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# Osmotic and hypoxic induction of the complement factor C9 in cultured human retinal pigment epithelial cells: Regulation of VEGF and NLRP3 expression

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**Purpose:** Variants of complement factor genes, hypoxia and oxidative stress of the outer retina, and systemic hypertension affect the risk of age-related macular degeneration. Hypertension often results from the high intake of dietary salt that increases extracellular osmolarity. We determined the effects of extracellular hyperosmolarity, hypoxia, and oxidative stress on the expression of complement genes in cultured (dedifferentiated) human RPE cells and investigated the effects of C9 siRNA and C9 protein on RPE cells.

**Methods:** Hyperosmolarity was induced by adding 100 mM NaCl or sucrose to the culture medium. Hypoxia was induced by culturing cells in 1% $O_2$ or by adding the hypoxia mimetic $CoCl_2$. Oxidative stress was induced by adding $H_2O_2$. Gene and protein expression levels were determined with real-time RT–PCR, western blot, and ELISA analyses. The expression of the nuclear factor of activated T cell 5 (NFAT5) and complement factor (C9) was knocked down with siRNA.

**Results:** Extracellular hyperosmolarity, hypoxia, and oxidative stress strongly increased the transcription of the C9 gene, while the expression of the C3, C5, CFH, and CFB genes was moderately altered or not altered at all. Hyperosmolarity also induced a moderate increase in the cytosolic C9 protein level. The hyperosmotic C9 gene expression was reduced by inhibitors of the p38 MAPK, ERK1/2, JNK, and PI3K signal transduction pathways and of the transcription factors STAT3 and NFAT5. The hypoxic C9 gene expression was reduced by a STAT3 inhibitor. The knockdown of C9 with siRNA decreased the hypoxic vascular endothelial growth factor (VEGF) and NLRP3 gene expression, the hypoxic secretion of VEGF, and the hyperosmotic expression of the NLRP3 gene. Exogenous C9 protein inhibited the hyperosmotic expression of the C9 gene, the hypoxic and hyperosmotic VEGF gene expression, and the hyperosmotic expression of the NLRP3 gene. Both C9 siRNA and C9 protein inhibited inflammasome activation under hyperosmotic conditions, as indicated by the decrease in the cytosolic level of mature IL-1β.

**Conclusions:** The expression of the C9 gene in cultured RPE cells is highly induced by extracellular hyperosmolarity, hypoxia, and oxidative stress. The data may support the assumption that C9 gene expression may stimulate the expression of inflammatory (NLRP3) and angiogenic growth factors (VEGF) in RPE cells. Extracellular C9 protein may attenuate this effect, in part via negative regulation of the C9 mRNA level.

Age-related macular degeneration (AMD) is the most common cause of irreversible blindness in the elderly in developed countries [1,2]. There are two forms, dry and wet AMD. The majority of patients suffer from the dry form. Normal aging and AMD are associated with an accumulation of lipofuscin within the RPE and the deposition of drusen beneath the RPE. In late-stage dry AMD, geographic atrophy of the RPE is associated with the degeneration of the neural retina. The wet form is characterized by choroidal neovascularization and subretinal edema induced by a dysfunction of the RPE, outer retinal hypoxia, and abnormalities in Bruch's membrane [3]. Dysfunction of the RPE and the development of edema result in a progressive decrease in visual acuity due to photoreceptor degeneration [4]. Hypoxia and oxidative damage to the RPE are important pathogenic conditions implicated in the development of AMD [5,6]. Vascular endothelial growth factor (VEGF) is the most relevant hypoxia-induced angiogenic factor that promotes choroidal neovascularization and edema [7].

AMD is also a chronic inflammatory disease characterized by local activation of the alternative complement cascade [8,9]. Complement proteins and their activation products, such as anaphylatoxins C3a and C5a and the membrane attack

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complex (MAC) C5b-9, accumulate in the drusen and ocular tissues of AMD patients [8-11]. Variants of complement genes, for example, of complement factors C2, C3, B (CFB), and H (CFH), are associated with the risk of AMD [12-15]. There is a significant association between a polymorphism of the C9 gene and AMD [16]; for example, a haploinsufficiency of C9 is associated with a reduced risk of AMD in the Japanese population [17]. C9 is the most abundant protein component of drusen [18]. It is also a constituent of the MAC; after assembly of the MAC, C9 can polymerize and form a cytolytic pore in the plasma membrane [19]. In mice, the MAC has been associated with the development of laser-induced choroidal neovascularization. For example, mice deficient in CD59, a complement regulator that prevents MAC assembly by the binding of C8 and C9 [20], showed accelerated laser-induced choroidal neovascularization and increased C9 deposition at the lesion that was associated with the reduced production of VEGF [21]. However, the associations between C9, MAC, and AMD in humans are little understood.

Inflammatory processes are known to be activated by cytosolic protein-signaling complexes, termed inflammasomes [22,23]. It has been shown that the nucleotide-binding oligomerization domain receptor-like receptor protein 3 (NLRP3) inflammasome is expressed in the RPE of eyes affected by AMD [24]. Activation of the NLRP3 inflammasome is implicated in mediating RPE cell degeneration in geographic atrophy [25,26] and may promote neovascular AMD pathologies, such as RPE barrier breakdown and choroidal neovascularization [27].

In addition to advanced age, race, genetic influences, sunlight exposure, smoking, and nutritional factors, systemic hypertension is a risk factor for AMD [28-30]. The most relevant condition that causes hypertension in salt-sensitive (normo- and hypertensive) individuals is increased extracellular osmolarity after the intake of dietary salt [31,32]. Plasma osmolarity and the blood pressure-raising effect of dietary salt increase with age [33,34]. It has been shown that high salt intake also induces vascular disease and mortality independent of blood pressure via a direct harmful effect on tissues and vessels [35,36]. The blood pressure-independent tissue damage caused by dietary salt results from the elevation of the extracellular osmolarity and the alteration in the transmembrane NaCl gradient [37-39]. Hyperosmotic stress has various effects on the retina and RPE, including opening of the outer blood–retinal barrier constituted by the RPE [40]. We recently found that hyperosmotic stress induces the production of VEGF and primes and activates the NLRP3 inflammasome in RPE cells [41,42]. However, it is not known whether changes in the osmotic conditions also regulate the expression and production of complement factors in RPE cells. In the present study, we determined the effect of hyperosmotic stress on complement gene expression in cultured (dedifferentiated) human RPE cells and found that the transcription of the C9 gene is strongly induced by extracellular hyperosmolarity, hypoxia, and oxidative stress. We compared the intracellular signaling mechanisms involved in mediating the hyperosmotic and hypoxic expression of the C9 gene. We also compared the effects of small interfering RNA (siRNA)-mediated knockdown of C9 gene expression and of exogenous C9 protein on the expression of VEGF and NLRP3 genes in cultured RPE cells.

## METHODS

*Materials:* Cell culture components and solutions were purchased from Gibco BRL (Paisley, UK). VEGF-$A_{165}$, platelet-derived growth factor (PDGF)-BB, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and transforming growth factor (TGF)-β1 were purchased from R&D Systems (Wiesbaden, Germany). Recombinant human C9 was purchased from Merck-Millipore (Darmstadt, Germany). Stattic was purchased from Enzo Life Science (Lörrach, Germany). Caffeic acid phenethyl ester and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)$^{1}$H-imidazole (SB203580) were obtained from Tocris (Ellisville, MO). Activated blood coagulation factor X (FXa), the hypoxia-inducible transcription factor (HIF)-1 inhibitor 3-[2-(4-adamantan-1-yl-phenoxy)-acetylamino]-4-hydroxybenzoic acid methyl ester, 2-(4-morpholinyl)-8-phenyl-$^{4}$H-1-benzopyran-4-one (LY294002), 2'-amino-3′-methoxyflavone (PD98059), rottlerin, and anthra(1–9-cd)pyrazol-6($^{2}$H)-one (SP600125) were obtained from Calbiochem (Bad Soden, Germany). Human-specific siRNA against nuclear factor of activated T cell 5 (NFAT5) and C9, as well as nontargeted control siRNA were obtained from Qiagen (Hilden, Germany). Rabbit IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). α-Thrombin, and all other agents used were from Sigma-Aldrich (Taufkirchen, Germany), unless stated otherwise. The following antibodies were used for western blot analysis: rabbit anti-human C9 (1:500; Antikoerper-Online, Aachen, Germany) and anti-rabbit IgG conjugated with alkaline phosphatase (1:3000; Chemicon, Hofheim, Germany). The following antibodies were used for immunocytochemistry: rabbit anti-C9 (1:50; Assay Biotechnology, Sunnyvale, CA), mouse anti-vimentin (1:200; V9; DakoCytomation, Glostrup, Denmark), Cy2-coupled goat anti-rabbit (1:400; Jackson ImmunoResearch, West Grove, PA), and Cy3-coupled goat anti-mouse (1:400; Jackson ImmunoResearch).

*Cell culture:* The use of human material was approved by the Ethics Committee of the University of Leipzig (#745, 07/25/2011), and the study was performed according to the Declaration of Helsinki for research involving human subjects. Post-mortem eyes of human cornea donors without reported eye disease were obtained within 48 h of death with the written informed consent of relatives for the use of retinal tissue in basic research. RPE cells were prepared and cultured as follows. After removing the vitreous and neural retina, RPE cells were mechanically harvested, separated by digestion with 0.05% trypsin and 0.02% EDTA, and washed two times with PBS. The cells were suspended in complete Ham F-10 medium containing 10% fetal bovine serum, glutamax II, and gentamycin and were cultured in laminin-coated T-75 tissue culture flasks (Greiner, Nürtingen, Germany) in 95% air/5% $CO_2$ at 37 °C. When the cultures reached confluence after 2–4 weeks, the cells were subcultivated at $5\times10^5$ cells in T-75 culture flasks or were used for the experiments. Subcultivated cells reached full confluence after 4–6 d. For the experiments, the cells were seeded at $1.5\times10^4$ cells/1 ml in 12-well plates or at $1\times10^5$ cells/2 ml in 6-well plates.

Cell lines from a total of 46 donors were used; each line was used in 3–10 different experiments. Cells of passages 2–5 were used. After 4 d of cultivation in serum-containing medium, the cultures reached near confluency. To synchronize the cells, near-confluent cultures were growth arrested in medium without serum for 16 h. At this time period, the cultures were confluent [43], and serum-free media with and without test substances were added. Hyperosmotic media were made up by adding 100 mM NaCl or sucrose. The hypoosmotic medium (60% osmolarity) was made up by adding distilled water. Hypoxia was produced by cell culture in 1% $O_2$. Chemical hypoxia was induced by adding $CoCl_2$ (150 µM), and oxidative stress was induced by adding $H_2O_2$ (20 µM). The cells were preincubated with pharmacological inhibitors for 30 min. The concentrations of the pharmacological inhibitors were adapted from the $IC_{50}$ values provided by the commercial suppliers. We routinely controlled the cultures with light microcopy to address cell proliferation and signs of cell toxicity. When signs of cell toxicity were present, we tested various concentrations of the compound to reduce the concentrations. This was done for Stattic (0.1-20 µM) and caffeic acid phenethyl ester (0.1-10 µM).

*RT–PCR analysis:* Total RNA was extracted from RPE cells using an RNeasy Mini Kit (Qiagen). The quality of the RNA was analyzed by agarose gel electrophoresis. The $A_{260}/A_{280}$ ratio of 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 µg total RNA using a RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas, St. Leon-Roth, Germany). PCR was performed using the Taq PCR Master Mix kit (Qiagen) and the primer pairs described in Table 1. Next, 1 µl of the first-strand mixture and 0.25 µM of each gene-specific sense and anti-sense primer were used for amplification in a final volume of 20 µl. Amplification was performed 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:* Semiquantitative real-time RT–PCR analysis was performed with the Single-Color Real-Time PCR Detection System (BioRad, Munich, Germany) using the primer pairs described in Table 1. The PCR solution contained 1 µl cDNA, a specific primer set (0.2 µM each), and 10 µl of a 2x mastermix (QuantiTect SYBR Green PCR Kit; Qiagen) in a final volume of 20 µl. The following conditions were used: initial denaturation and enzyme activation (one 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. mRNA expression levels were normalized to the level of β-actin transcripts. The changes in mRNA expression were calculated according to the $2^{-\Delta\Delta CT}$ method (CT, cycle threshold), with $\Delta CT=CT_{\text{target gene}} - CT_{\text{actb}}$ and $\Delta\Delta CT=\Delta CT_{\text{treatment}} - \Delta CT_{\text{control}}$.

*mRNA stability:* Cells were first treated with NaCl (100 mM) or vehicle (double-distilled water) for 2 h, followed by the addition of actinomycin D (5 µg/ml). Total RNA was isolated 1.5 h, 3 h, 4.5 h, and 6 h after the addition of actinomycin D, and mRNA expression was determined with real-time RT–PCR analysis. The vehicle for actinomycin D, ethanol (1:500), had no effect on the C9 mRNA stability (not shown).

*siRNA transfection:* Cells were seeded at $7\times10^4$ cells per well in 12-well culture plates and were allowed to grow to a confluence of 60-80%. Thereafter, the cells were transfected with NFAT5 siRNA and nontargeted siRNA (10 nM each) using HiPerfect reagent (Qiagen) in F-10 medium containing 10% fetal bovine serum (Invitrogen) according to the manufacturer's instructions. The target sequence of the NFAT5 siRNA was 5' CCC AGT CGG AAT CAT CACA 3'. After 24 h, the medium was removed and fresh medium without serum was added for 2 h. Thereafter, hyperosmotic medium (+ 100 mM NaCl) without serum was added for another 2 h. Total RNA was extracted, and NFAT5 and C9 mRNA levels were determined with real-time RT–PCR analysis. To

knockdown C9, cells were transfected with C9 siRNA and nontargeted siRNA (10 nM each). The target sequence of the C9 siRNA was 5' AAC CGT GAT TGA TGT GAC TGA 3'. After 48 h, the medium was removed and serum-free hyperosmotic medium (+ 100 mM NaCl) or isosmotic medium containing $CoCl_2$ (150 µM) was added for 6 h or 24 h, or the cells were cultured in 1% $O_2$.

*Western blot analysis:* Cells were seeded at $5\times10^5$ cells per well in 6-well plates in 1.5 ml complete medium and were allowed to grow to a confluence of ~80%. After growth arrest for 16 h, the cells were treated with $CoCl_2$ (150 µM) or hyperosmotic medium (+ 100 mM NaCl) for 20 min, 6 h, 12 h, and 24 h. Next, the medium was removed, the cells were washed twice with prechilled PBS (pH 7.4; Invitrogen), and the monolayer was scraped into 150 µl of lysis buffer (Mammalian Cell Lysis-1 Kit; Sigma). Total cell lysates were centrifuged at 10,000 × *g* for 10 min, and supernatants were analyzed by immunoblotting. Equal amounts of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Immunoblots were probed with primary and secondary antibodies, and immunoreactive bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

*ELISA:* To determine the level of VEGF in the cultured media, the cells were cultured at $7\times10^4$ cells per well in 12-well plates. At a confluence of 60-80%, the cells were transfected with C9 siRNA and nontargeted siRNA (10 nM each). After 48 h, the medium was removed and serum-free isosmotic medium containing $CoCl_2$ (150 µM) was added for 24 h, or the cells were cultured for 24 h in serum-free isosmotic medium in 1% $O_2$. The level of VEGF-$A_{165}$ in the cultured media (100 µl) was determined with ELISA (R&D Systems). To determine the cytosolic level of mature IL-1β, siRNA-transfected cells were stimulated for 6 h with a hyperosmotic medium (+ 100 mM NaCl). Nontransfected cells were cultured for 24 h in iso- and hyperosmotic media in the absence or presence of C9 protein (10 nM). The level of mature IL-1β in cell lysates was determined with ELISA (HSLB00C; R&D Systems).

*Cell viability:* Cell viability was determined using an MTT assay (Serva, Heidelberg, Germany). Cells were seeded at $5\times10^3$ cells per well in 96-well plates. After reaching confluency, the cells were synchronized in serum-free medium for 16 h. Next, 10 µl of MTT solution (5 mg/ml) were added to each well after a 20 h period of stimulation. After 4 h, the culture supernatants were removed and DMSO (100 µl) was

Table 1. Primer pairs used in PCR experiments.

| Gene and accession number | Primer sequences (5'→3') | Product (bp) |
|---|---|---|
| ACTB<br>NM_001101 | s ATGGCCACGGCTGCTTCCAGC<br>as CATGGTGGTGCCGCCAGACAG | 237 |
| GAPDH<br>NM_002046 | s GCAGGGGGGAGCCAAAAGGGT<br>as TGGGTGGCAGTGATGGCATGG | 219 |
| C3<br>NM_000064 | s CTGCAGACCTCAGTGACCAA<br>as GCTCCGTTTCATCCAGGTAA | 207 |
| C5<br>NM_001735 | s GTGTGTGGAAGGGTGGAAG<br>as GTTCTCTCGGGCTTCAACAG | 222 |
| C9<br>NM_001737 | s CAACTGGGCCTCTTCCATAA<br>as ACCTCCATTTTGGCATGTGT | 184 |
| CFB<br>BC007990 | s CTGGAGCACCCTGAAGACTC<br>as CCGGAGAGTGTAACCGTCAT | 172 |
| CFH<br>NM_000186 | s CAGCAGTACCATGCCTCAGA<br>as GGATGCATCTGGGAGTAGGA | 189 |
| NFAT5<br>NM_006599.3 | s TCACCATCATCTTCCCACCT<br>as CTGCAATAGTGCATCGCTGT | 174 |
| NLRP3<br>NM_183395.2 | s AGACAGCATTGAAGAGGAGTGG<br>as TTTGTTGAGGCTCACACTCTCA | 169 |
| $VEGFA_{188, 164, 120}$<br>NM_003376.5<br>NM_001287044.1<br>NM_001025370.2 | s CCTGGTGGACATCTTCCAGGAGTA<br>as CTCACCGCCTCGGCTTGTCACA | 479; 407; 275 |

s, sense. as, anti-sense.

added. Absorbance was recorded at 570 nm using a Spectra Max 50 ELISA reader (Molecular Devices, Sunnyvale, CA).

*Immunocytochemistry:* The cultures were fixed with 4% paraformaldehyde for 20 min at room temperature (RT). After several washing steps in prechilled PBS (pH 7.4; Invitrogen), the cultures were incubated in PBS containing DMSO (1%) and 0.3% Triton X-100 for 15 min at RT. Blocking of nonspecific binding of the antibodies was performed with PBS containing 1% DMSO, 0.3% Triton, and 5% normal goat serum for 2 h at RT. Thereafter, the cultures were incubated in a mixture of primary antibodies diluted in blocking solution at 4 °C overnight. After washing in PBS plus 1% DMSO and 0.3% Triton X-100, secondary antibodies and Hoechst 33342 (1:1000) were applied for 2 h at RT. After several washing steps, the coverslips were mounted with Fluorescence Mounting Medium (DakoCytomation). Images were taken with an epifluorescence microscope (Olympus BX40, Olympus, Essex, UK), a CCD camera (Olympus XM10), and cellSens software (Olympus).

*Statistical analysis:* For each test, at least three independent experiments using cell lines from different donors were performed. Data are expressed as means ± standard error of the mean (SEM). Statistical analysis was performed with Prism version 6.05 (Graphpad Software, San Diego, CA). Significance was determined by one-way ANOVA followed by a Bonferroni multiple comparison test and a Mann–Whitney *U* test and was accepted at $p<0.05$.

## RESULTS

*Expression of complement genes:* RT–PCR analysis was performed to determine the expression of complement genes in cultured human RPE cells and in RPE cells that were freshly isolated from eyes of post-mortem donors without reported eye disease. As shown in Figure 1A, freshly isolated and cultured cells contained transcripts of the complement factors 3, 5, 9, H, and B. The results agree with previous studies that described the expression of various complement genes in RPE cells [44,45].

*Osmotic regulation of complement gene expression:* Extracellular hyperosmolarity produced by adding 100 mM NaCl to the culture medium induced a very high increase in the expression of the C9 gene, while the expression of further complement genes investigated was moderately or not at all altered compared to the isosmotic control (Figure 1B). The hyperosmotic increase in the expression of the C9 gene was mediated by gene transcription (Figure 2A) but not by alteration of the mRNA stability (Figure 2B). The effect of high extracellular NaCl on the cellular C9 mRNA level was dose-dependent (Figure 1C). Upregulation of C9 gene expression was also observed in cells cultured in medium that was made hyperosmotic by adding 100 mM sucrose (Figure 1D). The sucrose-induced expression of the C9 gene (Figure 1D) displayed a different time dependence compared to the high NaCl-induced expression (Figure 1B), suggesting that both extracellular hyperosmolarity and alteration of the transmembrane NaCl gradient induce C9 gene expression. A hypoosmotic medium (60% osmolarity) induced a moderate transient increase in C9 gene expression (Figure 1D).

*Regulation of C9 gene expression:* To determine further conditions and factors that regulate the expression of the C9 gene, we treated RPE cells with different compounds. The hypoxia mimetic $CoCl_2$ [46] induced a strong time-dependent increase in the expression of the C9 gene, while the expression levels of further complement genes investigated were moderately or not at all altered (Figure 3A). Cell culturing in an 1%-$O_2$ atmosphere also induced a significant ($p<0.05$) but lower increase in the expression of the C9 gene (Figure 3B). The effects of $CoCl_2$-induced chemical hypoxia and high NaCl-induced hyperosmolarity on the expression of the C9 gene were additive (Figure 3C). Moreover, the kinetics of the transcriptional upregulation were different under both conditions. While the high NaCl-induced expression of the C9 gene peaked after 6 h, the hypoxic gene induction was largest after 24 h of stimulation (Figure 3C). The data could support the assumption that the hypoxic and hyperosmotic expression of the C9 gene is mediated by different signal transduction mechanisms. In addition to extracellular hyperosmolarity and hypoxia, the level of C9 mRNA was increased in response to oxidative stress induced by the addition of $H_2O_2$, while the expression levels of further complement genes were moderately or not at all altered (Figure 3D).

The cellular level of C9 mRNA remained largely unaltered in the presence of high (25 mM) glucose, the activated blood coagulation factor X, TGF-β1, IL-1β, and TNFα (Figure 3E). The following factors induced a delayed decrease in the cellular level of C9 mRNA: α-thrombin, VEGF, PDGF, arachidonic acid, and prostaglandin $E_2$ (Figure 3E). The data may support the assumption that the expression of the C9 gene in cultured RPE cells is rather selectively induced by extracellular hyperosmolarity, hypoxia, and oxidative stress.

*Intracellular signaling involved in C9 gene expression:* It has been shown that extracellular hyperosmolarity induces activation of different signal transduction molecules in RPE cells, for example, p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) [41]. To compare the intracellular signal transduction pathways involved in mediating the expression of the C9 gene under hyperosmotic and hypoxic conditions, we

tested pharmacological blockers of key intracellular signal transduction molecules. The hyperosmotic expression of the C9 gene was significantly ($p<0.05$) decreased by inhibitors of the p38 MAPK, ERK1/2, c-Jun $NH_2$-terminal kinase (JNK), and phosphatidylinositol-3 kinase (PI3K) signal transduction pathways (Figure 4A). In contrast, the hypoxic expression of the C9 gene was not inhibited by these compounds (Figure 4B). The data may support the assumption that the C9 gene in cultured RPE cells is transcriptionally activated by different signal transduction pathways under hyperosmotic and hypoxic conditions.

*Transcription factor activities involved in mediating C9 gene expression:* An HIF-1 inhibitor [47] and the inhibitor of the nuclear transcription factor NF-κB, caffeic acid phenethyl ester [48], had no effects on the level of C9 mRNA under hyperosmotic and hypoxic conditions (Figure 4A,B). However, the expression of the C9 gene under both conditions was significantly ($p<0.05$) decreased in the presence of the inhibitor of the signal transducer and the activator of transcription-3 (STAT3), Stattic (Figure 4A,B) [49].

In various cell systems, the transcriptional activity of NFAT5 is critical for cell survival under hyperosmotic conditions [50,51]. We recently found that hyperosmolarity but not hypoxia increases the levels of NFAT5 mRNA and protein and induces DNA binding of NFAT5 protein in RPE cells [41]. To determine whether the hyperosmotic induction of C9 gene expression is mediated by the transcriptional activity of NFAT5, we tested the NFAT5 inhibitor rottlerin [52]. Rottlerin almost completely inhibited the hyperosmotic induction of C9 gene transcription (Figure 5A). To confirm

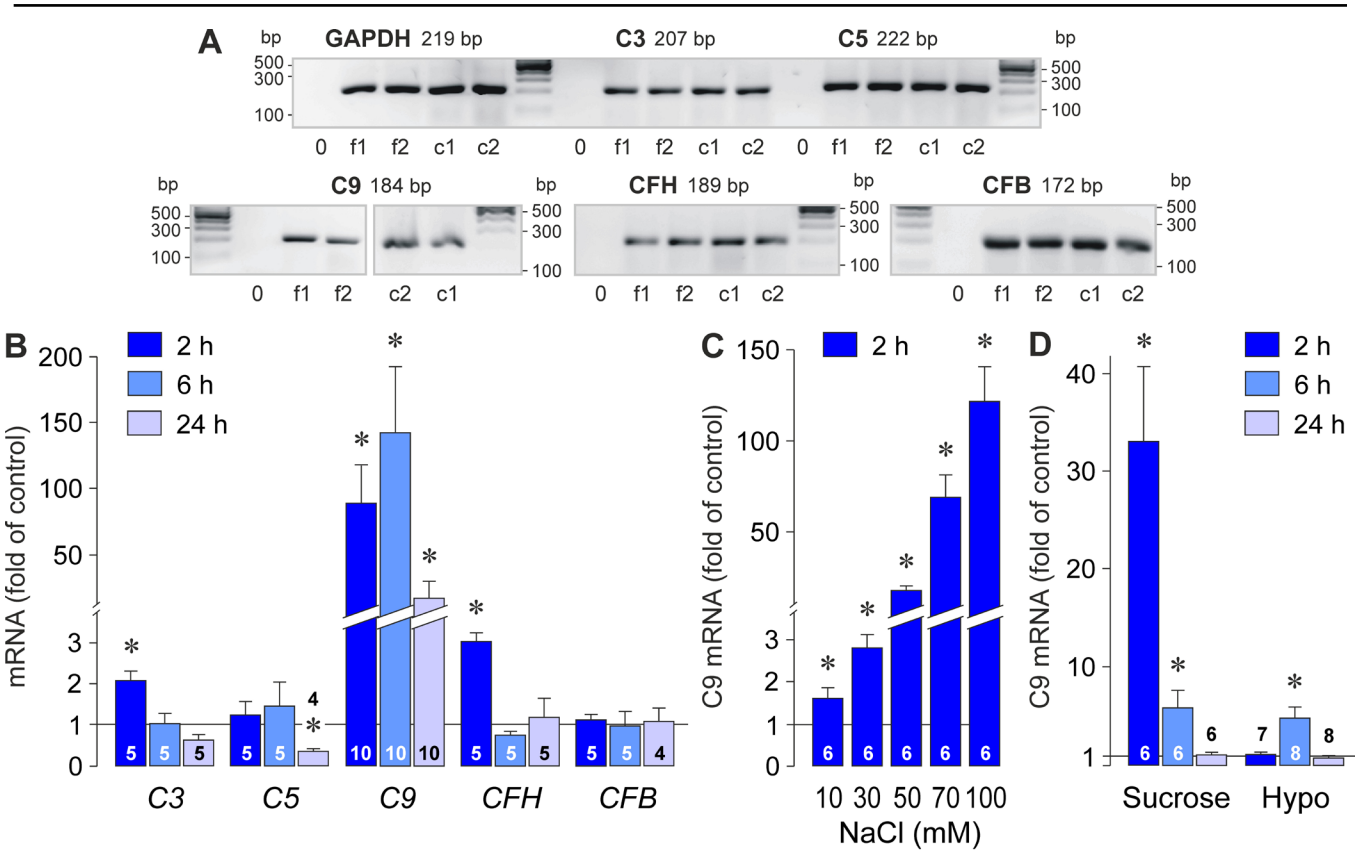


Figure 1. Osmolarity-dependent expression of complement factor genes in cultured human RPE cells. mRNA levels were determined with real-time RT–PCR analysis. **A**: Presence of complement gene transcripts in RPE cells. To confirm the correct lengths of PCR products, agarose gel electrophoresis was performed using products obtained from freshly isolated human RPE cells of two different post-mortem donors (f1, f2) and from cultured human RPE cells of the 5th (c1) and 4th (c2) passage, respectively. Negative controls (0) were obtained by using double-distilled water instead of cDNA as a template. GAPDH mRNA was used as a loading control. **B**: Relative expression levels of complement genes in cells cultured for 2 h, 6 h, and 24 h (as indicated by the panels of the bars) in hyperosmotic medium (+ 100 mM NaCl). **C**: Dose-dependent effect of high extracellular NaCl on the C9 mRNA level. Extracellular hyperosmolarity was induced by the addition of 10–100 mM NaCl to the culture medium. **D**: Effects of hyperosmotic (+ 100 mM sucrose) and hypoosmotic (Hypo; 60% osmolarity) media on the expression of the C9 gene. The numbers of independent experiments using cell lines from different donors are indicated in or above the bars. Significant difference versus isosmotic control: *$p<0.05$.

the involvement of NFAT5 activity using another method, we used siRNA to knock down NFAT5. Transfection with NFAT5 siRNA reduced the level of NFAT5 transcripts by approximately 50% in cells cultured in isosmotic medium (Figure 5B). Transfection with NFAT5 siRNA also reduced the level of C9 gene transcripts by approximately 50% in cells stimulated with a hyperosmotic medium (Figure 5C). However, nontargeted siRNA had no effect on the level of C9 gene transcripts in cells stimulated with hyperosmotic medium compared to nontransfected cells (Figure 5C). The data may support the assumption that the C9 gene in cultured RPE cells is (in part) transcriptionally activated by STAT3 and NFAT5 under hyperosmotic conditions and by STAT3 under hypoxic conditions.

*Effect of hyperosmolarity on cellular C9 protein level:* To determine whether extracellular hyperosmolarity induces the production of C9 protein in cultured RPE cells, we determined the level of C9 protein in cell lysates using western blot analysis. Lysates of RPE cells and of neural retinas obtained from different post-mortem donors contained C9 protein (Figure 6A). Stimulation of the cells with a hyperosmotic medium induced a moderate but significant ($p<0.05$) increase in the C9 protein level in RPE cell lysates (Figure 6B,C). Cultured RPE cells were labeled with an antibody against C9 (Figure 6D). A hyperosmotic medium did not apparently alter the distribution and level of C9 immunoreactivity in RPE cells when compared with the control (Figure 6D).

*Effects of C9 siRNA and protein on the expression of VEGF and NLRP3 genes:* The rather selective expression of the C9 gene under hyperosmotic conditions (Figure 1B) could suggest the functional role of C9, possibly in regulating the transcription or translation of other factors upregulated in response to osmotic stress. We recently found that in addition to the VEGF gene [41], the NLRP3 gene is transcriptionally activated in RPE cells by hyperosmotic stress (Figure 7F,G) [42]. VEGF and NLRP3 genes are also transcriptionally activated by chemical hypoxia (Figure 7C,E–G) [41]. To prove the assumption that C9 is involved in the regulation of VEGF and NLRP3 gene expression, we compared the effects of siRNA-mediated knockdown of C9 and the administration of exogenous C9 protein. siRNA-mediated knockdown of C9 significantly decreased ($p<0.05$) the cellular level of C9 gene transcripts under control, hyperosmotic, and hypoxic conditions (Figure 7A). Exogenous C9 protein induced a

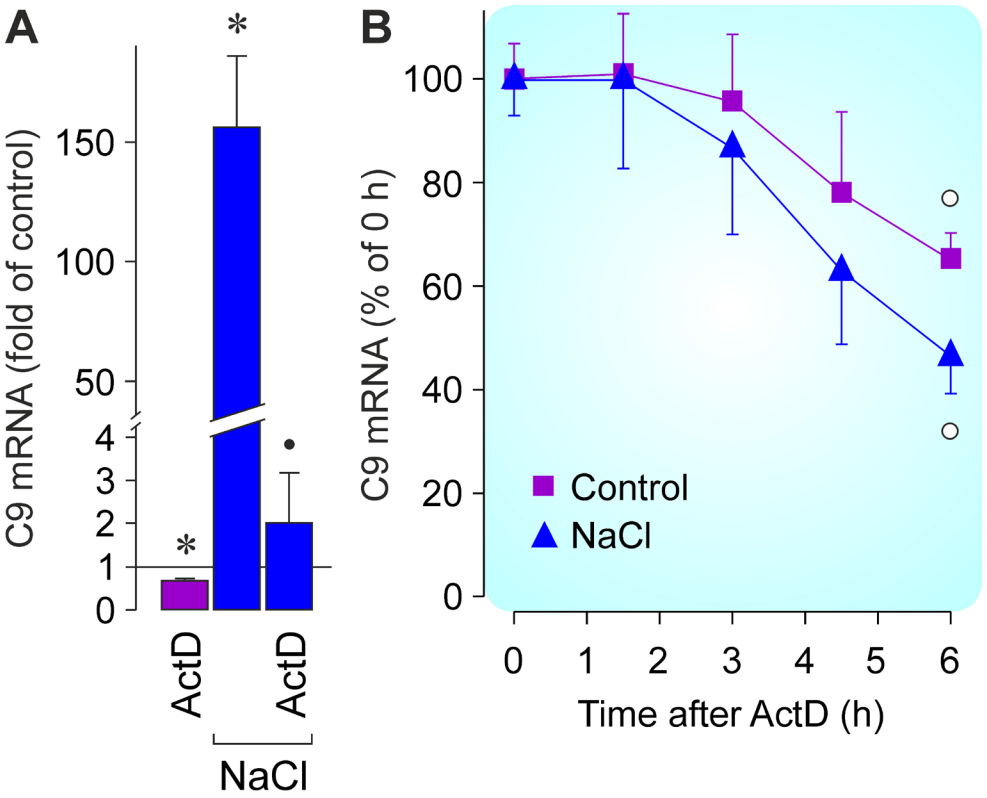


Figure 2. The hyperosmotic expression of the C9 gene is induced by the stimulation of gene transcription. The mRNA level was determined with real-time RT–PCR analysis. **A**: Inhibition of the RNA polymerase II by actinomycin D (ActD; 5 µg/ml) abrogated C9 gene expression induced by hyperosmotic (+ 100 mM NaCl) medium. The data were obtained after 6 h of stimulation. Actinomycin D was preincubated for 30 min. **B**: The stability of C9 mRNA did not significantly differ between isosmotic (control) and hyperosmotic (+ 100 mM NaCl) conditions. The cells were first treated with iso- and hyperosmotic media for 2 h, followed by the addition of actinomycin D (5 µg/ml). Total RNA was isolated at different time periods after the addition of actinomycin D. Means ± SEM of four independent experiments using cell lines from different donors. Significant difference versus isosmotic control: *$p<0.05$. Significant difference versus NaCl control: •$p<0.05$. Significant difference versus 0 h control: ○$p<0.05$.

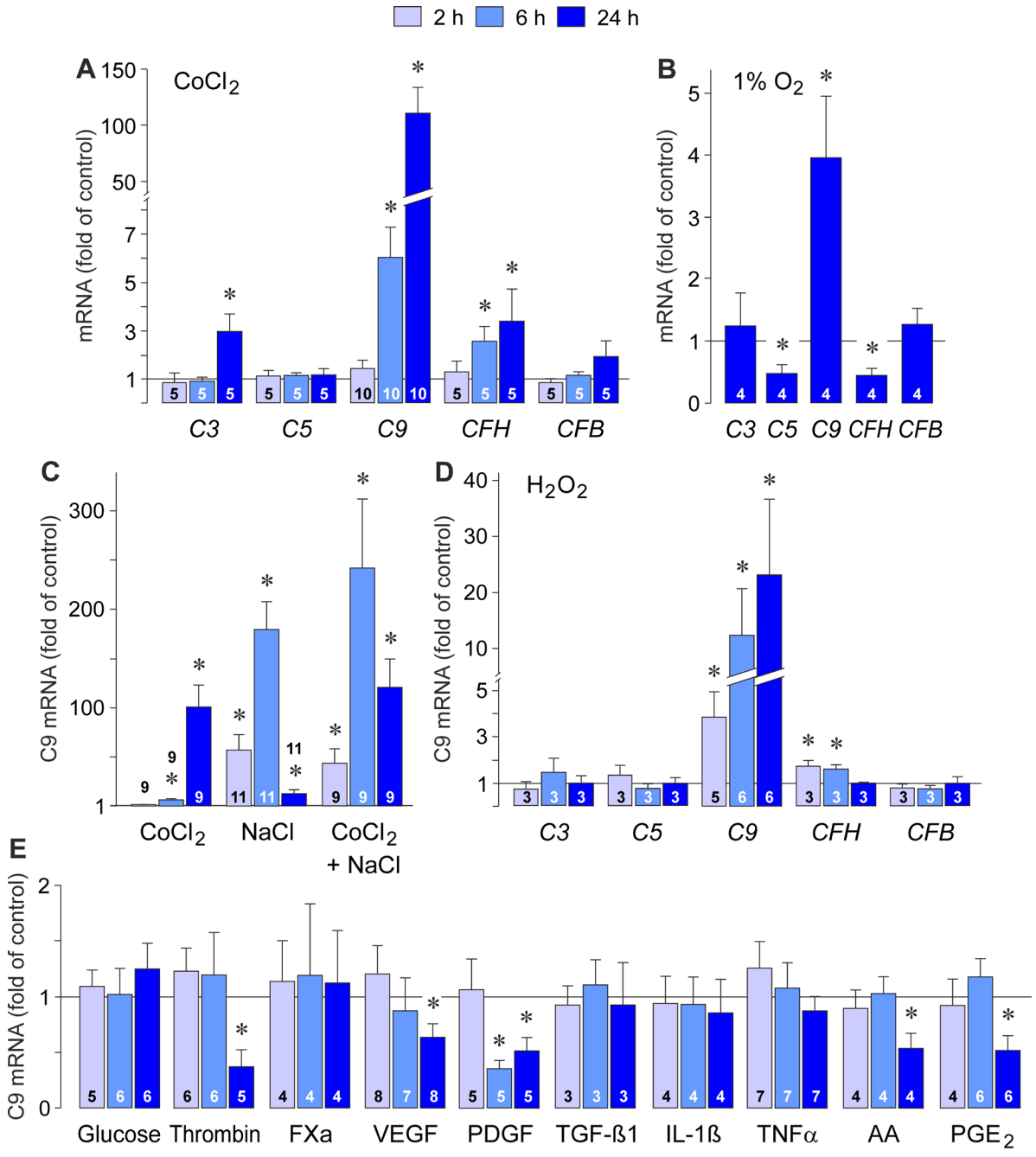


Figure 3. Transcriptional regulation of the C9 gene in cultured human RPE cells. The mRNA level was determined with real-time RT–PCR analysis after stimulation of the cells for 2 h, 6 h, and 24 h (as indicated by the panels of the bars). **A**: Effects of $CoCl_2$ (150 µM) on the expression levels of complement genes. **B**: Effects of cell culturing in 1% $O_2$ on the expression levels of complement genes. **C**: Effects of $CoCl_2$ (150 µM) and hyperosmotic medium (+ 100 mM NaCl) on the cellular level of C9 mRNA. **D**: Effects of oxidative stress induced by $H_2O_2$ (20 µM) on the expression levels of complement genes. **E**: The level of C9 mRNA was determined in cells stimulated with the following compounds: glucose (25 mM), thrombin (10 U/ml), the activated blood coagulation factor X (FXa; 1 U/ml), VEGF (10 ng/ml), PDGF-B (10 ng/ml), TGF-β1 (10 ng/ml), IL-1β (10 ng/ml), TNFα (10 ng/ml), arachidonic acid (AA; 5 µM), and prostaglandin $E_2$ ($PGE_2$; 10 ng/ml). The numbers of independent experiments using cell lines from different donors are indicated in or above the bars. Significant difference versus unstimulated control: *p<0.05.

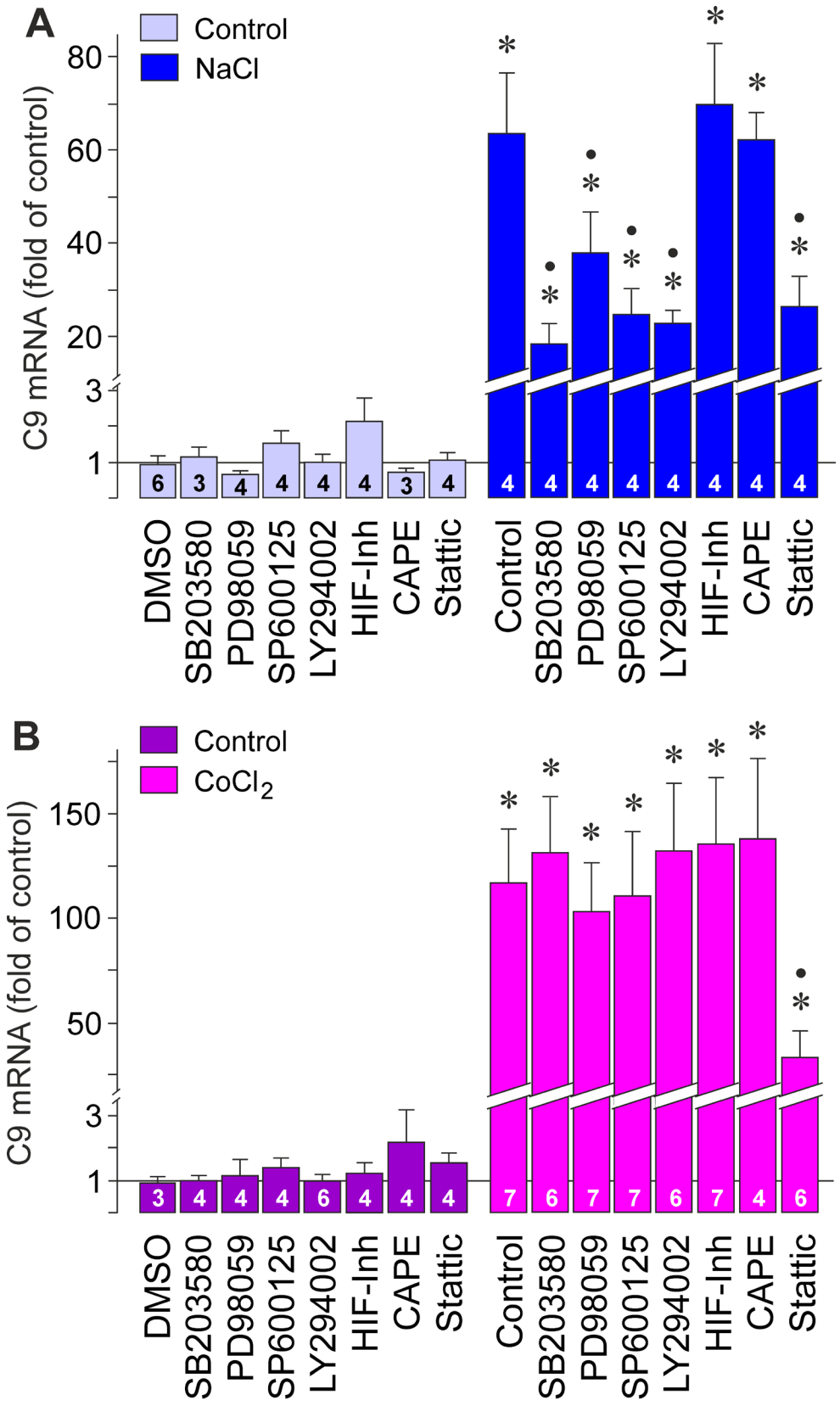


Figure 4. Involvement of signal transduction pathways and transcription factors in the hyperosmotic and hypoxic expression of the C9 gene. **A**: The mRNA level was determined with real-time RT–PCR analysis in cells cultured for 2 h under isosmotic control conditions (*left*) and hyperosmotic conditions (+ 100 mM NaCl; *right*). **B**: The mRNA level was determined in cells cultured for 24 h under control conditions (*left*) and in the presence of $CoCl_2$ (150 µM; *right*). The following blocking compounds were tested: the inhibitor of p38 MAPK activation, SB203580 (10 µM); the inhibitor of ERK1/2 activation, PD98059 (20 µM); the JNK inhibitor SP600125 (10 µM); the inhibitor of PI3K-related kinases, LY294002 (5 µM); an HIF-1 inhibitor (HIF-Inh; 5 µM); the NF-κB inhibitor caffeic acid phenethyl ester (CAPE; 1 µM); and the STAT3 inhibitor Stattic (1 µM). Vehicle controls were made with DMSO (1:1000). The numbers of independent experiments using cell lines from different donors are indicated in the bars. Significant difference versus unstimulated control: *p<0.05. Significant difference versus NaCl and $CoCl_2$ control: ●p<0.05.

dose-dependent decrease in the expression of the C9 gene under hyperosmotic conditions and had no effect under control and hypoxic conditions (Figure 7B).

siRNA-mediated knockdown of C9 also significantly decreased ($p<0.05$) the hypoxic expression of the VEGF gene and had no effect on VEGF gene expression under hyperosmotic conditions (Figure 7C). In addition, the knockdown of C9 significantly decreased ($p<0.05$) the hypoxic secretion of VEGF protein from RPE cells (Figure 7D). Exogenous C9 protein induced a dose-dependent decrease in the VEGF mRNA level under hyperosmotic conditions (Figure 7E). Under hypoxic conditions, C9 protein at a higher concentration induced a delayed decrease in VEGF gene expression (Figure 7E).

The expression level of NLRP3 mRNA was reduced in the presence of C9 siRNA under both hyperosmotic and hypoxic conditions (Figure 7F). However, the administration of C9 protein induced a delayed decrease in the NLRP3 mRNA level under hyperosmotic conditions and had no effect on the NLRP3 mRNA level under hypoxic conditions (Figure 7G). Under unstimulated control conditions, C9 siRNA and nontargeted siRNA did not alter the expression levels of VEGF and NLRP3 genes or the VEGF protein content of cultured media (data not shown). The data may support the assumption that C9 mRNA could play a regulatory role in cultured RPE cells and may stimulate the expression of VEGF and NLRP3 genes under certain conditions.

*Inflammasome activation:* NLRP3 inflammasome activation results in the proteolytic formation of mature IL-1β [22,23]. To investigate whether C9 affects inflammasome activation in RPE cells, we measured the cytosolic level of mature IL-1β with ELISA in RPE cells cultured under control and hyperosmotic conditions. Transfection of the cells with C9 siRNA or nontargeted siRNA did not alter the cytosolic level of mature IL-1β under control conditions (Figure 8A). As previously shown [42], high NaCl in the culture medium induced a significant ($p<0.05$) increase in the cytosolic level of mature IL-1β, suggesting inflammasome activation under hyperosmotic conditions (Figure 8A,B). The hyperosmotic increase in the IL-1β level was significantly ($p<0.05$) smaller in cells transfected with C9 siRNA compared to cells transfected with nontargeted siRNA (Figure 8A). The administration of exogenous C9 protein completely inhibited the hyperosmotic increase in the cytosolic level of mature IL-1β and had no

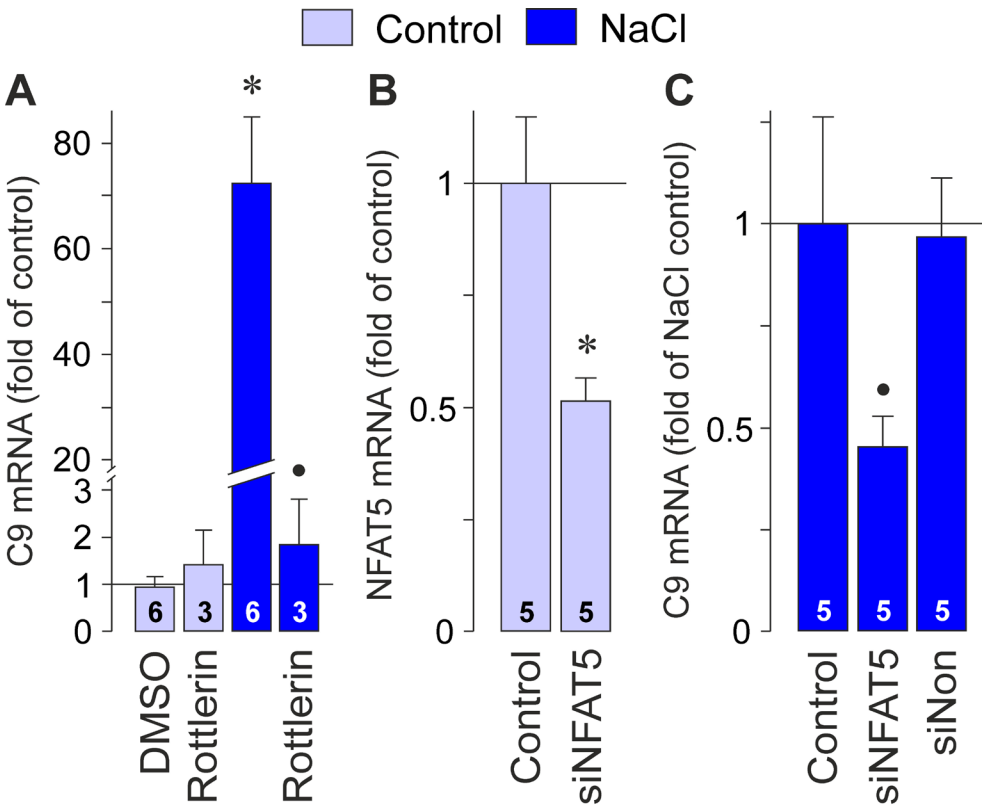


Figure 5. Dependence of hyperosmotic C9 gene expression on NFAT5 activity. **A**: The NFAT5 inhibitor rottlerin (10 µM) prevented the hyperosmotic (+ 100 mM NaCl) increase in the C9 mRNA level. The mRNA level was determined with real-time RT–PCR analysis after stimulation of the cells for 2 h. **B**: Transfection of RPE cells with NFAT5 siRNA (siNFAT5; 10 nM) resulted in a reduction of the NFAT5 mRNA level in RPE cells cultured in isosmotic medium for 24 h. **C**: Knocking down the gene expression of NFAT5 with siRNA (siNFAT5; 10 nM) reduced the level of C9 mRNA in cells cultured for 2 h in hyperosmotic (+ 100 mM NaCl) medium compared to nontransfected cells (control). Nontargeted siRNA (siNon; 10 nM) was used as a negative control. siRNA transfection was done 26 h before hyperosmotic stimulation. The numbers of independent experiments using cell lines from different donors are indicated in the bars. Significant difference versus unstimulated control: *$p<0.05$. Significant difference versus NaCl control: •$p<0.05$.

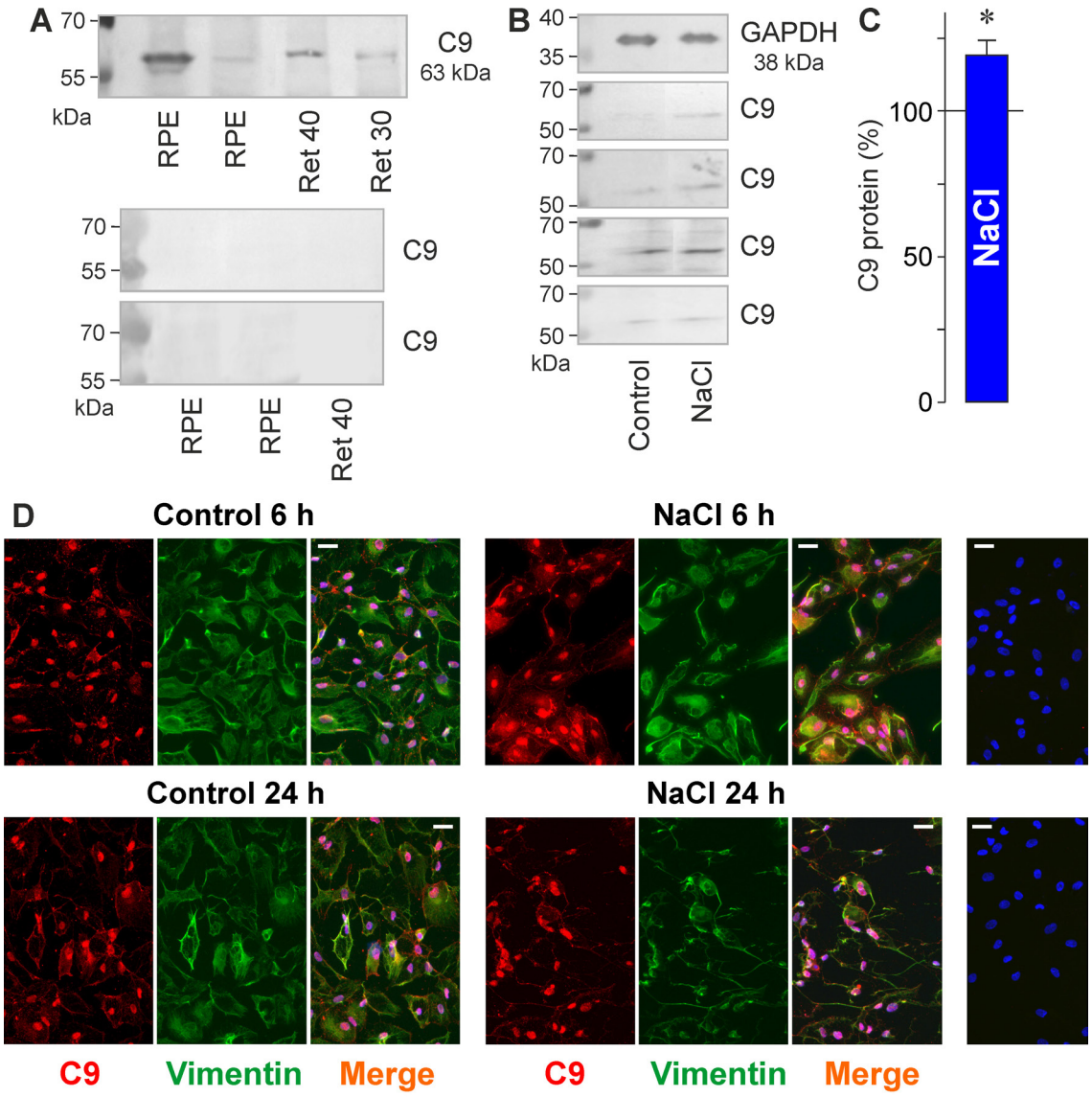


Figure 6. Hyperosmotic stress increases C9 protein expression in cultured human RPE cells. **A**: western blot analysis of C9 protein in lysates of cultured human RPE cells and human retinas (Ret) from post-mortem donors without reported eye disease (*above*). In the cases of RPE cells, 45 µg of total protein were used, while in the cases of retinas, either 40 µg (Ret 40) or 30 µg (Ret 30) of total protein were used. *Middle and below:* Negative controls done by omission of the first antibody (*middle*) and by using rabbit IgG (1:500) as the first antibody (*below*). **B**: western blot analysis of C9 protein in lysates of cells cultured in isosmotic (control) and hyperosmotic (+ 100 mM NaCl) media for 6 h. Results of four independent experiments using cells from different donors are shown. **C**: Mean ± SEM C9 protein content of cytosolic extracts of cells cultured for 6 h in hyperosmotic (+ 100 mM NaCl) medium in comparison to cells cultured in isosmotic medium (100%). The data were obtained using western blot analysis in six independent experiments using cell lines from different donors. Significant difference versus isosmotic control: *p<0.05. **D**: C9 immunoreactivity in cultured RPE cells. The cells were immunolabeled with antibodies against C9 (*red*) and vimentin (*green*). Cell nuclei were stained with Hoechst 33342 (*blue*). *Right side:* Negative controls were obtained by omitting the primary antibodies. (Note that the RPE monolayer is disrupted by the fixation procedure.) The cells were cultured for 6 h (*above*) and 24 h (*below*) in isosmotic control (*left side*) and hyperosmotic (+ 100 mM NaCl) media (*middle*). Bars, 20 µm.

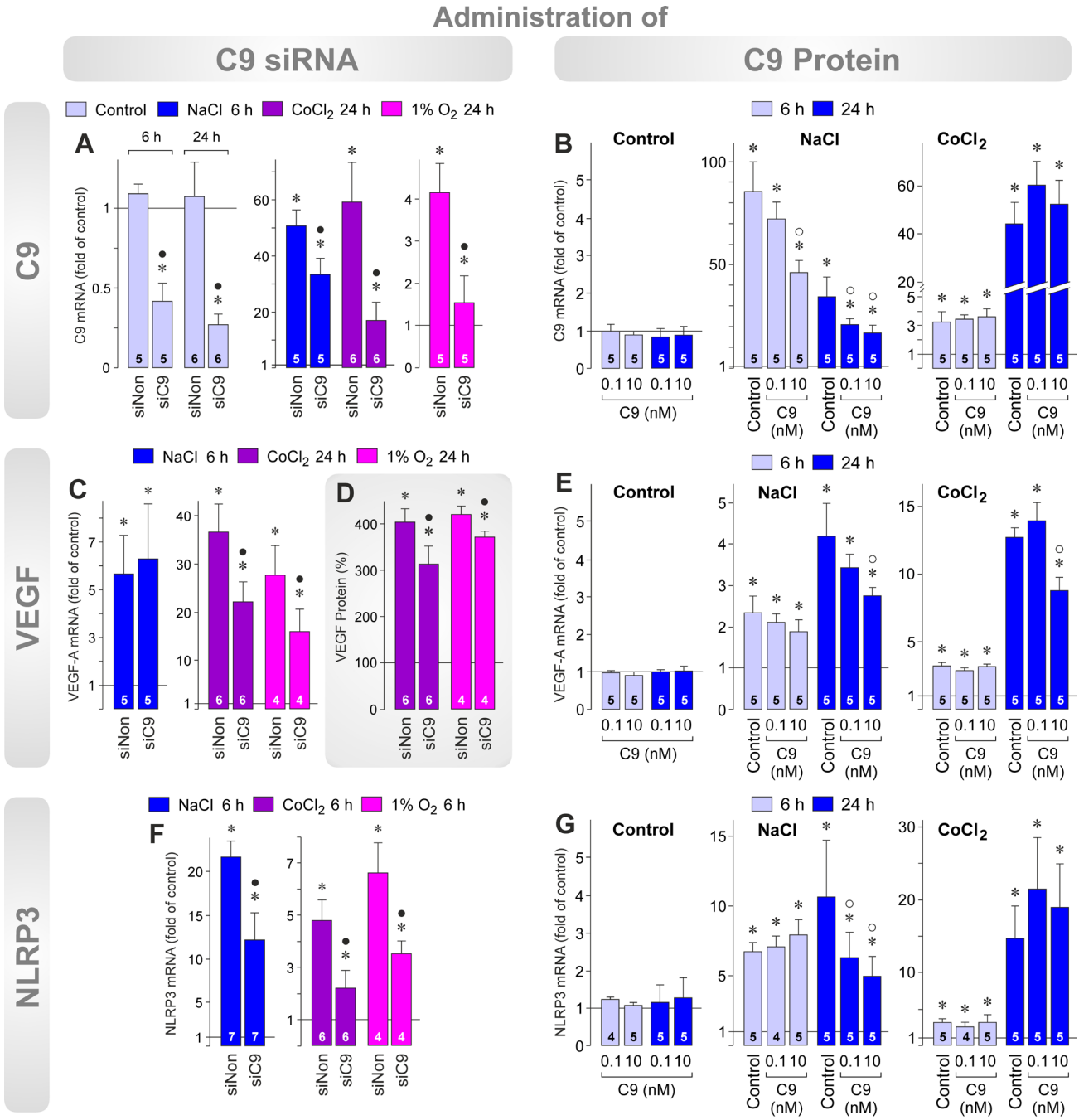


Figure 7. Effects of knockdown of C9 with siRNA (*left side*) and administration of C9 protein (*right side*) on the expression levels of C9 (**A, B**), VEGF (**C, E**), and NLRP3 genes (**F, G**) and on the secretion of VEGF protein (**D**). *Left side*: The cells were transfected with nontargeted siRNA (siNon; 10 nM) and C9 siRNA (siC9; 10 nM). After 48 h, the cells were stimulated with a hyperosmotic medium (+ 100 mM NaCl) for 6 h or an isosmotic medium containing $CoCl_2$ (150 µM) for 6 h or 24 h or were cultured for 6 h or 24 h in 1% $O_2$ (as indicated by the panels of the bars). mRNA levels were determined with real-time RT–PCR analysis and are expressed as folds of unstimulated control. The level of VEGF-$A_{165}$ protein in the cultured media was determined with ELISA and is expressed in percent of unstimulated control (100%). *Right side*: The cells were stimulated with a hyperosmotic medium (+ 100 mM NaCl) or an isosmotic medium containing $CoCl_2$ (150 µM) for 6 h and 24 h in the absence (control) and presence of C9 protein (0.1 and 10 nM, respectively). The numbers of independent experiments using cell lines from different donors are indicated in the bars. Significant difference versus unstimulated control: *p<0.05. Significant difference versus siNon: ●p<0.05. Significant difference versus NaCl and $CoCl_2$ control: ○p<0.05.

effect under control conditions (Figure 8B). The administration of C9 protein also did not alter the cytosolic level of mature IL-1β in cells cultured for 24 h in the presence of the hypoxia mimetic $CoCl_2$ (150 µM; data not shown). The data might be consistent with the assumption that C9 mRNA has a stimulatory effect, whereas exogenous C9 protein has an inhibitory effect on the hyperosmotic activation of the inflammasome in cultured RPE cells.

*Cell viability:* After assembly of the MAC, C9 can polymerize and form a cytolytic pore in the plasma membrane [19]. To investigate whether C9 affects the survival of RPE cells under hyperosmotic and hypoxic conditions, we determined the cell viability in the absence and presence of exogenous C9 protein. The addition of C9 protein under isosmotic control conditions did not alter the viability of the cells after 6 h of stimulation and induced a small decrease in the viability after 24 h of stimulation (Figure 9). As previously described [53], the addition of 100 mM NaCl or of the hypoxia mimetic $CoCl_2$ to the culture medium induced small but significant ($p<0.05$) decreases in the viability of RPE cells (Figure 9). Under both conditions, the administration of C9 protein resulted in further small decreases in cell viability; however, the C9-induced decrease was only significant ($p<0.05$) under high NaCl conditions (Figure 9). The data suggest that C9 protein induces a small decrease in the viability of cultured RPE cells under the conditions used.

## DISCUSSION

In the present study, we determined the effects of various pathogenic conditions on the expression of complement genes in cultured RPE cells. We found that extracellular hyperosmolarity very strongly stimulated transcription of the C9 gene, while the expression of various other complement factor genes was moderately or not at all altered (Figure 1B). C9 gene expression was also increased under hypoxic (Figure 3A,B) and oxidative stress conditions (Figure 3D). Various

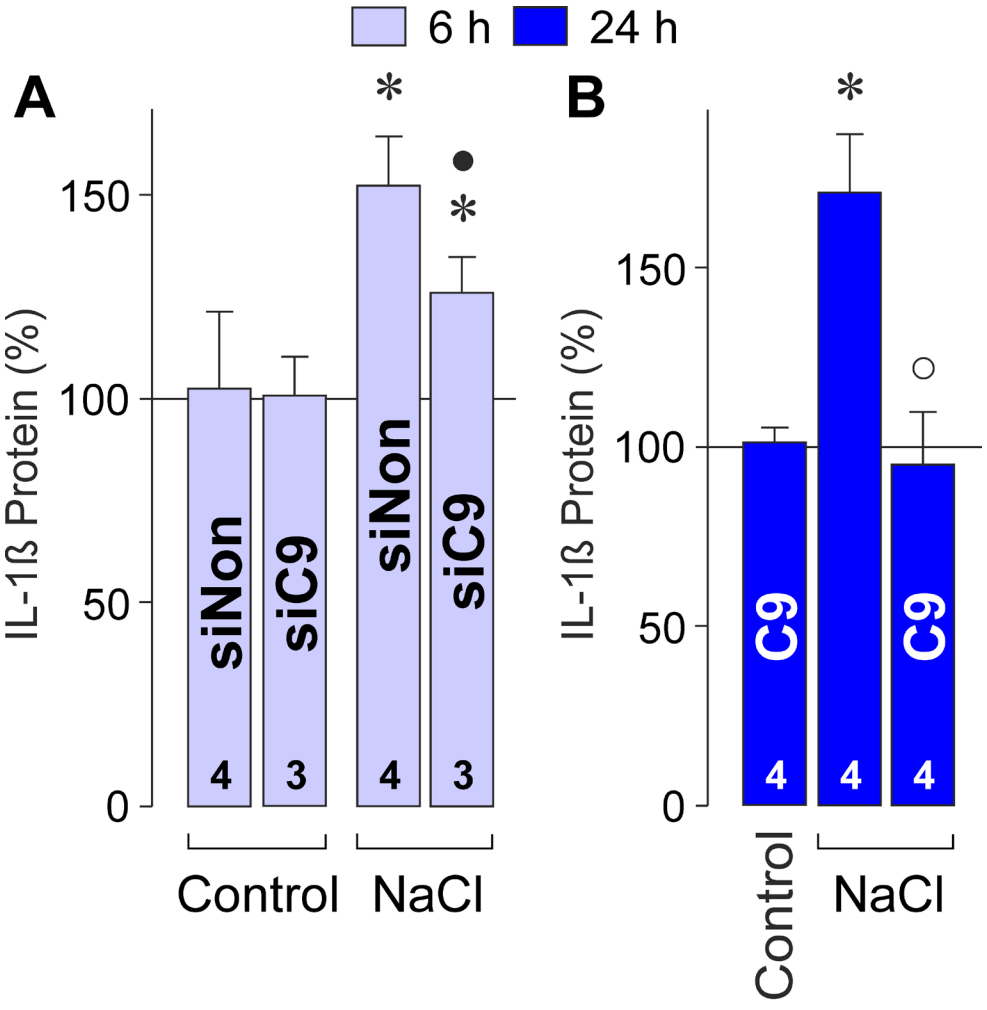


Figure 8. Effects of (**A**) knockdown of C9 with siRNA and (**B**) the administration of C9 protein on the cytosolic level of mature IL-1β. The cells were stimulated with a hyperosmotic medium (+ 100 mM NaCl) for (**A**) 6 h and (**B**) 24 h. The IL-1β level was measured with ELISA and is expressed as percent of unstimulated control (100%). **A**: The cells were transfected with nontargeted siRNA (siNon; 10 nM) and C9 siRNA (siC9; 10 nM). **B**: The cells were cultured in the absence and presence of C9 protein (10 nM). The numbers of independent experiments using cell lines from different donors are indicated in the bars. Significant difference versus unstimulated control: *$p<0.05$. Significant difference versus siNon: ●$p<0.05$. Significant difference versus NaCl control: ○$p<0.05$.

inflammatory, growth, and blood coagulation factors induced either no alteration or a decrease of in C9 gene expression (Figure 3E). The data may support the assumption that the expression of the C9 gene in cultured RPE cells is specifically increased by extracellular hyperosmolarity, hypoxia, and oxidative stress. The present results are in agreement with those of various studies of different cell and tissue systems that showed that free radicals increase the transcription of the C9 gene [54], that ischemia-reperfusion induces upregulation of C9 [55], and that inflammatory cytokines such as TNFα and IL-1β are ineffective in stimulating transcription of the C9 gene [56]. In contrast to the level of C9 mRNA, which increased greatly under hyperosmotic conditions (Figure 1B), the cytosolic level of C9 protein was only moderately increased (Figure 6B,C). Our findings are in line with those of a previous study that found that cultured astrocytes express C9 mRNA upon stimulation and that C9 protein is not detectable in cultured media [57].

We found that various inhibitors of intracellular signal transduction pathways decreased the hyperosmotic but not the hypoxic expression of the C9 gene (Figure 4A,B). This finding could be partially explained by the assumption that the expression of the C9 gene under both conditions is regulated differently by intracellular signal transduction mechanisms. Different activation mechanisms of the C9 gene under both conditions are also suggested by the finding that the increases in the C9 mRNA level under hyperosmotic and hypoxic conditions are additive and display different time dependencies (Figure 3C). The C9 gene transcription under both conditions was decreased by a STAT3 inhibitor (Figure 4A,B); this finding could be consistent with the assumption that the final step of C9 gene activation under both conditions is partially mediated by STAT3. The hyperosmotic transcription of the C9 gene is probably also dependent on the activity of NFAT5 (Figure 5A,C). However, it cannot be ruled out that further intracellular signal transduction pathways and transcription factors not investigated in the present study, for example, HIF-2 [58], contribute to the transcriptional activation of the C9 gene. Another limitation of the data is that it cannot be ruled out that various compounds used may also have unspecific effects on other unrelated proteins.

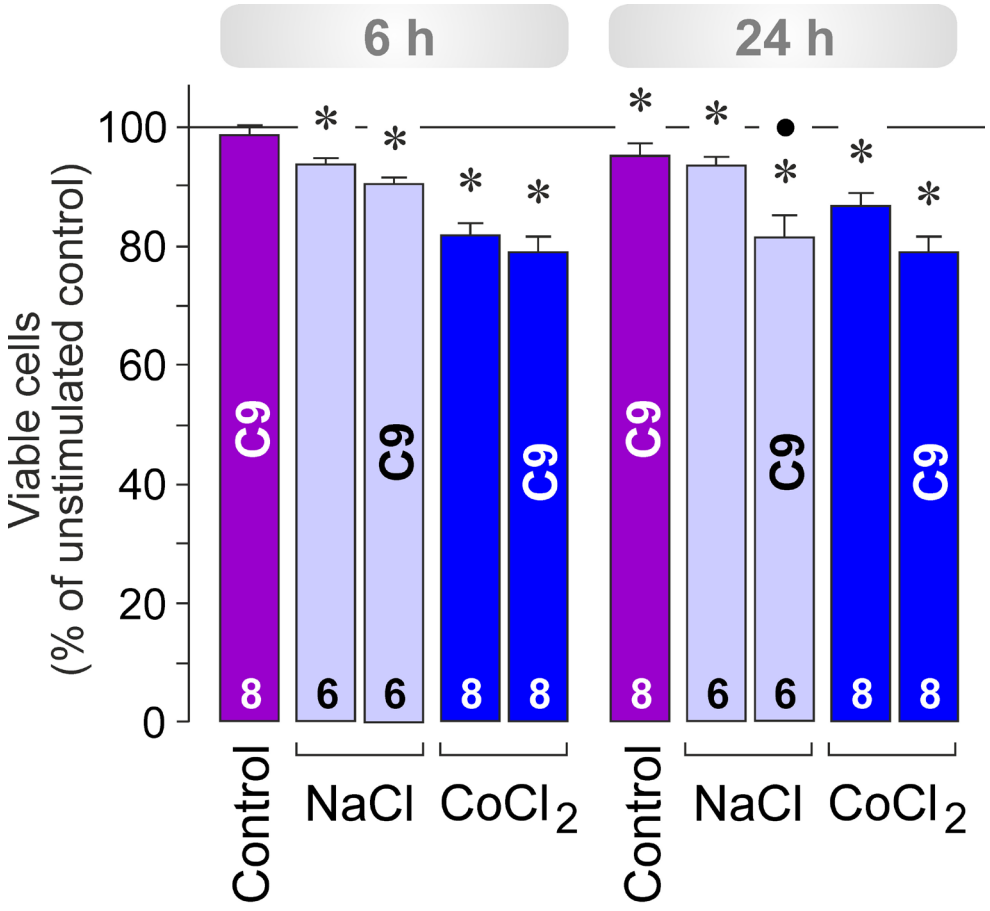


Figure 9. Effects of exogenous C9 protein on the viability of cultured human RPE cells. The cells were stimulated with a hyperosmotic medium (+ 100 mM NaCl) or an isosmotic medium containing $CoCl_2$ (150 µM) for 6 h and 24 h in the absence and presence of C9 protein (10 nM), respectively. The numbers of independent experiments using cell lines from different donors are indicated in the bars. Significant difference versus unstimulated control: *p<0.05. Significant difference versus NaCl control: ●p<0.05.

Extracellular hyperosmolarity, hypoxia, and oxidative stress induced strong increases in the C9 mRNA level in RPE cells (Figure 1B–D and Figure 3A, D). The rather selective activation of the C9 gene in comparison to other complement genes (Figure 1B and Figure 3A, D) could be consistent with the assumption of a functional role of C9 mRNA and/or C9 protein, possibly in regulating the transcription or translation of other proteins. To prove this assumption, we compared the effects of siRNA-mediated knockdown of C9 and the administration of exogenous C9 protein on the expression of key inflammatory (NLRP3) and angiogenic factor (VEGF) genes. We found that these procedures differentially altered the expression of both genes under hyperosmotic and hypoxic conditions. Generally, the data might be consistent with the assumptions that C9 mRNA facilitates the hypoxic expression and secretion of VEGF (Figure 7C,D) and the hyperosmotic and hypoxic expression of the NLRP3 gene (Figure 7F). However, C9 protein inhibited the hyperosmotic expression of VEGF and at higher concentrations and within longer time periods the hypoxic expression of VEGF (Figure 7E). It also reduced the hyperosmotic expression of NLRP3 in longer time periods (Figure 7G). The VEGF-induced downregulation of C9 gene expression (Figure 3E) possibly represents a negative feedback regulation to prevent the overproduction of VEGF. We also found that C9 mRNA and protein affect inflammasome activation under hyperosmotic conditions. The cytosolic level of mature IL-1β (which is produced by the activated inflammasome) is decreased in cells transfected with C9 siRNA (Figure 8A) and in the presence of exogenous C9 protein (Figure 8B). These findings may be consistent with the assumption that C9 mRNA stimulates while exogenous C9 protein inhibits inflammasome activation. The effects on inflammasome activation could be partially explained by the effects of C9 siRNA and C9 protein on NLRP3 mRNA expression (Figure 7F,G), for example, the priming of the NLRP3 inflammasome.

The inhibitory effect of C9 protein on the NLRP3 mRNA level under hyperosmotic conditions (Figure 7G) could partially result from the inhibitory effect of C9 protein on the level of C9 mRNA (Figure 7B). However, C9 protein had no effect on the hypoxic upregulation of C9 mRNA (Figure 7B); this could partially explain the finding that C9 protein did not alter the hypoxic upregulation of NLRP3 gene expression (Figure 7G). However, the findings that C9 protein did not alter the level of C9 mRNA (Figure 7B) but decreased the level of VEGF transcripts under hypoxic conditions (Figure 7E) may suggest that C9 protein induces further signal transduction mechanisms independent of the regulation of C9 gene expression. It cannot be ruled out that some of the effects of C9 protein on RPE cells resulted from MAC formation. We found that the presence of C9 protein was associated with a slight decrease in cell viability (Figure 9), which might be mediated by MAC-mediated pore formation [19]. It has been found that sublytic concentrations of MACs (that cause decreases in cell viability of <5%) may induce the production of proinflammatory factors and VEGF and may induce NLRP3 inflammasome activation [59,60]. However, we found that the addition of C9 protein to the culture medium resulted in the decreased expression of VEGF and NLRP3 genes under certain conditions (Figure 7E,G) and in decreased inflammasome activation (Figure 8B). Perhaps protective responses are activated in the cells that inhibit MAC formation, as previously described [61].

The mechanisms of the effects of the siRNA-mediated knockdown of C9 and of exogenous C9 protein on the expression of VEGF and NLRP3 genes (Figure 7C,E–G) are unclear. siRNA-mediated knockdown of C9 will result in a decrease in the C9 protein level. We found that exogenous C9 protein had no effect or induced decreases of the VEGF and NLRP3 gene expression under various conditions (Figure 7E,G). Therefore, an siRNA-mediated decrease in the C9 protein level should result in increased VEGF and NLRP3 gene expression. However, this was not observed; we found C9 protein-induced decreases of the VEGF and NLRP3 gene expression in siRNA-transfected cells under various conditions (Figure 7C,F). Therefore, we assume that the effects of C9 siRNA cannot be explained by a decrease in the C9 protein level. We also found that under hyperosmotic conditions, the cytosolic level of C9 protein was only moderately increased (Figure 6B,C), while the level of C9 mRNA increased greatly (Figure 1B). It seems conceivable that C9 mRNA itself may have a role in regulating the expression of other genes. It could be that C9 transcripts influence molecules of intracellular signal transduction pathways that regulate the expression of VEGF and NLRP3 genes. The mechanisms of such possible regulation remain to be proven in future investigations.

In summary, we found that various pathogenic conditions (extracellular hyperosmolarity, hypoxia, and oxidative stress) stimulate C9 gene expression in cultured RPE cells. Based on the present data, it is conceivable that C9 gene expression stimulates the expression of inflammatory (NLRP3) and angiogenic growth factors (VEGF) in RPE cells. Extracellular C9 protein may attenuate this effect, in part via negative regulation on the C9 mRNA level. Further research is required to support the assumption of the possible regulatory role of C9 in the expression of VEGF and NLRP3 in RPE cells. It should be kept in mind that the present data were obtained for cultured, dedifferentiated cells and therefore

must be confirmed using differentiated cells or in in vivo experiments.

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