



Hypoxic expression of NLRP3 and VEGF in cultured retinal pigment epithelial cells: contribution of P2Y₂ receptor signaling

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Abstract

Retinal hypoxia is a major condition of the chronic inflammatory disease age-related macular degeneration. Extracellular ATP is a danger signal which is known to activate the NLRP3 inflammasome in various cell systems. We investigated in cultured human retinal pigment epithelial (RPE) cells whether hypoxia alters the expression of inflammasome-associated genes and whether purinergic receptor signaling contributes to the hypoxic expression of key inflammatory (NLRP3) and angiogenic factor (VEGF) genes. Hypoxia and chemical hypoxia were induced by a 0.2% O₂ atmosphere and addition of CoCl₂, respectively. Gene expression was determined with real-time RT-PCR. Cytosolic NLRP3 and (pro-) IL-1β levels, and the extracellular VEGF level, were evaluated with Western blot and ELISA analyses. Cell culture in 0.2% O₂ induced expression of NLRP3 and pro-IL-1β genes but not of the pro-IL-18 gene. Hypoxia also increased the cytosolic levels of NLRP3 and (pro-) IL-1β proteins. Inflammasome activation by lysosomal destabilization decreased the cell viability under hypoxic, but not control conditions. In addition to activation of IL-1 receptors, purinergic receptor signaling mediated by a pannexin-dependent release of ATP and a release of adenosine, and activation of P2Y₂ and adenosine A₁ receptors, was required for the full hypoxic expression of the NLRP3 gene. P2Y₂ (but not A₁) receptor signaling also contributed to the hypoxic expression and secretion of VEGF. The data indicate that hypoxia induces priming and activation of the NLRP3 inflammasome in cultured RPE cells. The hypoxic NLRP3 and VEGF gene expression and the secretion of VEGF are in part mediated by P2Y₂ receptor signaling.

Keywords Retinal pigment epithelium · P2Y₂ · NLRP3 · VEGF · Inflammasome · Hypoxia

Abbreviations

AIM	absent in melanoma	IL	interleukin
AMD	age-related macular degeneration	NFAT	nuclear factor of activated T cell
AOPCP	adenosine-5'-O-(α,β -methylene)-diphosphate	NLRC	NLR family CARD domain-containing protein
AP	activator protein	NLRP	nucleotide-binding oligomerization domain receptors-like receptor protein
ASC	apoptosis-associated speck-like protein containing a caspase-recruitment domain	RPE	retinal pigment epithelium
ATP	adenosine 5'-triphosphate	siRNA	small interfering RNA
CAPE	caffeic acid phenethyl ester	STAT	signal transducer and activator of transcription
CREB	cAMP response element-binding protein	VEGF	vascular endothelial growth factor
DPCPX	8-cyclopentyl-1,3-dipropylxanthine		
HIF	hypoxia-inducible transcription factor		

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Introduction

Age-related macular degeneration (AMD) is the main cause of visual impairment and blindness in people aged over 65 years in developed countries [1, 2]. The majority of patients suffer from the dry form of AMD which is, in the late stage, characterized by geographic atrophy, i.e., a degeneration of the retinal pigment epithelium (RPE) associated with a degeneration of photoreceptors [3]. The remaining patients suffer from the

neovascular form characterized by choroidal neovascularization and subretinal edema. Normal aging and AMD are associated with a decrease in the choroidal blood flow [4, 5] and a dysfunction of the RPE. The dysfunction of RPE cells results in an impaired degradation of peroxidized photoreceptor lipoproteins, accumulation of lipofuscin in the RPE [6, 7], and deposition of drusen beneath the RPE [8, 9]. Accumulated lipoproteins constitute a hydrophobic barrier that adversely affects the transport of oxygen and nutrients from the choriocapillaris to the photoreceptors [10]. Inadequate choroidal perfusion and accumulation of lipoproteins lead to hypoglycemia and hypoxia of the outer retina resulting in overproduction of angiogenic factors by RPE cells that stimulate the growth of choroidal vessels [10]. Vascular endothelial growth factor (VEGF) is the most relevant angiogenic factor induced by retinal hypoxia [11]. In addition to glial cells and macrophages, RPE cells are a source of VEGF [12].

AMD was shown to be associated with chronic local and systemic inflammation [13–16]. Inflammatory processes are initiated by activation of inflammasomes which are cytosolic protein-signaling complexes that recognize pathogen- and damage-associated molecular patterns [17]. The activated inflammasome mediates the proteolytic activation of caspase-1 that catalyzes the maturation of the inflammatory cytokines interleukin (IL)-1 β and IL-18 [17]. The RPE of patients with geographic atrophy or neovascular AMD contains the NLRP3 (nucleotide-binding oligomerization domain receptors-like receptor protein 3) inflammasome [18]. Activation of the NLRP3 inflammasome is assumed to be involved in mediating the degeneration of the RPE and the development of choroidal neovascularization [19–21].

Activation of the inflammasome is a two-step process triggered by a priming signal followed by the action of an activation signal [17]. Priming of the inflammasome involves the gene expression and production of inflammasome receptor and pro-IL-1 β proteins [17]. The NLRP3 inflammasome in RPE cells was shown to be activated by different factors that are assumed to be implicated in the pathogenesis of AMD like complement factors, lipofuscin-mediated photooxidative damage, peroxidized lipids, lysosomal destabilization, and overexpressed VEGF [18–25]. The NLRP3 inflammasome in RPE cells is also transiently primed and activated by elevation of the extracellular osmolarity; the hyperosmotic activation of the NLRP3 inflammasome is associated with a decrease of the RPE cell viability [26]. Among other factors like advanced age, race, genetic factors, sunlight exposure, and cigarette smoking, systemic hypertension is a risk factor of AMD [27, 28]. The main condition that causes acute hypertension is increased extracellular osmolarity following intake of dietary salt (NaCl) [29].

Retinal hypoxia is a major pathogenic condition of AMD; however, it is not known whether hypoxia also induces priming and activation of the NLRP3 inflammasome in RPE cells.

Therefore, the first aim of the present study was to determine whether the gene expression of inflammasome-associated proteins in cultured human RPE cells is altered under hypoxic conditions. We found that the expression levels of NLRP3 and pro-IL-1 β genes are elevated under low-O₂ conditions, suggesting that hypoxia induces priming of the NLRP3 inflammasome in cultured RPE cells. We also found a hypoxic increase of the cytosolic level of (pro-) IL-1 β , suggesting that hypoxia may also induce activation of the inflammasome in RPE cells. The hyperosmotic priming of the NLRP3 inflammasome in RPE cells was shown to be in part dependent on autocrine/paracrine purinergic (P2) receptor signaling induced by a pannexin-mediated release of adenosine 5'-triphosphate (ATP) [26]. Extracellular ATP is a danger signal that is released in large amounts by stressed cells, e.g., in response to inflammation, oxidative stress, nutrient starvation, ischemia-hypoxia, and cell injury [30]. However, it is not known whether the hypoxic and hyperosmotic activation of the inflammasome in RPE cells is mediated by similar or different signal transduction mechanisms including extracellular ATP signaling. The second aim of the present study was to determine whether autocrine/paracrine purinergic receptor signaling contributes to the hypoxia-induced expression of key inflammatory (NLRP3) and angiogenic factor (VEGF) genes in cultured RPE cells.

Materials and methods

Human material

The use of human material was approved by the Ethics Committee of the University of Leipzig (approval #745, 07/25/2011) and was performed according to the Declaration of Helsinki. Postmortem eyes from human cornea donors without reported eye disease were obtained within 48 h of death; the written informed consent for the use of retinal tissue in basic science was obtained from the relatives of each donor.

Cell lines from a total of 26 donors (14 females, 12 males) were used. The age of the donors ranged from 38 to 90 years (means \pm SD, 60.8 \pm 14.8 years for females and 59.6 \pm 15.1 years for males). The donors suffered from following end-stage diseases: multiple organ failure ($n = 9$), subarachnoid hemorrhage ($n = 4$), cerebral hemorrhage ($n = 4$), cardiac infarction ($n = 3$), pulmonary embolism ($n = 2$), lung failure and hepatocerebral syndrome due to chronic liver cirrhosis ($n = 1$), traumatic brain injury ($n = 1$), cerebral edema ($n = 1$), and heparin-induced thrombocytopenia, renal failure, and bronchopneumonia ($n = 1$). Data obtained with cells of younger and aged donors, and with cells of females and males, were not significantly different (not shown).

Materials

Cell culture components and solutions were obtained from Gibco BRL (Paisley, UK). The recombinant human IL-1 receptor antagonist was purchased from R&D Systems (Abingdon, UK). Stattic was from Enzo Life Science (Lörrach, Germany). 666-15, A-438079, AR-C 118925XX, ARL-67156, caffeic acid phenethyl ester (CAPE), MRS2179, the pannexin-blocking peptide ¹⁰panx, the scrambled control peptide ¹⁰panxScr, and SR11302 were obtained from Tocris (Ellisville, MO). 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), 8-(3-chlorostyryl) caffeine (CSC), the hypoxia-inducible transcription factor (HIF)-1 inhibitor 3-[2-(4-adamantan-1-yl-phenoxy)-acetyl-amino]-4-hydroxybenzoic acid methyl ester, and SU1498 were obtained from Calbiochem (Bad Soden, Germany). Ac-YVAD-CMK was obtained from Enzo (Lausen, Switzerland). L-Leucyl-L-leucine methyl ester was from Bachem (Weil am Rhein, Germany). Adenosine-5'-O-(α,β -methylene)-diphosphate (AOPCP), apyrase, and all other agents used were from Sigma-Aldrich (Taufkirchen, Germany), unless stated otherwise. Human-specific small interfering RNA (siRNA) against nuclear factor of activated T cell 5 (NFAT5) and non-targeted control siRNA were obtained from Santa Cruz (Dallas, TX). The following antibodies were used: a mouse anti-human NLRP3 (1:1000; #ALX-804-819, Enzo), a rabbit anti-human β -actin (1:1000; #8457, Cell Signaling, Frankfurt/M., Germany), an anti-mouse IgG conjugated with alkaline phosphatase (1:2000; #7056, Cell Signaling), and an anti-rabbit IgG conjugated with alkaline phosphatase (1:2000; #7054, Cell Signaling).

Cell culture

Preparation and culture of RPE cells were previously described [31]. Cell lines of passages 2–5 were used; each line was used in 3–10 different experiments. When a confluency of approximately 90% was achieved, the cells were cultured in serum-free medium for 16 h. During this time period, the cultures reached 100% confluency. Thereafter, test substances were added to the serum-free medium. Hypoxia was induced by two methods: cell culture in a 0.2%-O₂ atmosphere and addition of CoCl₂ (150 μ M) to the culture medium. The hyperosmotic medium was made up by addition of 100 mM NaCl to the culture medium. The cells were preincubated with pharmacological inhibitors for 30 min.

RT-PCR analysis

The RNeasy Mini Kit (Qiagen, Hilden, Germany) was used to extract the total RNA. The quality of the RNA was analyzed with agarose gel electrophoresis. The A₂₆₀/A₂₈₀ ratio of the optical density was measured using the GeneQuantpro device

(Pharmacia, Uppsala, Sweden), and was between 2.0 and 2.2 for all RNA samples, indicating sufficient quality. After treatment with DNase I (Roche, Mannheim, Germany), cDNA was synthesized from 1 μ g of total RNA using the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas, St. Leon-Roth, Germany). PCR was performed with the Taq PCR Master Mix kit (Qiagen) and the primer pairs described in Table 1. One microliter of the first-strand mixture and 0.25 μ M of each gene-specific sense and anti-sense primers were used for amplification in a final volume of 20 μ l. Amplification was done for 40 cycles with the PTC-200 Thermal Cycler (MJ Research, Watertown, MA). Each cycle consisted of 30 s at 94 °C, 60 s at 58 °C, and 1 min at 72 °C.

Real-time RT-PCR analysis

Semi-quantitative real-time RT-PCR analysis was performed with the Single-Color Real-Time PCR Detection System (BioRad, Munich, Germany) and the primer pairs described in Table 1. The PCR solution contained 1 μ l cDNA, specific primer set (0.2 μ M each), and 10 μ l of a 2x mastermix (QuantiTect SYBR Green PCR Kit; Qiagen) in a final volume of 20 μ l. The following conditions were used: initial denaturation and enzyme activation (1 cycle at 95 °C for 15 min); denaturation, amplification, and quantification (45 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min); and melting curve (55 °C with the temperature gradually increased (0.5 °C) up to 95 °C). The amplified samples were analyzed with standard agarose gel electrophoresis. The mRNA expression levels were normalized to the level of β -actin mRNA. Changes in mRNA expression were calculated according to the 2^{- $\Delta\Delta$ CT} method [32].

siRNA transfection

Cells were seeded at 7 \times 10⁴ cells per well in 12-well culture plates and were allowed to grow up to a confluency of 60–80%. Thereafter, the cells were transfected with NFAT5 siRNA and non-targeted siRNA (5 nM each), respectively, using HiPerfect reagent (Qiagen) in F-10 medium containing 10% fetal bovine serum (Invitrogen, Paisley, UK) according to the manufacturer's instructions. After 48 h (a time point at which the cultures were confluent), the medium was removed and fresh medium without serum was added for 2 h. Thereafter, CoCl₂ (150 μ M) was added for further 6 h. Total RNA was extracted, and the NLRP3 mRNA level was determined with real-time RT-PCR analysis.

Western blot analysis

Cells were seeded at 5 \times 10⁵ cells per well in 6-well plates in 2 ml complete medium and were allowed to grow up in serum-containing medium. After cell culture in serum-free

Table 1 Primer pairs used in PCR experiments. s, sense. as, anti-sense

Gene and accession number	Primer sequences (5' → 3')	Product (bp)
ACTIN	s ATGGCCACGGCTGCTTCCAGC	237
NM_001101	as CATGGTGGTGCCGCCAGACAG	
VEGFA _{188, 164, 120}	s CCTGGTGGACATCTTCCAGGAGTA	479, 407, 275
NM_003376.5	as CTCACCGCCTCGGCTTGTCA	
NM_001287044.1		
NM_001025370.2		
IL-1 β	s GGGCCTCAAGGAAAAGAATC	205
NM_000576	a TTCTGCTTGAGAGGTGCTGA	
IL-18	s AATGCACCCCGACCATATTT	203
NM_001243211.1	as CCTGGGACACTTCTCTGAAAGA	
ASC	s CTGACGGATGAGCAGTACCAG	222
NM_145182.2	as GGATGATTTGGTGGGATTGCC	
NLRP2 NM_001174083.1	s ACAACTTTGAGACACCCCAAGT	161
	as TTCACCCCTGTATTCCCAATGG	
NALP3	s AGACAGCATTGAAGAGGAGTGG	169
NM_183395.2	as TTTGTTGAGGCTCACACTCTCA	
AIM2	s GTGCTGCACCAAAAAGTCTCTC	169
NM_004833.1	as TGAAACATCTCCTGCTTGCCT	
NRF2	s AGCTAGATAGTGCCCTGGAA	179
NM_001145412	as GGTTTCCGATGACCAGGACT	
CASP1	s GCACACGCTTGTCTCATTATC	244
NM_033293.3	as CCTTCCCGAATACCATGAGACAT	
CASP4	s GACAGCACAATGGGCTCTATCT	152
NM_033306.2	as AGTCGTTCTATGGTGGGCATTT	
NFAT5	s TCACCATCATCTTCCCACCT	174
NM_006599.3	as CTGCAATAGTGCATCGCTGT	

medium for 16 h, the cells were cultured in the presence of CoCl_2 (150 μM) for 6, 16, and 24 h, respectively. Then, the medium was removed, the cells were washed twice with prechilled phosphate-buffered saline (pH 7.4; Invitrogen), scraped into 150 μl of lysis buffer (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1% protease inhibitor cocktail), and agitated at 4 °C for 30 min. Cell lysates were centrifuged at 13,000 $\times g$ for 10 min, and supernatants were analyzed with immunoblotting. Equal amounts of protein (35 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis. Immunoblots were probed with primary and secondary antibodies; immunoreactive bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

ELISA

Cells were cultured at 3×10^3 cells per well in 12-well plates. At a confluency of about 90%, the cells were cultured in serum-free medium for 16 h; within this time period, the cultures reached 100% confluency. Subsequently, the medium was changed, and the cells were cultured in 0.2% O_2 or treated with CoCl_2 (150 μM). Culture supernatants (1 ml) and cell lysates (150 μl) were collected after 6 and 24 h. The cytosolic

level of IL-1 β (which may include both pro-IL-1 β and mature IL-1 β) and the level VEGF-A₁₆₅ in the culture supernatants (100 μl) were determined with ELISA (#HSLB00C; DVE00; R&D Systems).

Cell viability

A trypan blue exclusion assay was used to investigate the cell viability. The cells were seeded at 5×10^4 cells per well in 6-well plates. After reaching a confluency of about 90%, the cells were cultured in serum-free medium for 16 h; during this period, the cultures reached 100% confluency. Thereafter, the cells were cultured for 24 h in serum-free medium in a 0.2% O_2 atmosphere or in the presence of CoCl_2 (150 μM). After trypsinization, the cells were stained with trypan blue (0.4%). The numbers of viable (non-stained) and dead (stained) cells were counted using a hemocytometer.

Statistical analysis

At least three independent experiments with cell lines from different donors were performed for each test. Data are shown as means \pm SEM. Statistical analysis was made with Prism (Graphpad Software, San Diego, CA). Significant differences

were evaluated with one-way ANOVA followed by Bonferroni's multiple comparison test and with Mann-Whitney U test, respectively, and were accepted at $p < 0.05$.

Results

Expression of inflammasome-associated genes

We investigated with RT-PCR analysis whether cultured human RPE cells contain gene transcripts of inflammasome-associated proteins. We found in agreement with a previous study [26] that the cells contained transcripts of the inflammasome sensor proteins NLRP2, NLRP3, and absent in melanoma 2 (AIM2), of the adaptor protein apoptosis-associated speck-like protein containing a caspase-

recruitment domain (ASC), of pro-IL-1 β , pro-IL-18, and the procaspases 1 and 4 (Fig. 1a). Transcripts of other inflammasome sensor proteins (NLRP1, NLRP6, NLRP7, NLRP12, NLRC4) were not detected in the RNAs extracted from different cell lines cultured under control and hypoxic conditions (not shown).

Hypoxic regulation of inflammasome-associated gene expression

Real-time RT-PCR analysis was carried out to investigate whether hypoxia induces alterations in the expression of inflammasome-associated genes in cultured RPE cells. Hypoxia was induced by cell culture in a 0.2% O_2 atmosphere or by addition of the hypoxia mimetic $CoCl_2$ [33]. Cell culture in 0.2% O_2 induced significant ($p < 0.05$) increases in the

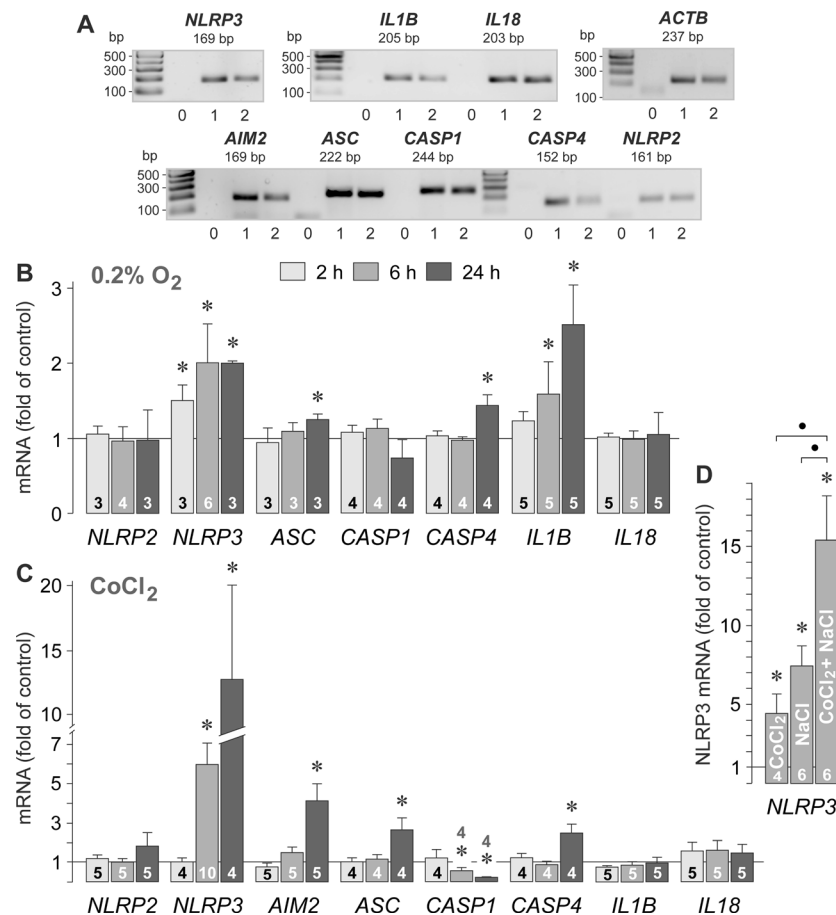


Fig. 1 Hypoxic regulation of the gene expression of inflammasome-associated proteins in cultured human RPE cells. mRNA levels were determined with real-time RT-PCR analysis in cells cultured 2, 6, and 24 h (as indicated by the panels of the bars) and are expressed as folds of unstimulated control. **a** Presence of gene transcripts of inflammasome-associated proteins in RPE cells. The correct lengths of PCR products were confirmed with agarose gel electrophoresis using products obtained from cell lines of two different donors (1, 2). Negative controls (0) were done with double-distilled water instead of cDNA as template. *ACTB* mRNA was used as loading control. **b, c** Effects of cell culture in 0.2%

O_2 (**b**) and of addition of $CoCl_2$ (150 μ M; **c**), respectively, on the gene expression of inflammasome-associated proteins. **d** Effects of $CoCl_2$ (150 μ M) on the expression of *NLRP3* in iso- and hyperosmotic media. Hyperosmolarity was induced by addition of 100 mM NaCl to the culture medium. The numbers of independent experiments using cell lines from different donors are indicated in or above the bars. Significant differences were evaluated with one-way ANOVA followed by Bonferroni's multiple comparison test. Significant difference vs. unstimulated control: * $p < 0.05$. Significant difference between two groups (as indicated by the brackets): • $p < 0.05$

expression levels of NLRP3 and pro-IL-1 β genes (Fig. 1b). Addition of CoCl₂ to the culture medium resulted in significant ($p < 0.05$) increases in the expression of NLRP3, AIM2, ASC, and caspase-4 genes, while the level of caspase-1 gene transcripts was time-dependently decreased; the expression of the pro-IL-1 β gene remained unaltered (Fig. 1c). The expression levels of NLRP2 and IL-18 genes did not change under both conditions (Fig. 1b, c). Extracellular hyperosmolarity was shown to induce priming of the NLRP3 inflammasome in RPE cells [26]. We found that the effects of high NaCl-induced extracellular hyperosmolarity and CoCl₂-induced hypoxia on the expression of the NLRP3 gene were additive (Fig. 1d). The time-dependencies of the NLRP3 gene expression under both conditions were also different; the CoCl₂-induced expression of the NLRP3 gene was highest after 24 h of stimulation (Fig. 1c), whereas the hyperosmotic expression of the NLRP3 gene was shown to peak after 6 h of stimulation [26]. The data may support the assumption that different signal transduction mechanisms mediate the transcriptional induction of the NLRP3 gene under hypoxic and hyperosmotic conditions.

Hypoxia-induced increases of NLRP3 and IL-1 β protein levels

Western blot analysis was performed to investigate whether hypoxia also increases the level of NLRP3 protein in cultured RPE cells. Under unstimulated control conditions, the level of NLRP3 protein in the cells was low (Fig. 2a). The cellular level of NLRP3 protein increased time-dependently in the presence, but not in the absence, of the hypoxia mimetic CoCl₂ (Fig. 2a, b). Increases of the cellular NLRP3 protein level were observed after 16 and 24 h of stimulation (Fig. 2a, b); this time dependency fits well with that of the CoCl₂-induced expression of the NLRP3 gene (Fig. 1c).

In order to investigate whether hypoxia also induces activation of the NLRP3 inflammasome in cultured RPE cells, we determined with ELISA the cytosolic level of (pro-) IL-1 β protein. Cell culture in 0.2% O₂ had a biphasic effect on the (pro-) IL-1 β protein level in cultured RPE cells; it induced a small but significant ($p < 0.05$) decrease after 6 h and a high increase after 24 h (Fig. 2c). In agreement with a previous study [26], the concentration of IL-1 β in the media obtained from cells cultured 6 or 24 h under control and CoCl₂-stimulated conditions was below the detection threshold of the ELISA used (not shown).

RPE cell viability

It was suggested that activation of the NLRP3 inflammasome contributes to the degeneration of the RPE in patients with AMD [19, 21]. We investigated whether activation of the NLRP3 inflammasome under hypoxic conditions is

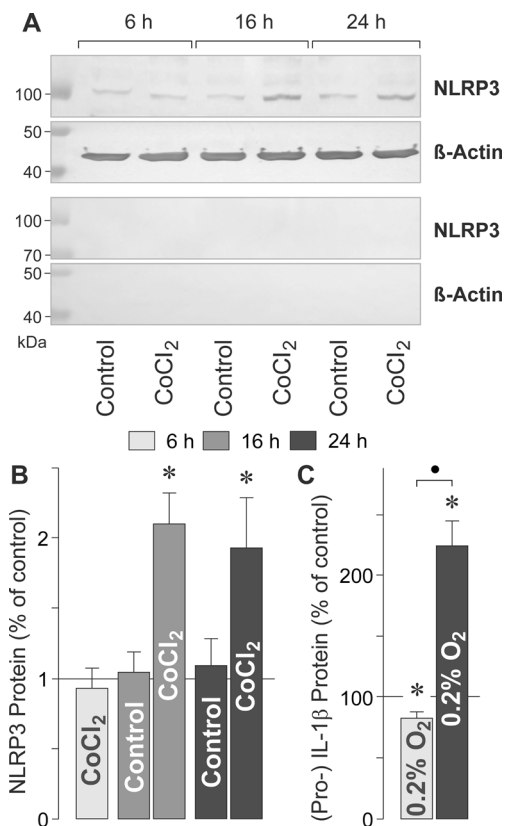


Fig. 2 Hypoxia-induced increases of the cytosolic levels of NLRP3 (a, b) and (pro-) IL-1 β proteins (c) in cultured RPE cells. **a** The cells were cultured 6, 16, and 24 h in the absence (control) and presence of the hypoxia mimetic CoCl₂ (150 μ M). The level of NLRP3 protein (110 kDa) in cell lysates was determined with Western blot analysis (above). Equal amounts of total protein (35 μ g) were used for separation. β -Actin (45 kDa) was used as loading control. Negative controls were done by omission of the first antibodies (below). **b** Cytosolic level of the NLRP3 protein, as determined by densitometric analysis of Western blot data. The data were normalized to the level of β -actin protein and are expressed as percent of unstimulated control measured after 6 h of stimulation (100%). **c** The cytosolic level of (pro-) IL-1 β protein of cells which were cultured 6 and 24 h in 0.2% O₂. The protein level was determined with ELISA and is expressed as percent of unstimulated control (100%; 3.20 ± 0.67 pg/ml). Means \pm SEM of 3 (b) and 4 (c) independent experiments using cell lines from different donors. Significant difference vs. unstimulated 6-h control (evaluated with one-way ANOVA followed by Bonferroni's multiple comparison test): * $p < 0.05$. Significant difference between 6-h and 24-h groups (evaluated with Mann-Whitney U test): • $p < 0.05$

associated with a decrease in the viability of cultured RPE cells. In addition to hypoxia, inflammasome activation was induced by addition of L-leucyl-L-leucine methyl ester which produces permeabilization of lysosome membranes [34] and activation of the NLRP3 inflammasome in RPE cells [18, 35]. We found that cell culture for 24 h in a 0.2%-O₂ atmosphere induced a significant ($p < 0.05$) decrease in the viability of RPE cells (Fig. 3). The decrease in cell viability after 24 h of hypoxia corresponds with the time period of inflammasome activation as indicated by the increase of the cytosolic (pro-) IL-1 β level (Fig. 2c). In agreement with a previous study [26],

the presence of L-leucyl-L-leucine methyl ester in the culture medium did not alter the cell viability under control conditions. However, under hypoxic conditions, additional activation of the NLRP3 inflammasome by L-leucyl-L-leucine methyl ester was associated with a significant ($p < 0.05$) decrease of the RPE cell viability (Fig. 3). The decrease of the cell viability induced by cell culture in 0.2%-O₂ was significantly ($p < 0.05$) greater in the presence than in the absence of L-leucyl-L-leucine methyl ester (Fig. 3). The data may suggest that inflammasome activation might be one factor that mediates the hypoxic decrease of the cell viability.

Transcription factor activities involved in mediating the hypoxia-induced expression of the NLRP3 gene

Pharmacological blockers were tested in CoCl₂-stimulated cell cultures to investigate which transcription factors are involved in mediating the hypoxic expression of the NLRP3 gene. The CoCl₂-induced expression of the NLRP3 gene was significantly ($p < 0.05$) decreased by a HIF-1 inhibitor [36] and the inhibitor of the cAMP response element-binding protein (CREB), 666-15 (Fig. 4a). The CoCl₂-induced expression of the NLRP3 gene was not altered in the presence of inhibitors of signal transducer and activator of transcription 3 (STAT3), Stattic [37], nuclear factor (NF)-κB,

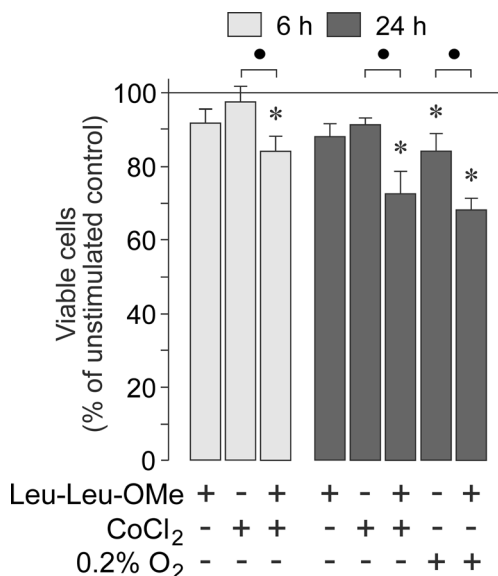


Fig. 3 Effect of lysosomal destabilization with L-leucyl-L-leucine methyl ester (Leu-Leu-OMe; 1 mM) on the viability of cultured RPE cells under hypoxic conditions. The cells were cultured 6 and 24 h in 0.2% O₂ and in the presence of CoCl₂ (150 μM), respectively. Means ± SEM of 4 independent experiments using cell lines from different donors. Significant differences were evaluated with one-way ANOVA followed by Bonferroni's multiple comparison test. Significant difference vs. unstimulated control: * $p < 0.05$. Significant difference between two groups (as indicated by the brackets; evaluated with Mann-Whitney U test): * $p < 0.05$

CAPE [38], and activator protein-1 (AP-1), SR11302 (Fig. 4a).

In order to investigate whether the hypoxic expression of the NLRP3 gene in cultured RPE cells might be also dependent on the transcriptional activity of NFAT5, we knocked down NFAT5 with siRNA. Transfection of the cells with NFAT5 siRNA resulted in a decrease of the NFAT5 mRNA level by approximately 50% under unstimulated control conditions (Fig. 4b). On the other hand, knocking down NFAT5 had no effect on the CoCl₂-induced expression of the NLRP3 gene (Fig. 4c). The data may suggest that the hypoxic expression of the NLRP3 gene in cultured RPE cells is (in part) dependent on the activities of HIF-1 and CREB.

Receptor signaling implicated in the regulation of the hypoxic expression of NLRP3 and VEGF genes

Inflammasome activation results in the production of IL-1β [17]. An IL-1 receptor antagonist was tested in CoCl₂-stimulated cell cultures to investigate whether autocrine/paracrine IL-1 receptor activation is required for the hypoxic expression of the NLRP3 gene. The CoCl₂-induced expression of the NLRP3 gene was significantly ($p < 0.05$) decreased in the presence of the IL-1 receptor antagonist (Fig. 5a). On the other hand, the caspase-1 inhibitor Ac-YVAD-CMK (as well as the inhibitor of VEGF receptor-2, SU1498) did not alter the CoCl₂-induced expression of the NLRP3 gene (Fig. 5a). The data may support the assumption that hypoxia induces a release of IL-1β from cultured RPE cells; autocrine/paracrine activation of IL-1 receptors is likely involved in mediating the hypoxic expression of the NLRP3 gene.

VEGF is the most relevant hypoxia-induced angiogenic factor of the retina [11]. The expression of the VEGF gene in cultured RPE cells was significantly ($p < 0.05$) increased in the presence of the VEGF receptor-2 antagonist SU1498 under both control and CoCl₂-stimulated conditions (Fig. 5b). The IL-1 receptor antagonist increased the VEGF gene expression under unstimulated control conditions (Fig. 5b) and after 24 h of stimulation with CoCl₂ (Fig. 5c). The data may support the assumption that autocrine/paracrine VEGF and IL-1β signaling inhibits the VEGF gene expression in cultured RPE cells. The caspase-1 inhibitor Ac-YVAD-CMK had no effects on the levels of NLRP3 and VEGF mRNAs under control and CoCl₂-stimulated conditions (Fig. 5a, b). The caspase-1 inhibitor did also not alter the VEGF gene expression and secretion during a 24-h culture in a 0.2%-O₂ atmosphere (not shown).

Purinergic receptor signaling is involved in mediating the hypoxic expression of the NLRP3 gene

It has been shown in various cell systems that ATP released from stressed cells is a danger signal which contributes to the

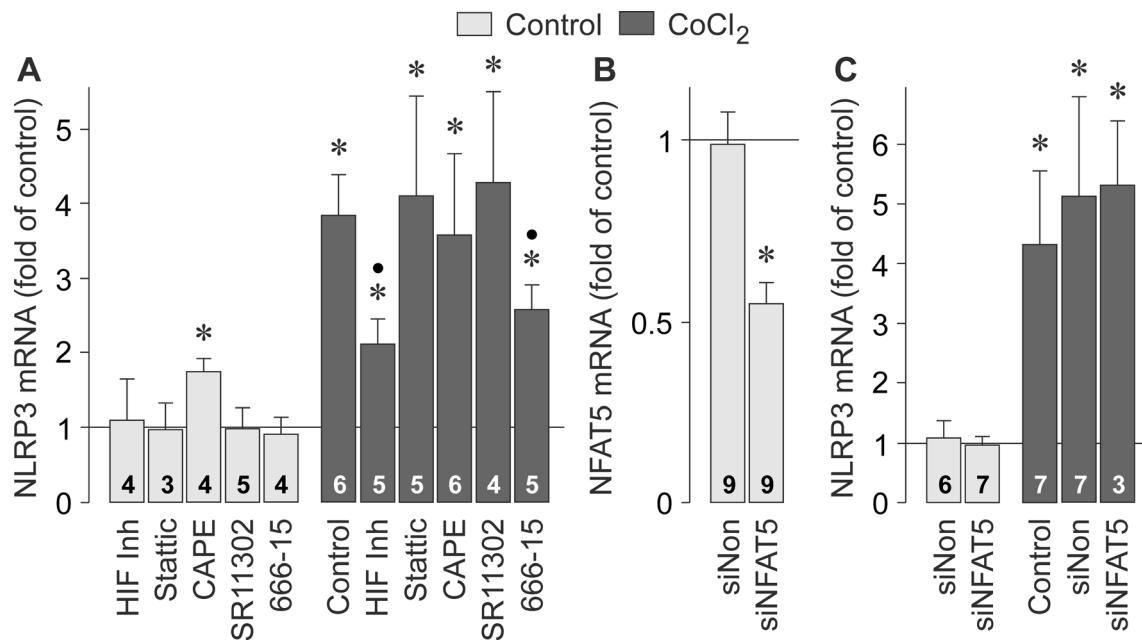


Fig. 4 Transcription factor activities involved in mediating the hypoxic expression of the NLRP3 gene in cultured RPE cells. The mRNA levels were determined with real-time RT-PCR analysis in cells cultured 6 h in the absence (control) and presence of CoCl₂ (150 μM; as indicated by the panels of the bars), and are expressed as folds of unstimulated control. **a** The following pharmacological agents were tested: a HIF-1 inhibitor (HIF-Inh; 5 μM), the STAT3 inhibitor Stattic (1 μM), the NF-κB inhibitor CAPE (5 μM), the AP-1 inhibitor SR11302 (5 μM), and the CREB inhibitor 666-15 (250 nM). **b** Transfection of RPE cells with NFAT5 siRNA (siNFAT5; 5 nM) resulted in a reduction of the NFAT5 mRNA level in cells cultured 48 h in control medium. As negative

control, non-targeted siRNA (siNon; 5 nM) was used. **c** Knocking down the gene expression of NFAT5 with siRNA (siNFAT5; 5 nM) did not alter the level of NLRP3 mRNA in cells cultured 6 h in the presence of CoCl₂ (150 μM) compared to non-transfected cells (control). siRNA transfection was done 50 h before addition of CoCl₂. The numbers of independent experiments using cell lines from different donors are indicated in the bars. Significant differences were evaluated with one-way ANOVA followed by Bonferroni's multiple comparison test. Significant difference vs. unstimulated control: **p* < 0.05. Significant difference vs. CoCl₂ control: •*p* < 0.05

activation of the NLRP3 inflammasome [39]. RPE cells express multiple purinergic receptor subtypes including metabotropic P2Y and ionotropic P2X receptors, as well as adenosine receptors [19, 26, 40]. Pharmacological receptor antagonists were tested in CoCl₂-stimulated cell cultures to investigate whether purinergic receptor signaling is involved in mediating the hypoxic NLRP3 gene expression in cultured RPE cells. The control and CoCl₂-induced expression of the NLRP3 gene was significantly (*p* < 0.05) decreased in the presence of the ATP-hydrolyzing enzyme apyrase (Fig. 6a). This finding may support the assumption that a release of endogenous ATP is required for the full induction of the constitutive and hypoxic transcription of the NLRP3 gene in cultured RPE cells. We found in agreement with a previous study [26] that the constitutive expression of the NLRP3 gene is significantly (*p* < 0.05) decreased in the presence of the P2Y₁ receptor antagonist MRS2179 (Fig. 6a). This finding might indicate that the transcription of the NLRP3 gene under control conditions is (at least in part) induced by autocrine/paracrine activation of P2Y₁ receptors. However, the CoCl₂-induced expression of the NLRP3 gene was not altered by the P2Y₁ receptor antagonist MRS2179 but was significantly (*p* < 0.05) decreased by the P2Y₂ receptor antagonist AR-C 118925XX and by the adenosine A₁ receptor antagonist DPCPX (Fig. 6a).

The P2X₇ receptor antagonist A-438079, the ecto-ATPase inhibitor ARL-67156, and the ectonucleotidase inhibitor AOPCP had also no effects on the CoCl₂-induced expression of the NLRP3 gene (Fig. 6a). The data could be explained with the assumption that a release of ATP and adenosine from cultured RPE cells and autocrine/paracrine activation of P2Y₂ and adenosine A₁ receptors contribute to the induction of the NLRP3 gene expression under hypoxic conditions. The adenosine A_{2A} receptor antagonist CSC increased significantly (*p* < 0.05) the expression level of the NLRP3 gene under CoCl₂-induced conditions (Fig. 6a). We found that the pannexin-blocking peptide ¹⁰panx decreased the CoCl₂-induced expression of the NLRP3 gene to a similar extent like apyrase, while a scrambled control peptide had no effect (Fig. 6a). The data might indicate that hypoxia induces a pannexin-dependent release of ATP from cultured RPE cells.

Purinergic receptor signaling is involved in the hypoxic expression of the VEGF gene

The hypoxic expression of the VEGF gene was significantly (*p* < 0.05) suppressed by the ATP/ADP phosphohydrolase apyrase and the P2Y₂ receptor antagonist AR-C 118925XX (Fig. 6b). In addition, the pannexin-blocking peptide ¹⁰panx

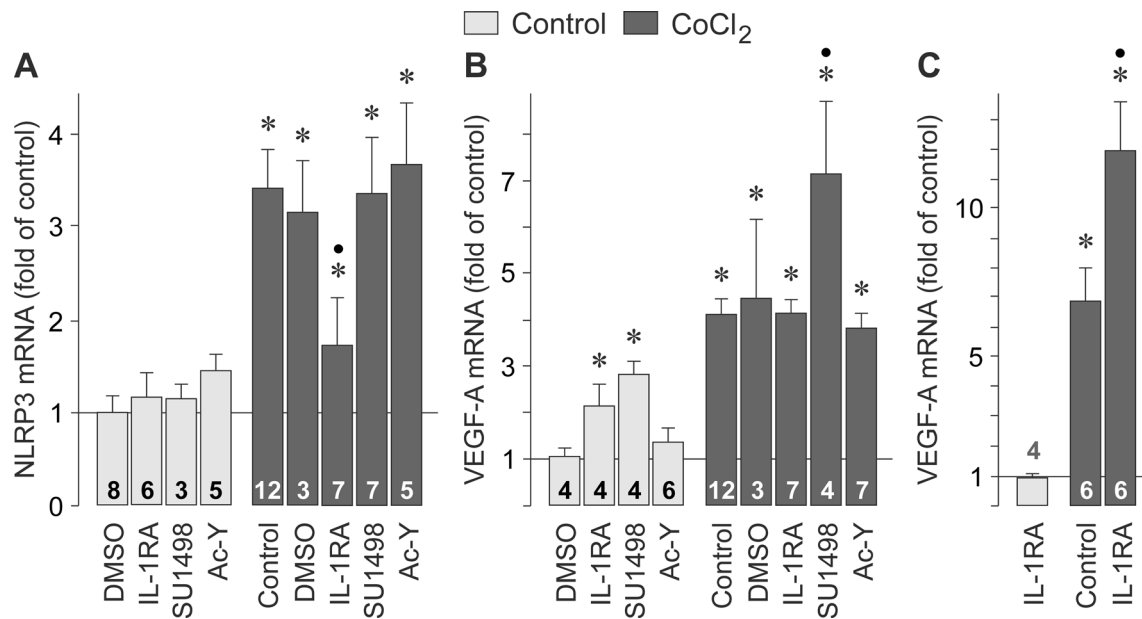


Fig. 5 Effects of IL-1 and VEGF receptor signaling on the hypoxic expression of NLRP3 (a) and VEGF genes (b, c) in cultured RPE cells. The mRNA levels were determined with real-time RT-PCR analysis in cells cultured 6 (a, b) and 24 h (c) in the absence (control) and presence of CoCl₂ (150 μM; as indicated by the panels of the bars), and are expressed as folds of unstimulated control. The following agents were tested: a recombinant human IL-1 receptor antagonist (IL-1RA; 1 μg/ml), the inhibitor of VEGF receptor-2, SU1498 (10 μM), and the caspase-1

inhibitor Ac-YVAD-CMK (Ac-Y; 500 nM). Vehicle control was made with dimethyl sulfoxide (DMSO; 1:1000). The numbers of independent experiments using cell lines from different donors are indicated in or above the bars. Significant differences were evaluated with one-way ANOVA followed by Bonferroni's multiple comparison test. Significant difference vs. unstimulated control: **p* < 0.05. Significant difference vs. CoCl₂ control: •*p* < 0.05

decreased significantly (*p* < 0.05) the hypoxic expression of the VEGF gene (Fig. 6b). The adenosine A_{2A} receptor antagonist CSC increased the hypoxic expression of the VEGF gene under low-O₂ conditions, but not in the presence of CoCl₂ (Fig. 6b). The data may support the assumption that a pannexin-dependent release of ATP and autocrine/paracrine P2Y₂ receptor signaling also contribute to the full induction of the VEGF gene expression in cultured RPE cells under hypoxic conditions.

Purinergic receptor signaling is involved in the hypoxic secretion of VEGF

In order to investigate whether purinergic receptor signaling is required for the hypoxic secretion of VEGF from cultured RPE cells, the level of VEGF protein in the cultured media was measured with ELISA. As shown in Fig. 7, hypoxia induced by a 0.2% O₂ atmosphere or by addition of CoCl₂ to the medium induced significant (*p* < 0.05) increases in the VEGF content of the cultured media. The hypoxic secretion of VEGF was significantly (*p* < 0.05) decreased in the presence of the ATP/ADP phosphohydrolase apyrase and the P2Y₂ receptor antagonist AR-C 118925XX, respectively (Fig. 7). The hypoxic secretion of VEGF was not altered in the presence of the adenosine A₁ receptor antagonist DPCPX, an IL-1 receptor antagonist, and the caspase-1 inhibitor Ac-YVAD-CMK (Fig. 7). The data may be consistent with the assumption that autocrine/paracrine P2Y₂

receptor activation is required for the full secretion of VEGF from cultured RPE cells under hypoxic conditions.

Discussion

It was suggested that activation of the NLRP3 inflammasome, which is expressed in the RPE of patients with AMD [18], is implicated in mediating the degeneration of the RPE in geographic atrophy [19, 21] and the development of choroidal neovascularization [20]. A main pathogenic condition of AMD is hypoxia of the outer retina resulting from the decrease of the choroidal blood flow and accumulation of lipoproteins in and beneath the RPE [4–9]. Hypoxia of the outer retina stimulates the production of angiogenic factors by the RPE that induce choroidal neovascularization [10]. In the present study, we investigated whether hypoxia induces priming and activation of the NLRP3 inflammasome in cultured RPE cells. We found that cell culture in a low-O₂ atmosphere is associated with a priming of the NLRP3 inflammasome, i.e., expression of NLRP3 and pro-IL-1β genes (Fig. 1b). In addition, the gene of the adaptor protein ASC (which binds procaspase-1 during inflammasome formation) is transcriptionally activated under hypoxic conditions (Fig. 1b, c). The expression of NLRP2 and pro-IL-18 genes was not altered (Fig. 1b). The finding that the IL-18 gene expression was not altered under hypoxic conditions (Fig. 1b, c) is in agreement with various

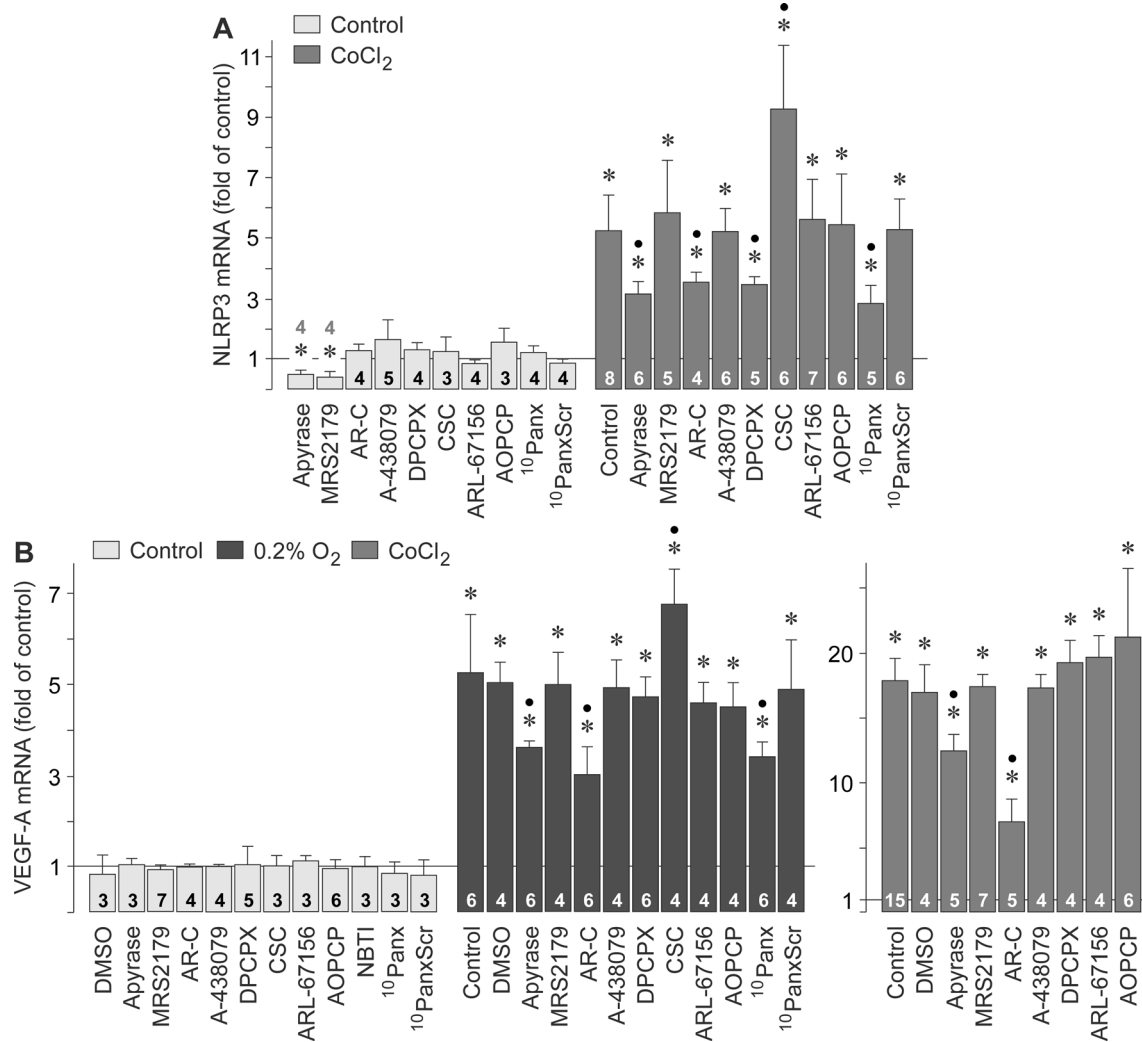


Fig. 6 Involvement of purinergic receptor signaling in mediating the hypoxic expression of NLRP3 (a) and VEGF genes (b) in cultured RPE cells. mRNA levels were determined with real-time RT-PCR analysis. mRNA levels are expressed as folds of unstimulated control. **a** The cells were cultured 6 h in the absence (control) and presence of CoCl₂ (150 μM). **b** The cells were cultured 24 h in a 0.2% O₂ atmosphere and in the presence of CoCl₂ (150 μM), respectively. The following agents were tested: the ATP/ADP phosphohydrolase apyrase (10 U/ml), the P2Y₁ receptor antagonist MRS2179 (30 μM), the P2Y₂ receptor antagonist AR-C 118925XX (AR-C; 10 μM), the P2X₇ receptor antagonist A-438079 (50 nM), the adenosine A₁ receptor antagonist DPCPX

(50 nM), the adenosine A_{2A} receptor antagonist CSC (200 nM), the ecto-ATPase inhibitor ARL-67156 (50 μM), the ectonucleotidase inhibitor AOPCP (250 μM), the pannexin-blocking peptide ¹⁰panx (200 μM), and the scrambled control peptide ¹⁰panxScr (200 μM). Vehicle control was made with dimethyl sulfoxide (DMSO; 1:1000). The numbers of independent experiments using cell lines from different donors are indicated in or above the bars. Significant differences were evaluated with one-way ANOVA followed by Bonferroni's multiple comparison test. Significant difference vs. unstimulated control: **p* < 0.05. Significant difference vs. low-O₂ and CoCl₂ control, respectively: •*p* < 0.05

studies which showed that IL-18, but not IL-1β, is constitutively expressed in RPE cells [41] and that NLRP3 inflammasome activation in a RPE cell line results in a production of IL-1β, but not of IL-18 [42]. The finding that hypoxia does not induce expression of the procaspase-1 gene (Fig. 1b) is consistent with a study in macrophages which showed that lipopolysaccharide-induced priming is characterized by NLRP3 and pro-IL-1β gene expression, but no alteration of the procaspase-1 mRNA level [43].

Caspase-1, which cleaves pro-IL-1β, might be activated by various molecular pathways mediated, for example, by

the activated NLRP3 inflammasome, the AIM2 inflammasome, and by a direct interaction of caspase-4 and procaspase-1 [17, 44–46]. Caspase-4 may be also essential for efficient NLRP3 inflammasome-dependent production of IL-1β [44]. We found that hypoxia also activates the transcription of AIM2 and procaspase-4 genes (Fig. 1b, c). Further research is required to investigate whether hypoxia also induces AIM2 inflammasome- and caspase-4-mediated production of IL-1β in RPE cells and whether these pathways play a role in mediating the retinal inflammation in AMD.

We found that hypoxia had a biphasic effect on the (pro-) IL-1 β protein level in cultured RPE cells, i.e., a decrease after 6 h and a strong increase after 24 h of hypoxia (Fig. 2c). Because the increase of the NLRP3 protein level was observed after 16 and 24 h of hypoxic stimulation (Fig. 2a, b), we assume that the increase in the cytosolic (pro-) IL-1 β level (Fig. 2c) is caused by both production of pro-IL-1 β and inflammasome-mediated production of mature IL-1 β . The decrease in the cytosolic (pro-) IL-1 β level after 6 h of hypoxia (Fig. 2c) might be caused by the secretion of stored preformed IL-1 β . Hypoxia-mediated secretion of IL-1 β from RPE cells is also suggested by the effects of an IL-1 receptor antagonist on the expression of VEGF and NLRP3 genes (Fig. 5a, c). We did not detect IL-1 β in the media obtained from cells cultured 6 or 24 h under control and CoCl₂-stimulated conditions with the ELISA (not shown). Because we found effects of an IL-1 receptor antagonist on the hypoxic gene expression (Fig. 5a, c), we assume that the main part of the secreted IL-1 β reentered the cells via receptor-mediated endocytosis and was degraded intracellularly. The effect of the IL-1 receptor antagonist on the gene expression of VEGF under control conditions (Fig. 5b) may suggest that there is a constitutive secretion of IL-1 β from the cells. This may correspond with our finding that there is a low level of NLRP3 protein in the cells under unstimulated control conditions (Fig. 2a).

We found that the caspase-1 inhibitor Ac-YVAD-CMK had no effects on the levels of NLRP3 and VEGF mRNAs under

control and hypoxic conditions (Fig. 5a, b). It might be conceivable that the effects of the IL-1 receptor antagonist on the hypoxic expression of VEGF and NLRP3 genes (Fig. 5a, c) are caused by the secretion of stored preformed IL-1 β and that IL-1 β produced by newly formed inflammasome protein complexes does not contribute to the regulation of the NLRP3 and VEGF gene expression under the present study conditions. Whether newly formed IL-1 β regulates the NLRP3 and VEGF gene expression during longer time periods remains to be proven in future investigations. It was described that NLRP3 inflammasome activation in RPE cells induced by lysosomal membrane permeabilization is associated with a reduction of the constitutive secretion of VEGF from RPE cells [35]. We found that the IL-1 receptor antagonist increased the level of VEGF mRNA under control and hypoxic conditions (Fig. 5b, c). This suggests that autocrine/paracrine IL-1 receptor signaling induced by a release of IL-1 β suppresses the expression of the VEGF gene. On the other hand, the secretion of VEGF is independent on both inflammasome activation and IL-1 receptor signaling (Fig. 7).

Both cell culture in a low-O₂ atmosphere and chemical hypoxia stimulated the expression of the NLRP3 gene (Fig. 1c). However, there were various differences in the effects of both conditions, e.g., on the expression of pro-IL-1 β and procaspase-1 genes (Fig. 1b, c). We also found that the level of VEGF mRNA is lower in the presence of a low-O₂ atmosphere than in the presence of CoCl₂ (Fig. 6b). The reasons for

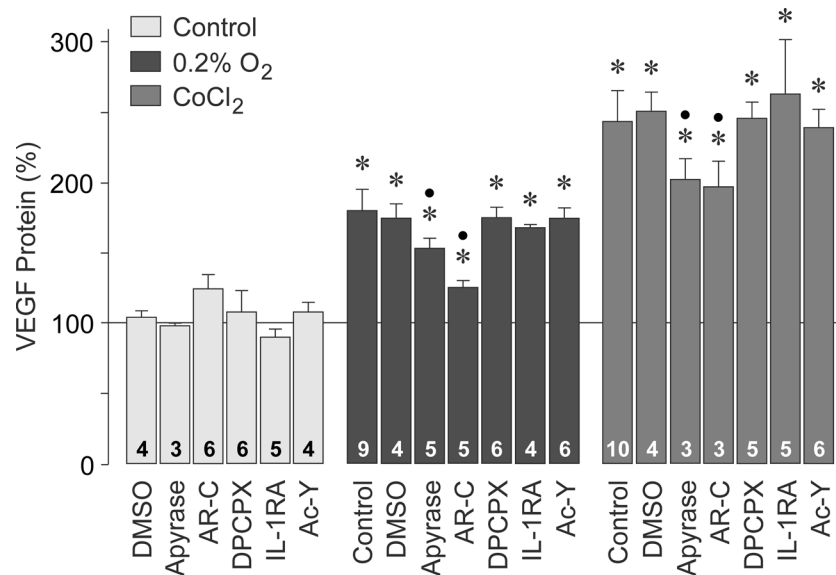


Fig. 7 Involvement of P2Y₂ receptor signaling in mediating the hypoxic secretion of VEGF from cultured RPE cells. The cells were cultured 24 h in a 0.2%-O₂ atmosphere and in the presence of CoCl₂ (150 μ M), respectively. The level of VEGF-A₁₆₅ protein in the cultured media was determined with ELISA and is expressed in percent of the unstimulated control (100%). The following agents were tested: the ATP/ADP phosphohydrolase apyrase (10 U/ml), the P2Y₂ receptor antagonist AR-C 118925XX (AR-C; 10 μ M), the adenosine A₁ receptor antagonist DPCPX (50 nM), a recombinant human IL-1 receptor antagonist (IL-

1RA; 1 μ g/ml), and the caspase-1 inhibitor Ac-YVAD-CMK (Ac-Y; 500 nM). Vehicle control was made with dimethyl sulfoxide (DMSO; 1:1000). The numbers of independent experiments using cell lines from different donors are indicated in the bars. Significant differences were evaluated with one-way ANOVA followed by Bonferroni's multiple comparison test. Significant difference vs. unstimulated control: * p < 0.05. Significant difference vs. low-O₂ and CoCl₂ control, respectively: • p < 0.05

the differences are unclear but could be explained partially with the assumptions that CoCl_2 induces (in addition to hypoxia) oxidative stress and that it also interacts with calcium channels and calcium-dependent enzymes. Oxidative stress was shown to activate the NLRP3 inflammasome in RPE cells [23, 47]. Further investigations are required to explore the (partially different) mechanisms of low- O_2 and CoCl_2 on the priming and activation of the NLRP3 inflammasome in RPE cells.

We described recently that the NLRP3 inflammasome in cultured RPE cells is primed and activated following elevation of the extracellular osmolarity [26]. As suggested by the additive effects of extracellular hyperosmolarity and hypoxia on the expression level of the NLRP3 gene (Fig. 1d) and the different time-dependencies of both effects, it is likely that the hyperosmotic and hypoxic expressions of the NLRP3 gene in cultured RPE cells are mediated by different signal transduction mechanisms. The hypoxic expression of the NLRP3 gene is (at least in part) dependent on the transcriptional activities of HIF-1 and CREB (Fig. 4a), whereas the hyperosmotic expression of the NLRP3 gene is dependent on the activities of HIF-1 and NFAT5 [26]. The present data are in agreement with previous studies which showed in various cell systems that HIF activity is implicated in the induction of NLRP3 inflammasome activation [48, 49]. In addition, we found that an IL-1 receptor antagonist decreased the hypoxic expression of the NLRP3 gene (Fig. 5a), suggesting that autocrine/paracrine IL-1 β signaling is a priming signal for hypoxic RPE cells.

The findings that apyrase and a pannexin-blocking peptide decreased the hypoxic expression of the NLRP3 gene (Fig. 6a) may support the assumption that hypoxia induces a pannexin-mediated release of ATP from cultured RPE cells. Similar effects of both compounds were found in respect to the hyperosmotic expression of the NLRP3 gene [26]. Autocrine/paracrine activation of P2Y₁ receptors is implicated in mediating both the constitutive and the hyperosmotic expression of the NLRP3 gene [26]. On the other hand, the present data may indicate that activation of P2Y₂ receptors contributes to the induction of NLRP3 gene expression under hypoxic conditions (Fig. 6a). The decreasing effect of the A₁ receptor antagonist DPCPX on the expression of the NLRP3 gene (Fig. 6a) may support the assumption that hypoxia also induces a release of adenosine from cultured RPE cells. Apparently, adenosine has contrary effects under hypoxic conditions; via activation of A₁ receptors, it stimulates the expression of the NLRP3 gene, and via activation of A_{2A} receptors, it decreases the expression of the NLRP3 gene (Fig. 6a). Because inhibition of the ecto-ATPase and ectonucleotidase did not alter the expression level of the NLRP3 gene under hypoxic conditions (Fig. 6a), it is assumed that adenosine is not formed by extracellular degradation of ATP. Interestingly, we found that autocrine/paracrine P2Y₂ receptor signaling also contributes to the stimulation of the expression of the VEGF gene (Fig. 6b) and the secretion of VEGF from cultured RPE cells under hypoxic conditions

(Fig. 7). Because inhibition of adenosine A₁ receptors by DPCPX had no effects on the hypoxic expression of the VEGF gene (Fig. 6b), it may be conceivable that the effects of P2Y₂ receptor activation on the expression of NLRP3 and VEGF genes are mediated by different intracellular signal transduction pathways.

In summary, we show that hypoxia induces priming and activation of the NLRP3 inflammasome in cultured RPE cells. Because outer retinal hypoxia is a pathogenic condition of AMD which also stimulates the progression of AMD to the neovascular stage [10], hypoxic priming and activation of the NLRP3 inflammasome in RPE cells could represent a mechanism which contributes to the development of dry and neovascular AMD. Hypoxic inflammasome activation may render RPE cells more susceptible to inflammatory stimuli and other pathogenic factors involved in mediating the degeneration of the RPE. This assumption is supported by the finding that inflammasome activation resulting from lysosomal destabilization induces RPE cell death under hypoxic but not under control conditions (Fig. 3). We found that autocrine/paracrine purinergic receptor signaling is involved in mediating the hypoxic priming of the NLRP3 inflammasome in RPE cells and the gene expression and secretion of VEGF. Because both effects may contribute to the development and progression of AMD, P2Y receptors may represent a target for the development of pharmacological approaches to treat age-related retinal inflammation and degeneration. However, because the present data were obtained in cultured, dedifferentiated RPE cells, the data must be confirmed with differentiated cells or in vivo experiments.

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Authors' contribution LK, AB, and MH conceived and designed the experiments. FD, PP, and MH performed the experiments. FD, AB, and MH analyzed and interpreted the data. PW, LK, and MH supervised research personnel. FD, AB, and MH drafted the manuscript. PW and LK made critical revision of the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest Fabian Doktor declares that he has no conflict of interest.

Philipp Prager declares that he has no conflict of interest.

Peter Wiedemann declares that he has no conflict of interest.

Leon Kohen declares that he has no conflict of interest.

Andreas Bringmann declares that he has no conflict of interest.

Margrit Hollborn declares that she has no conflict of interest.

Ethical approval All experimental protocols in this study were approved by the Ethics Committee of the University of Leipzig (approval #745, 07/25/2011).

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