate that certain pathologic stimuli may preferentially induce either the basal or the apical secretion of VEGF, using different intracellular pathways. Considering the stimuli described, RPE cells are thought to protect photoreceptors from oxidative stress, as well as conduct autoprotective functions [46,47], while hyperthermia could indicate a problem with the choroidal circulation. Therefore, the side of VEGF release may be orchestrated by the need the stimulus indicates. Further research is required to further investigate these issues.

In contrast to p38, VEGFR-2 seems to be involved only in apical VEGF-A regulation. Surprisingly, this effect of apical regulation was seen only in RPE/choroid organ culture, while in RPE cell culture, no significant effect was seen. However, after 72 h, the inhibition of VEGFR-2 displayed an effect on the apical, but not the basal, VEGF-A secretion in primary RPE cells (data not shown). Moreover, western blot studies indicate that VEGFR-2 inhibition reduces VEGF-A protein; therefore, an effect was seen in the RPE cell culture. Possibly, the full effect can be seen only in optimal polarized and differentiated RPE cells, which is given in the RPE/choroid organ culture. These data indicate the importance of tissue interaction and strongly indicate a delicate interdependency between the different cell types. Apical regulation via VEGFR-2 indicates a truly autocrine effect of RPE-derived VEGF-A, and may be connected to its protective function on the RPE against oxidative stress [47].

In addition, we investigated the regulation of PIGF secretion. To our knowledge, this is the first time the regulation of PIGF secretion has been investigated in the RPE. Generally, little is known about PIGF regulation in other cell types [48]. Our data indicate that within the limitations of this system NF- κ B and SP-1 are important for PIGF regulation, similarly to their effect on VEGF-A. An influence of NF- κ B on the expression of PIGF has been previously shown in embryonic kidney 293 cells [49]. The MAPK p38 seems to be of minor

importance; however, some effect of p38 inhibition is seen on the basal side. The MAPK p38 has been shown before to increase PIGF mRNA half-life in human coronary artery smooth muscle cells [50]. More importantly, PIGF is regulated on the basal side via the VEGFR-2 (in contrast to VEGF-A, in which VEGFR-2 regulates only the apically secreted VEGF-A). PIGF does not bind VEGFR-2, strongly indicating that VEGF-A may upregulate PIGF expression via VEGFR-2 activation. This further supports the idea of synergistic effects of PIGF and VEGF-A [14,16] and may result in an elevated proangiogenic effect via concomitant VEGF-A and PIGF upregulation. Thus, the inhibition PIGF may be a useful approach in the treatment of pathological angiogenesis, since PIGF is most likely not involved in neuroprotective effects. Expression of PIGF on the apical side is minor.

In conclusion, we have shown that VEGF-A and PIGF secretion is apically and basally differentially regulated, with VEGFR-2 involved in apical VEGF-A and basal PIGF expression. Moreover, both factors are regulated by NF- κ B and SP-1, and regulation of PIGF by VEGF-A may be indicated.

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