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Osmotic induction of cyclooxygenase-2 in RPE cells: Stimulation of inflammasome activation

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Purpose: Systemic hypertension is a risk factor of age-related macular degeneration, a disease associated with chronic retinal inflammation. The main cause of acute hypertension in the elderly is consumption of dietary salt (NaCl) resulting in increased extracellular osmolarity. The aim of the present study was to determine whether extracellular osmolarity regulates the expression of cyclooxygenase (*COX*) genes in cultured human retinal pigment epithelial (RPE) cells, and whether COX activity is involved in mediating the osmotic expression of key inflammatory (*NLRP3* and *IL1B*) and angiogenic factor (*VEGFA*) genes.
Methods: Extracellular hyperosmolarity was induced by addition of NaCl or sucrose. Gene expression was determined with real-time reverse transcription (RT)–PCR. Cytosolic interleukin-1β (IL-1β) and extracellular vascular endothelial growth factor (VEGF) levels were evaluated with enzyme-linked immunosorbent assay (ELISA).
Results: Extracellular hyperosmolarity induced a dose-dependent increase in *COX2* gene expression when >10 mM NaCl was added to the culture medium, while *COX1* gene expression was increased at higher doses (>50 mM of added NaCl). Extracellular hypo-osmolarity decreased *COX2* gene expression. High extracellular osmolarity also induced increases in the COX2 protein level. NaCl-induced expression of *COX2* was mediated by various intracellular signal transduction molecules (p38 mitogen-activated protein kinase [p38 MAPK], extracellular signal-regulated kinases 1 and 2 [ERK1/2], and phosphatidylinositol-3 kinase [PI3K]), intracellular calcium signaling involving activation of phospholipase Cγ (PLCγ) and protein kinase Cα/β (PKCα/β), and the activity of nuclear factor of activated T cell 5 (NFAT5). Inhibition of fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), and interleukin-1 (IL-1) receptor activities decreased NaCl-induced *COX2* gene expression. Selective inhibition of COX2 activity decreased osmotic expression of the *VEGFA*, *IL1B*, and *NLRP3* genes, and blocked the NaCl-induced increase in the cytosolic IL-1β level.
Conclusions: The expression of *COX2* in RPE cells is osmoresponsive, and depends on NFAT5. COX2 activity stimulates hyperosmotic expression of angiogenic (*VEGFA*) and inflammatory factor (*IL1B* and *NLRP3*) genes, and activation of the NLRP3 inflammasome in RPE cells.

Age-related macular degeneration (AMD) is the most common cause of irreversible loss of central vision and blindness in people aged over 65 years in developed countries [1,2]. Approximately 90% of patients with AMD suffer from the dry form of the disease which is characterized, in the late stage, by geographic atrophy, that is, degeneration of the RPE associated with degeneration of photoreceptors. The remaining patients suffer from the wet form characterized by choroidal neovascularization lesions that later develop into fibrous scars. AMD is associated with systemic and local inflammation [3–5]. Findings of various studies suggested that the nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome is expressed in the RPE of eyes affected by geographic atrophy or wet AMD, that NLRP3 inflammasome activation is implicated in mediating RPE degeneration in geographic atrophy, and that NLRP3 inflammasome activation may also promote choroidal neovascularization [6–9]. Activated inflammasomes mediate the proteolytic activation of caspase-1 that catalyzes the maturation of the inflammatory cytokines interleukin-1β (IL-1β) and IL-18 [10]. Activation of the NLRP3 inflammasome is a two-step process that involves priming, that is, the gene expression and production of NLRP3 and pro-IL-1β proteins, and the assembly of the inflammasome [10].

AMD is a multifactorial disease. In addition to advanced age, race, and genetic factors, lifestyle factors (like sunlight exposure, cigarette smoking, and nutrition) influence the risk of AMD. In addition, systemic hypertension is a risk factor of AMD [11–13]. The main cause of acute hypertension in the elderly is the increase in extracellular osmolarity following intake of dietary salt (NaCl) [14–16]. High extracellular NaCl induces priming and activation of the NLRP3 inflammasome, and stimulates the production of vascular endothelial growth factor (VEGF) in RPE cells [17,18]. VEGF is the main angiogenic factor that promotes the development of choroidal neovascularization [19]. High extracellular NaCl also induces expression of calcium-dependent phospholipase A$_2$ (PLA$_2$)

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isoforms in RPE cells [20]. PLA_2 produces arachidonic acid which is the precursor of prostaglandin synthesis by cyclooxygenases (COXs). Prostaglandins are lipid mediators that play important roles in several biologic processes, including vasodilation, inflammation, immunity, platelet aggregation, and angiogenesis [21]. However, it is not known whether high NaCl also induces expression of COX in RPE cells. It was shown that RPE cells express the constitutive (COX1) and inducible (COX2) isoforms of the enzyme [22]. COX2 was detected in the RPE and vascular endothelial cells in human choroidal neovascularization membranes [23]. Experimental choroidal neovascularization lesions are smaller in COX2-null mice compared to wild-type mice; the reduction is associated with reduced retinal VEGF and IL-1β expression [24]. Inhibition of COX2 activity was shown to inhibit the development of experimental choroidal neovascularization and subretinal fibrosis; the former is mediated by attenuation of macrophage infiltration and downregulation of VEGF in the RPE, and the latter is mediated by downregulation of transforming growth factor-β2 (TGF-β2) [25]. Pharmacological inhibition of COX2 activity decreases the secretion of VEGF and TGF-β2 from mouse RPE cells [25]. The aims of the present study were to determine whether extracellular osmolarity regulates the expression of COX1 and COX2 in cultured human RPE cells, and to investigate whether COX activity is involved in mediating the osmotic expression of key inflammatory (*NLRP3*; Gene ID 114548; OMIM 606416) and angiogenic factor (*VEGF;* Gene ID7422; OMIM 192240) genes. High osmolarity was induced by addition of NaCl (up to 100 mM) to the culture medium. It is generally accepted that the highest pathological blood osmolarity in human subjects is around 360 mosm/kg H_2O [26,27] which can be induced by an increase of the extracellular NaCl concentration by about 40 mM. However, the local extracellular NaCl concentration in the interstitium may be considerably higher (up to 250 mM) than the plasma concentration of NaCl (about 140 mM) [28,29].

METHODS

Human material: The study followed the tenets of the Declaration of Helsinki as well as the ARVO statement for the use of human subjects. The use of human material was approved by the Ethics Committee of the University of Leipzig (approval #745, 07/25/2011). Eyes were obtained from 39 post-mortem Caucasian cornea donors (11 women and 28 men) without reported eye disease within 48 h of death. Written informed consent for the use of retinal cells in basic research was obtained from the relatives of each donor. The age of the donors varied between 19 and 84 years (mean ± standard deviation [SD], 61.7±17.8 years for women, and 60.3±20.3 years for men). There were no statistically significant differences between data obtained in cells from younger and aged donor eyes, and in cells from both sexes (data not shown).

Materials: All cell culture materials were obtained from Gibco BRL (Paisley, UK). Recombinant human basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), heparin-binding epidermal growth factor-like growth factor (HB-EGF), hepatocyte growth factor (HGF), IL-1β, IL-1 receptor antagonist (IL1-RA), pigment epithelium-derived factor (PEDF), platelet-derived growth factor-BB (PDGF), TGF-β1, and VEGF-A_{165} were purchased from R&D Systems (Abingdon, UK). Recombinant human placental growth factor-2 (PlGF-2) was from Reliatech (Braunschweig, Germany). The following compounds were obtained from Calbiochem (Bad Soden, Germany): cyclosporin A, Gö6976, H-89, the hypoxia-inducible transcription factor (HIF)-1 inhibitor, LY294002, human recombinant matrix metalloproteinase-2 (MMP-2), PD98059, PP2, SP600125, SU1498, and U73122. The compounds 666–15, amiloride, caffeic acid phenethyl ester (CAPE), GSK650394, NS-398, SB203580, and SR11302 were purchased from Tocris (Ellisville, MO). Ac-YVAD-CMK, AG1478, and Stattic were from Enzo Life Science (Lausen, Switzerland). Dithiothreitol was from Carl Roth (Karlsruhe, Germany), and PD173074 was kindly provided by Pfizer (Karlsruhe, Germany). Human-specific small interfering RNA (siRNA) against nuclear factor of activated T cell 5 (NFAT5) and nontargeted control siRNA were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). AG1296, BAPTA/AM, 1,10-phenanthroline, SB431542, triamcinolone acetonide, VU0285655–1, VU0359595, and all other agents used were from Sigma-Aldrich (Taufkirchen, Germany), unless stated otherwise. The following antibodies were used: rabbit anti-human β-actin (1:1,000; Cell Signaling, Frankfurt, Germany), rabbit anti-COX2 (1:1,000; Cell Signaling), and anti-rabbit immunoglobulin G (IgG) conjugated with alkaline phosphatase (1:2,000; Cell Signaling).

Cell culture: Preparation and culture of RPE cells were described previously [30]. Briefly, the vitreous and the retina were removed from the eyeballs, and the RPE cells were mechanically harvested. After separation of the cells by digestion with 0.05% trypsin and 0.02% EDTA, and dual washing with PBS (Invitrogen, Paisley, UK; 1X; 155 mM NaCl, 1.54 mM KH_2PO_4, 2.71 mM Na_2HPO_4-$7H_2O$, pH7.2) the cells were suspended in complete Ham F-10 medium that contained 10% fetal bovine serum, GlutaMAX II, and penicillin/streptomycin. The cells were cultured in tissue culture flasks (Greiner, Nürtingen, Germany) in 95% air/5% CO_2 at

37 °C. The epithelial nature of the RPE cells was routinely identified with immunocytochemistry using the monoclonal antibodies AE1 (recognizing most of the acidic type I keratins) and AE3 (recognizing most of the basic type II keratins), both from Chemicon (Hofheim, Germany). Approximately 98% of the cultured cells were also vimentin-positive, as previously shown [17,20], indicating sufficient purity of the cell cultures. Addition of 100 mM NaCl to the culture medium induced a decrease in cell viability by 5–8% after 6 and 24 h of stimulation [31]. The pharmacological agents used did not statistically significantly ($p>0.05$) alter the viability of the cells (data not shown).

Cell lines of passages 3–5 were used. When a confluency of about 90% was achieved after 4 days of cultivation, the cells were cultured in serum-free medium for 16 h. During this period, the cultures reached 100% confluency. Thereafter, test substances were added to the serum-free medium. Extracellular hyperosmolarity was induced with the addition of NaCl or sucrose to the culture medium. A decrease in extracellular osmolarity to 60% of control was achieved with the addition of distilled water to the medium. Hypoxia was induced with the addition of the hypoxia mimetic $CoCl_2$ (150 µM) or with cell culture in 1% O_2 atmosphere. Pharmacological inhibitors were preincubated for 30 min.

RNA extraction and cDNA synthesis: Total RNA was extracted with the InviTrap Spin Universal RNA Mini Kit (Stratec Molecular, Berlin, Germany). The A_{260}/A_{280} ratio of the optical density of the RNA samples (measured with NanoDrop1000; peQLab, Erlangen, Germany) was between 1.95 and 2.05, indicating adequate RNA quality. The RNA samples were treated with DNase I, and cDNA was synthesized from 0.5 µg RNA with a reverse transcription kit (ThermoFisher Scientific).

Real-time reverse transcription PCR: Real-time reverse transcription PCR (RT–PCR) was performed with the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Munich, Germany). The primer sequences are given in Table 1. The amplification reaction mixture (15 µl) consisted of 7.5 µl of 2×iQ SYBR Green Supermix (Bio-Rad), a specific primer set (0.2 µM each), and 1 µl (1.25 ng) cDNA. The following protocol was used: one cycle of denaturation at 95 °C for 3 min, 45 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 20 s, extension at 72 °C for 45 s, and a melting curve at 55 °C with the temperature gradually increased for 0.5 °C up to 95 °C. To prove the correct lengths of the PCR products, the samples were analyzed with agarose gel electrophoresis. RT–PCR for β-actin mRNA was used as an internal control. The results were analyzed with the $2^{-\Delta\Delta CT}$ method.

Western blotting: The cells were seeded at 5×10^5 cells per well in six-well plates, and were cultured in fetal bovine serum (10%)-containing F-10 medium. When a confluency of 80–90% was achieved, the cells were growth arrested for 16 h in serum-free medium. Thereafter, NaCl (100 mM),

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

Gene / Accession number	Gene ID	OMIM number	Primer sequences (5′→3′)	Product (bp)
ACTIN NM_001101	60	102630	s ATGGCCACGGCTGCTTCCAGC as CATGGTGGTGCCGCCAGACAG	237
COX1 NM_080591.2	5742	176805	s TTGACCGCTACCAGTGTGAC as ACGGATAAGGTTGGAGCGCACT	221
COX2 NM_000963.3	5743	600262	s TGAGCATCTACGGTTTGCTG as TGCTTGTCTGGAACAACTGC	158
IL1B NM_000576.2	3553	147720	s TGGGCCTCAAGGAAAAGAATC as CTTTCTGTTCCCTTTCTGCCA	358
NLRP3 NM_183395.2	114548	606416	s AGACAGCATTGAAGAGGAGTGG as TTTGTTGAGGCTCACACTCTCA	169
TGFB2 NM_001135599.2	7042	190220	s ACGTCTCAGCAATGGAGAAGA as ATTCGCCTTCTGCTCTTGTTT	195
SCNN1A NM_001038.5	6337	600228	s TACCAGCTCTCTGCTGGTTACTC as GAGGGAGACTCAGAATTGGTTTT	173
$\text{VEGFA}_{188, 164, 120}$ NM_003376.5 NM_001287044.1 NM_001025370.2	7422	192240	s CCTGGTGGACATCTTCCAGGAGTA as CTCACCGCCTCGGCTTGTCACA	479; 407; 275

sucrose (200 mM), or $CoCl_2$ (150 µM) was added for further 6 or 24 h. After medium was removed, the cells were washed twice with prechilled PBS (pH 7.4; Invitrogen, Paisley, UK) and scraped into 180 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 1% phosphatase inhibitor cocktail). The cell lysates were centrifuged at 20,124 ×*g* for 10 min. Equal amounts of cytosolic protein (40 µg) were separated with 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Immunoreactive bands were probed with primary and secondary antibodies, and visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

Enzyme-linked immunosorbent assay: Cells were seeded at 15 × 10^3 cells per well in 12-well plates. After reaching a confluency of about 90%, the medium was changed to a serum-free medium for 16 h. NaCl (100 mM) was added to the serum-free medium for 6 or 24 h. The levels of VEGF-A_{165} in the cultured media (100 µl) and of mature IL-1β in the cell lysates (150 µl) were quantified with enzyme-linked immunosorbent assay (ELISA; R&D Systems).

Small interfering RNA transfection: Cells were seeded at 7 × 10^4 cells per well in 12-well plates. When confluency of 60–80% was achieved, NFAT5 siRNA or nontargeted siRNA (5 nM each) was transfected into the cells with HiPerfect reagent (Qiagen, Hilden, Germany) in serum (10%)-containing F-10 medium. After 48 h, the cells were cultured for 2 h in serum-free medium. Thereafter, the cells were cultured for 6 h in serum-free iso- or hyperosmotic medium (+ 100 mM NaCl). After RNA extraction, the level of *COX2* (Gene ID 5743; OMIM 600262) mRNA was evaluated with real-time RT–PCR.

Statistical analysis: Each test involved at least three experiments with cell lines of different donors. Data are shown as mean ± standard error of the mean (SEM). Statistical analysis was performed with Prism (GraphPad Software, San Diego, CA). Comparisons between groups were performed with one-way ANOVA followed by Bonferroni's multiple comparison test and the Mann–Whitney *U* test. A p value of less than 0.05 was considered statistically significant.

RESULTS

Regulation of COX1 and COX2 gene expression: Cultured human RPE cells contained *COX1* (Gene ID 5742; OMIM 176805) and *COX2* transcripts (Figure 1A). To investigate whether the expression of *COX* genes is regulated by extracellular osmolarity, we stimulated the cells with hyper- and hypoosmotic media. Hyperosmotic media were made up with the addition of NaCl or sucrose. As shown in Figure 1B, the addition of 100 mM NaCl to the culture medium induced time-dependent increases in the expression of the *COX1* and *COX2* genes. NaCl-induced expression of the *COX1* gene developed slowly and peaked after 24 h of stimulation, whereas that of the *COX2* gene developed rapidly and peaked after 6 h of stimulation (Figure 1B). NaCl-induced expression of both genes was dose-dependent. *COX2* gene expression increased when more than 10 mM NaCl was added to the culture medium (Figure 1C, D). *COX1* gene expression increased at higher doses, after the addition of more than 50 mM NaCl to the medium (Figure 1C, D). Cell culture in a hypoosmotic medium was associated with a decrease in *COX2* gene expression (Figure 1E). The addition of 200 mM sucrose to the culture medium (which produced the same increase in osmolarity as 100 mM NaCl) also induced an increase in the *COX2* gene expression (Figure 1F). However, although the *COX2* gene expression level was similar after 2 h of stimulation with 100 mM NaCl and 200 mM sucrose, the level was lower after 6 and 24 h of stimulation with 200 mM sucrose, compared to that after the addition of 100 mM NaCl (Figure 1B, F). The data suggest that the early expression of *COX2* in RPE cells depends on the extracellular osmolarity. After longer time periods (6 and 24 h) of hyperosmotic stimulation, *COX2* gene expression depends on the increase in extracellular osmolarity and alteration of the transmembrane NaCl gradient. Expression of the *COX1* gene at higher NaCl doses (Figure 1D) may suggest that *COX1* in RPE cells is normally not regulated by extracellular osmolarity.

COX2 gene expression also increased under hypoxic conditions (Figure 1G). It was described that *COX2* gene expression in RPE cells is induced by growth factors and proinflammatory cytokines [21,22,32]. We found that various growth factors (EGF, HB-EGF, bFGF, TGF-β1, HGF, and VEGF) and IL-1β induced moderate increases in *COX2* gene expression with different time-dependencies (Figure 1H). The strongest increases in *COX2* gene expression were found in response to PDGF, TGF-β1, and IL-1β (Figure 1H).

NaCl-induced regulation of COX2 protein expression: Expression of the *COX2* gene was increased under high-NaCl conditions (Figure 1B–D). To determine whether NaCl-induced extracellular hyperosmolarity also induces an increase in the COX2 protein level in RPE cells, cell lysates were analyzed with western blotting. The COX2 protein level was low in cells cultured under unstimulated control conditions (Figure 2A). The addition of 100 mM NaCl to the culture medium induced a statistically significant ($p<0.05$) increase in the cytosolic level of the COX2 protein; this increase was observed after 6 and 24 h of stimulation (Figure 2A,B). The COX2 protein level was also increased when 200 mM sucrose was added to the culture medium (Figure 2A,B), suggesting

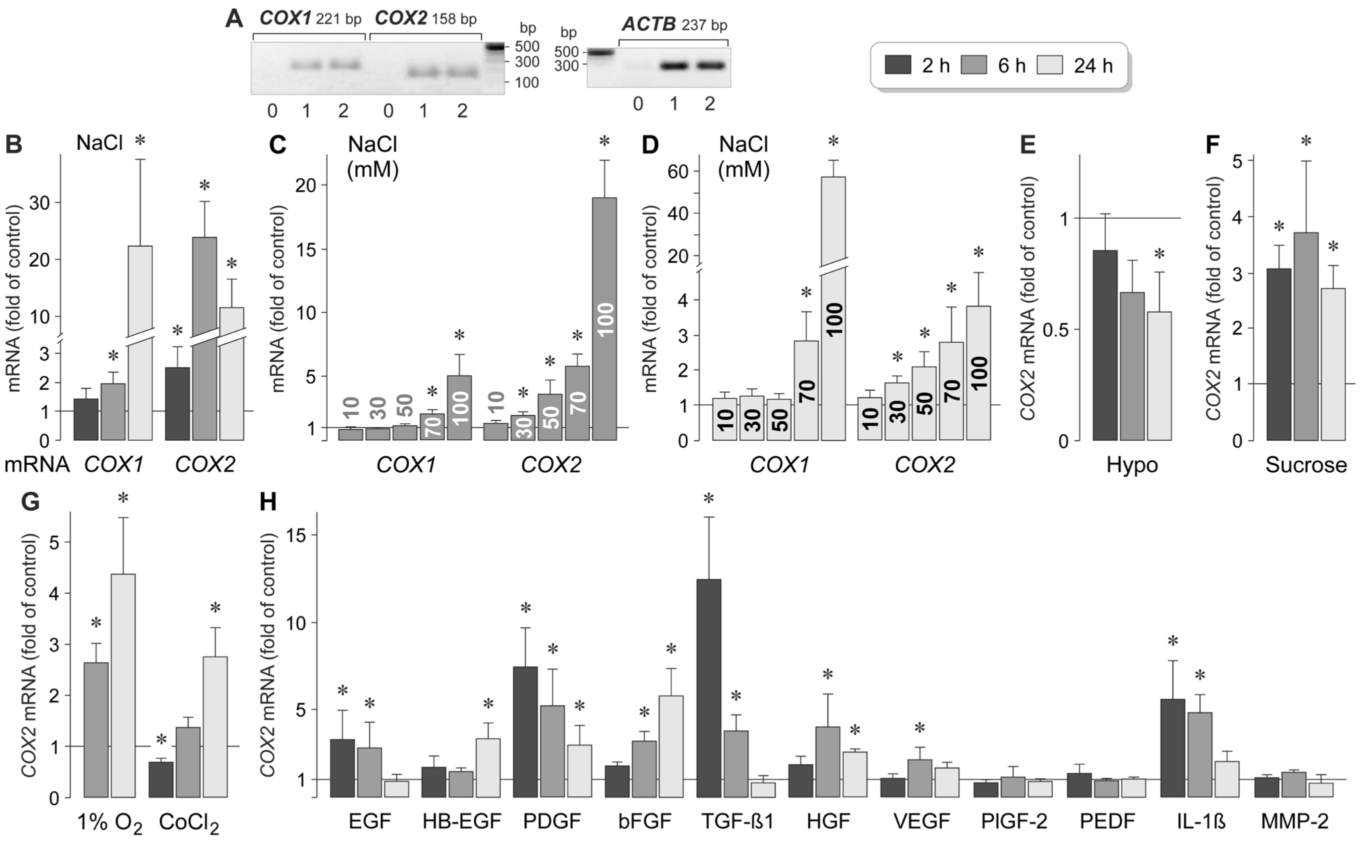

Figure 1. Regulation of cyclooxygenases -1 and -2 (*COX1* and *COX2*) gene expression in retinal pigment epithelial (RPE) cells. **A**: Presence of *COX1* and *COX2* transcripts in RPE cells. To confirm the correct lengths of the PCR products, agarose gel electrophoresis was performed using products obtained from two RPE cell lines (1, 2) derived from different post-mortem donors. Negative controls (0) were done by adding double-distilled water instead of cDNA as the template. The β-actin (*ACTB*) mRNA level was used to normalize the *COX1* and *COX2* mRNA levels. **B**–**H**: *COX1* and *COX2* mRNA levels, as determined with real-time reverse transcription (RT)–PCR after stimulation of the cells for 2, 6, and 24 h (as indicated by the panels of the bars). The mRNA levels are expressed as folds of unstimulated control. **B**: Effects of extracellular hyperosmolarity induced by addition of high (+ 100 mM) NaCl on the *COX1* and *COX2* gene expression levels. **C, D**: Dose-dependencies of the effects of high extracellular NaCl on the *COX1* and *COX2* mRNA levels. Ten to 100 mM NaCl were added to the culture medium, as indicated in the bars. The data were obtained after stimulation of the cells for 6 h (**C**) and 24 h (**D**). **E**: Effect of extracellular hypo-osmolarity (Hypo; 60% osmolarity) on the *COX2* gene expression level. **F**: Effect of the addition of 200 mM sucrose on the *COX2* gene expression level. **G**: Effects of hypoxia induced by cell culture in 1% O_2, and by addition of 150 µM $CoCl_2$, on the *COX2* gene expression level. **H**: Effects of inflammatory and growth factors on the expression of *COX2*. The following factors were tested: endothelial growth factor (EGF), heparin-binding epidermal growth factor-like growth factor (HB-EGF), platelet-derived growth factor-BB (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), placental growth factor-2 (PlGF-2), pigment epithelium-derived factor (PEDF), interleukin-1β (IL-1β), and matrix metalloproteinase-2 (MMP-2). Each factor was applied at 10 ng/ml. Each bar represents data obtained in three to ten independent experiments with cell lines from different donors. Each experiment was performed with cell lines from three to six donors; in total, cell lines from 21 different donors were used for all experiments shown. Statistically significant difference versus unstimulated control: *p<0.05.

that RPE cells produce the COX2 protein in response to extracellular hyperosmolarity. In the presence of the hypoxia mimetic $CoCl_2$ [33], the cytosolic level of COX2 protein was increased after 6 h, and returned to the control level after 24 h of stimulation (Figure 2A,B).

Intracellular signaling involved in mediating NaCl-induced expression of the COX2 gene: To investigate the intracellular signaling involved in mediating NaCl-induced expression of the *COX2* gene in RPE cells, we tested pharmacological blockers of key intracellular signal transduction molecules in cultures that were stimulated 6 h with high (+ 100 mM) NaCl. As shown in Figure 3, expression of the *COX2* gene under unstimulated control conditions was statistically significantly (p<0.05) increased in the presence of inhibitors

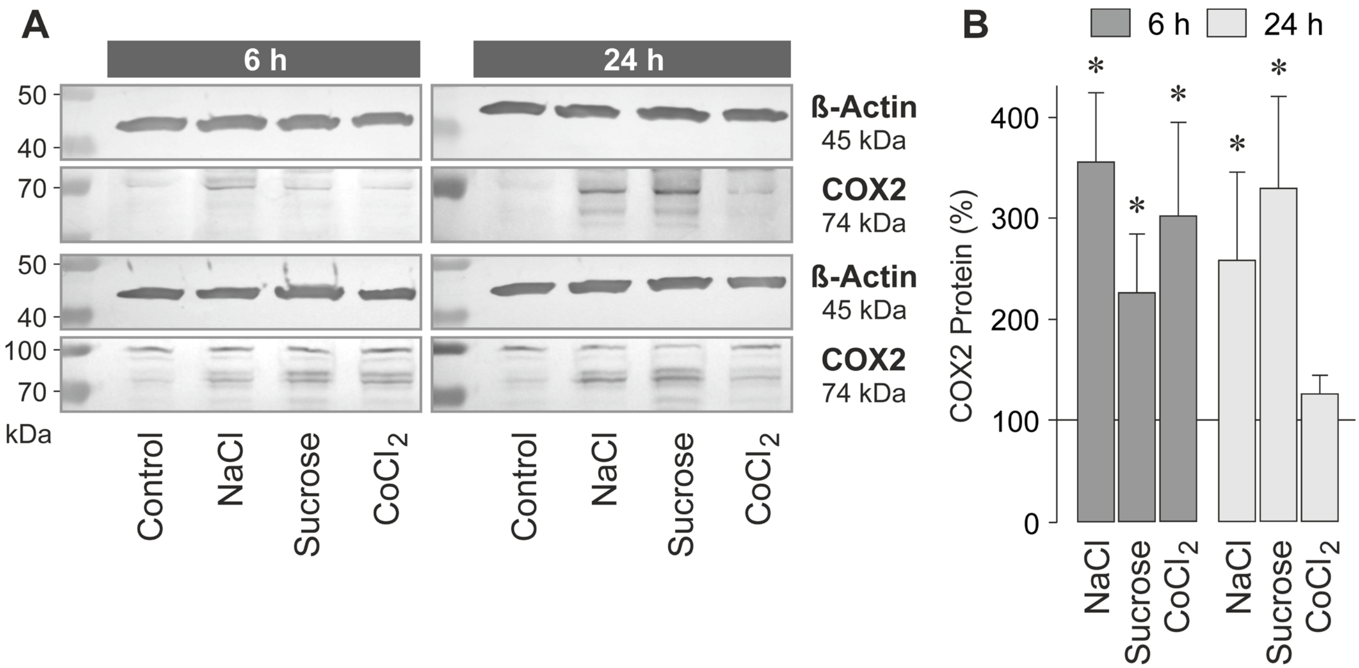

Figure 2. Regulation of cyclooxygenase-2 (COX2) protein expression in retinal pigment epithelial (RPE) cells. The cells were stimulated for 6 and 24 h with high (+ 100 mM) NaCl, 200 mM sucrose, and the hypoxia mimetic $CoCl_2$ (150 µM), respectively. Protein levels were determined with western blotting of cell lysates. **A:** Examples of western blotting of two cell lines (*above* and *below*). The levels of the β-actin and COX2 proteins are shown. **B:** Mean ± standard error of the mean (SEM) cytosolic level of the COX2 protein. The data are normalized to the level of β-actin protein, and are expressed as a percent of the unstimulated control (100%). Each bar represents data obtained in four to five independent experiments with cell lines from different donors. Each experiment was performed with cell lines from four to five donors. Statistically significant difference versus unstimulated control: *p<0.05.

of extracellular signal-regulated kinases 1 and 2 (ERK1/2) activation (PD98059), c-Jun NH_2-terminal kinases (JNKs; SP600125), and phospholipase Cγ (PLCγ; U73122). These findings may suggest that activation of the ERK1/2 and JNK signal transduction cascades, as well as intracellular calcium signaling, inhibits the constitutive expression of the *COX2* gene in RPE cells. In addition, the selective COX2 antagonist NS-398 [34] increased statistically significantly ($p<0.05$) expression of the *COX2* gene under unstimulated control conditions (Figure 3). This finding may suggest that prostaglandins exert negative feedback regulation on the constitutive *COX2* gene expression.

COX2 gene expression under high-NaCl conditions was statistically significantly ($p<0.05$) decreased by inhibitors of the activation of p38 mitogen-activated protein kinase (p38 MAPK; SB203580), ERK1/2 (PD98059), and phosphatidylinositol-3 kinase (PI3K)-related kinases (LY294002; Figure 3). NaCl-induced *COX2* gene expression was also decreased by the cell-permeable calcium chelator BAPTA/AM, the PLCγ inhibitor U73122, and the inhibitor of protein kinase Cα/β (PKCα/β), Gö6976 (Figure 3). Inhibitors of JNKs (SP600125), the phospholipase D1 (PLD1; VU0359595), the PLD2 (VU0285655–1), the serum and glucocorticoid-regulated kinase (SGK; GSK650394), protein kinase A (PKA; H-89), Src tyrosine kinases (PP2), and COX2 (NS-398) did not alter the cellular level of *COX2* transcripts under high-NaCl conditions (Figure 3). The finding that the caspase-1 inhibitor Ac-YVAD-CMK had no effect (Figure 3) suggests that NaCl-induced *COX2* gene expression did not depend on inflammasome activation. NaCl-induced *COX2* gene expression was also not altered in the presence of the reducing agent dithiothreitol and the inhibitor of mitochondrial permeability transition, cyclosporin A (Figure 3). These findings suggest that oxidative stress and a loss of mitochondrial integrity are not involved in mediating NaCl-induced *COX2* gene expression. The data may suggest that NaCl-induced *COX2* gene expression in RPE cells depends on activation of various signal transduction cascades (p38 MAPK, ERK1/2, and PI3K), and on intracellular calcium signaling that involves the activation of PLCγ and PKCα/β. We also found that the anti-inflammatory glucocorticoid triamcinolone acetonide decreased statistically significantly ($p<0.05$) the level of *COX2* transcripts under high-NaCl conditions (Figure 3).

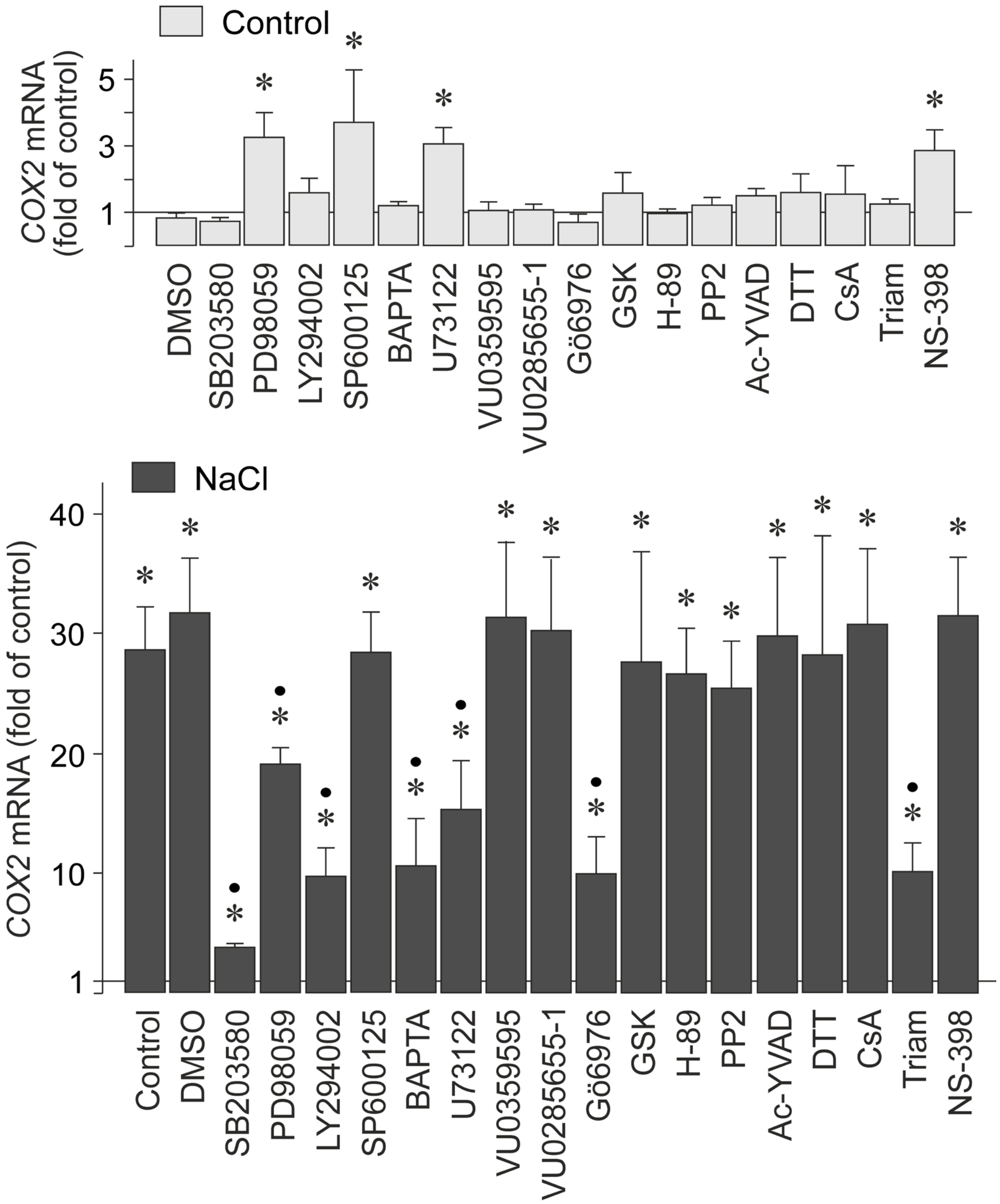

Figure 3. Intracellular signaling involved in mediating NaCl-induced expression of cyclooxygenase-2 (*COX2*) in retinal pigment epithelial (RPE) cells. The mRNA level was determined with real-time reverse transcription (RT)–PCR in cells cultured 6 h in iso- (control) and hyperosmotic (+ 100 mM NaCl) media, and is expressed as folds of the unstimulated control. The following compounds were tested: the inhibitor of p38 mitogen-activated protein kinase (p38 MAPK) activation, SB203580 (10 µM), the inhibitor of extracellular signal-regulated kinases 1 and 2 (ERK1/2) activation, PD98059 (20 µM), the inhibitor of phosphatidylinositol-3 kinase (PI3K)-related kinases, LY294002 (5 µM), the c-Jun NH_2-terminal kinase (JNK) inhibitor SP600125 (10 µM), the cell-permeable calcium chelator BAPTA/AM (5 µM), the phospholipase Cγ (PLCγ) inhibitor U73122 (4 µM), the phospholipase D1 (PLD1) inhibitor VU0359595 (150 nM), the phospholipase D2 (PLD2) inhibitor VU0285655–1 (500 nM), the inhibitor of protein kinase Cα/β (PKCα/β), Gö6976 (1 µM), the serum and glucocorticoid-regulated kinase (SGK) inhibitor GSK650394 (GSK; 1 µM), the protein kinase A inhibitor H-89 (1 µM), the inhibitor of Src tyrosine kinases, PP2 (100 nM), the caspase-1 inhibitor Ac-YVAD-CMK (500 nM), the reducing agent dithiothreitol (DTT; 3 mM), the inhibitor of mitochondrial permeability transition, cyclosporin A (CsA; 1 µM), the anti-inflammatory glucocorticoid triamcinolone acetonide (Triam; 50 µM), and the COX2 antagonist NS-398 (50 µM). Each bar represents data obtained in three to 20 independent experiments with cell lines from different donors. Each experiment was performed with cell lines from three to seven donors; in total, cell lines from 26 different donors were used for all experiments shown. Statistically significant difference versus unstimulated control: *$p<0.05$. Statistically significant difference versus NaCl control: ●$p<0.05$.

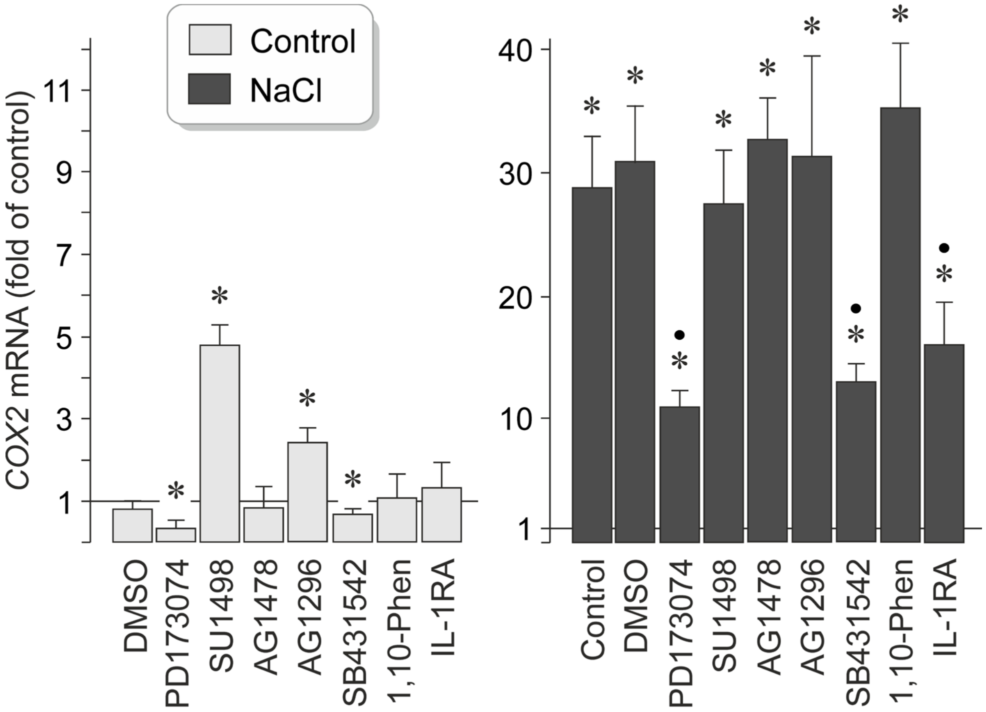

Figure 4. Receptor-mediated signaling involved in mediating NaCl-induced expression of cyclooxygenase-2 (*COX2*) in retinal pigment epithelial (RPE) cells. The mRNA level was determined with real-time reverse transcription (RT)–PCR in cells cultured 6 h in iso- (control) and hyperosmotic (+ 100 mM NaCl) media, and is expressed as folds of the unstimulated control. The following agents were tested: the inhibitor of the fibroblast growth factor (FGF) receptor kinase, PD173074 (500 nM), the blocker of vascular endothelial growth factor (VEGF) receptor-2, SU1498 (10 µM), the blocker of the endothelial growth factor (EGF) receptor tyrosine kinase, AG1478 (600 nM), the inhibitor of the platelet-derived growth factor (PDGF) receptor tyrosine kinase, AG1296 (10 µM), the inhibitor of transforming growth factor-β1 (TGF-β1) superfamily activin receptor-like kinase receptors, SB431542 (10 µM), the broad-spectrum matrix metalloproteinase inhibitor 1,10-phenanthroline (1,10-Phen; 10 µM), and a recombinant human interleukin-1 receptor antagonist (IL-1RA; 1 µg/ml). Vehicle control was made with dimethyl sulfoxide (DMSO; 1:1000). Each bar represents data obtained in three to 24 independent experiments with cell lines from different donors. Each experiment was performed with cell lines from three to seven donors; in total, cell lines from 23 different donors were used for all experiments shown. Statistically significant difference versus unstimulated control: *p<0.05. Statistically significant difference versus NaCl control: ●p<0.05.

Extracellular signaling involved in mediating NaCl-induced expression of the COX2 gene: High extracellular NaCl has been shown to induce the release of growth factors, like VEGF, bFGF, and TGF-β1, from RPE cells [17,35]. Because various growth factors induced expression of the *COX2* gene in RPE cells (Figure 1H), we determined whether autocrine or paracrine growth factor receptor signaling is required for NaCl-induced *COX2* gene expression. As shown in Figure 4, NaCl-induced *COX2* gene expression was statistically significantly (p<0.05) decreased in the presence of inhibitors of the FGF receptor kinase (PD173074), and of TGF-β1 superfamily activin receptor-like kinase receptors (SB431542). Inhibitors of the VEGF receptor-2 (SU1498), the EGF receptor tyrosine kinase (AG1478), and the PDGF receptor tyrosine kinase (AG1296) did not have an effect (Figure 4). In addition, the broad-spectrum matrix metalloproteinase inhibitor 1,10-phenanthroline had no effect (Figure 4), suggesting that shedding of growth factors from the extracellular matrix is not involved in mediating NaCl-induced expression of the *COX2* gene. We also found that the recombinant human IL-1 receptor antagonist statistically significantly (p<0.05) decreased NaCl-induced *COX2* gene expression (Figure 4). This finding may suggest that NaCl-induced extracellular hyperosmolarity induces the release of IL-1β from RPE cells which activates IL-1 receptors in an autocrine or paracrine manner.

Transcription factor activity that mediates NaCl-induced expression of the COX2 gene: High extracellular NaCl has been shown to induce expression and activation of various transcription factors in RPE cells, including HIF-1α, nuclear factor (NF)-κB, activator protein-1 (AP-1), and NFAT5 [17,20,36]. Pharmacological blockers were used to determine which transcription factors mediate NaCl-induced expression of the *COX2* gene in RPE cells. As shown in Figure 5A, *COX2* gene expression under control and high-NaCl conditions was not altered in the presence of inhibitors of HIF-1 [37], signal transducer and activator of transcription 3 (STAT3) [38], NF-κB [39], AP-1, and cAMP response element-binding protein (CREB).

In various cell systems, cellular survival under hyperosmotic conditions depends on the transcriptional activity of NFAT5 [40]. In RPE cells, high extracellular NaCl induces NFAT5 gene and protein expression, and DNA binding of NFAT5 [17]. To investigate whether NFAT5 activity is involved in mediating NaCl-induced expression of the *COX2* gene in RPE cells, NFAT5 was knocked down by transfection of the cells with NFAT5 siRNA. As negative control, nontargeted scrambled siRNA was used. It was shown that transfection with NFAT5 siRNA reduces the level of NFAT5 transcripts by about 50% in RPE cells cultured under control and high-NaCl conditions, whereas transfection with nontargeted siRNA has no effect [17]. Cells transfected with NFAT5 siRNA also contained a statistically significantly ($p<0.05$) lower level of *COX2* transcripts under high-NaCl conditions than cells transfected with nontargeted siRNA (Figure 5B). The data may suggest that NaCl-induced expression of the *COX2* gene in RPE cells is (at least in part) mediated by the activity of NFAT5.

COX2 activity involved in mediating NaCl-induced expression of angiogenic and inflammatory factors: Previous studies suggested that COX2 activity plays a role in inducing experimental choroidal neovascularization and subretinal fibrosis, via stimulation of the retinal expression of VEGF, IL-1β, and TGF-β2 [24,25]. We used the selective COX2 antagonist NS-398 [34] to investigate whether COX2 activity contributes to NaCl-induced expression of these factors in cultured human RPE cells. It was shown that the expression of *VEGFA*, *IL1B* (Gene ID 3553; OMIM 147720), and *TGFB2* (Gene ID 7042; OMIM 190220), as well as the *NLRP3* gene, is increased in RPE cells in response to high NaCl [17,18,35]. As shown in Figure 6, inhibition of COX2 activity with NS-398 decreased statistically significantly ($p<0.05$) NaCl-induced expression of the *VEGFA*, *NLRP3*, and *IL1B* genes, and had no effect on NaCl-induced expression of the *TGFB2* gene. NS-398 also suppressed the constitutive expression of the *IL1B* gene (Figure 6). As previously described [17,18], the anti-inflammatory glucocorticoid triamcinolone acetonide decreased statistically significantly ($p<0.05$) NaCl-induced expression of the *VEGFA* gene, and had no effect on NaCl-induced expression of the *NLRP3* gene (Figure 6). Triamcinolone also decreased statistically significantly ($p<0.05$) NaCl-induced expression of the *IL1B* and *TGFB2* genes (Figure 6).

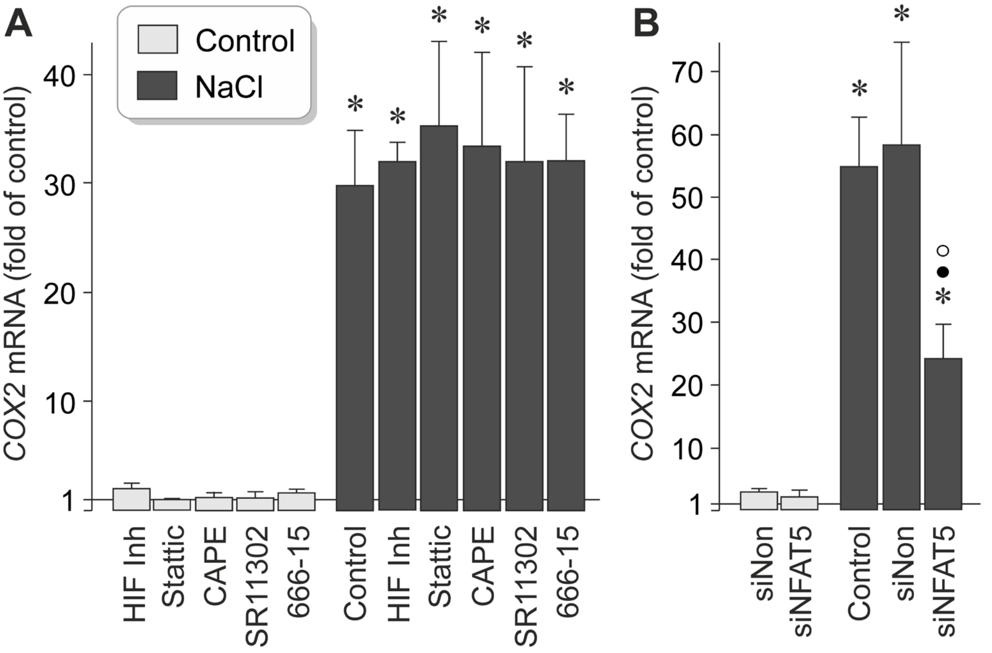

Figure 5. Transcription factor activity involved in mediating NaCl-induced expression of cyclooxygenase-2 (*COX2*) in retinal pigment epithelial (RPE) cells. The mRNA level was determined with real-time reverse transcription (RT)–PCR in cells cultured 6 h in iso- (control) and hyperosmotic (+ 100 mM NaCl) media, and is expressed as folds of the unstimulated control. **A:** The following compounds were tested: a hypoxia-inducible transcription factor (HIF)-1 inhibitor (HIF-Inh; 5 µM), the signal transducer and activator of transcription 3 (STAT3) inhibitor Stattic (1 µM), the nuclear factor (NF)-κB inhibitor CAPE (5 µM), the activator protein-1 (AP-1) inhibitor SR11302 (5 µM), and the cAMP response element-binding protein (CREB) inhibitor 666–15 (250 nM). **B:** Knocking down nuclear factor of activated T cell 5 (NFAT5) with small interfering RNA (siRNA) reduced the level of *COX2* mRNA under hyperosmotic conditions. Nontargeted siRNA (siNon) had no effects. Each bar represents data obtained in three to nine independent experiments with cell lines from different donors. Each experiment was performed with cell lines from three to six donors; in total, cell lines from 21 different donors were used for all experiments shown. Statistically significant difference versus unstimulated control: *$p<0.05$. Statistically significant difference versus NaCl control: ●$p<0.05$. Statistically significant difference versus nontargeted siRNA: ○$p<0.05$.

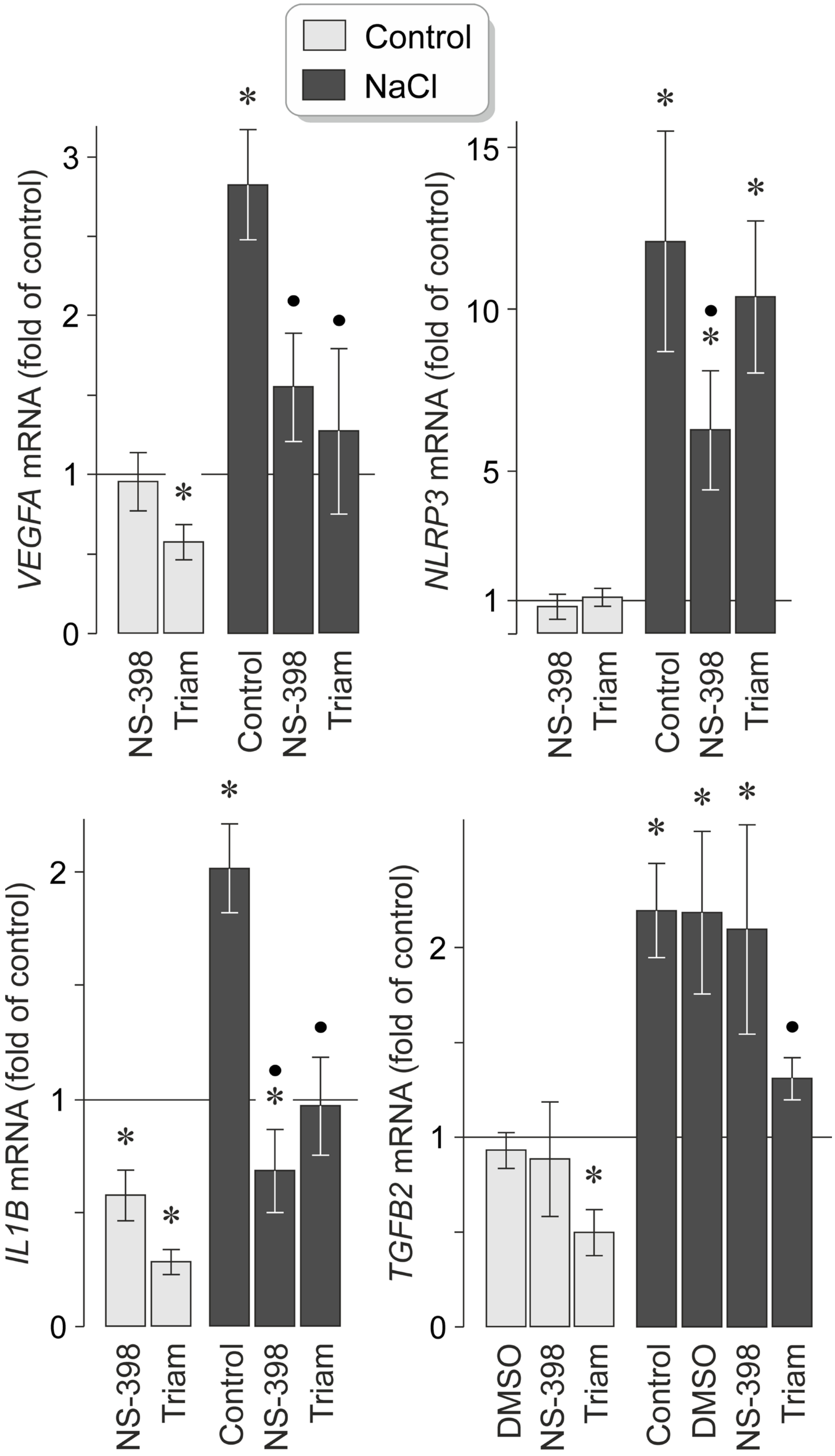

Figure 6. Effects of cyclooxygenase-2 (COX2) inhibition by NS-398 (50 µM) and the anti-inflammatory glucocorticoid triamcinolone acetonide (Triam; 50 µM) on NaCl-induced expression of the vascular endothelial growth factor A (*VEGFA*), nucleotide-binding oligomerization domain receptors-like receptor protein 3 (*NLRP3*), interleukin-1ß (*IL1B*), and transforming growth factor-ß2 (*TGFB2*) genes in retinal pigment epithelial (RPE) cells. The mRNA levels were determined with real-time reverse transcription (RT)–PCR in cells cultured 6 h in iso- (control) and hyperosmotic (+ 100 mM NaCl) media, and are expressed as folds of the unstimulated control. Vehicle control was made with dimethyl sulfoxide (DMSO; 1:1,000). Each bar represents data obtained in four to ten independent experiments with cell lines from different donors. Each experiment was performed with cell lines from four to six donors; in total, cell lines from nine donors were used for all experiments shown. Statistically significant difference versus unstimulated control: *$p<0.05$. Statistically significant difference versus NaCl control: ●$p<0.05$.

We used ELISA to investigate whether COX2 activity influences the secretion of VEGF and the level of mature IL-1β protein in RPE cells. As shown in Figure 7A, inhibition of COX2 activity with NS-398 had no effect on the constitutive and NaCl-induced secretion of VEGF from RPE cells. However, NaCl-induced secretion of VEGF was fully prevented with triamcinolone acetonide, as previously described [17]. NS-398 prevented a NaCl-induced increase in the cytosolic level of the mature IL-1β protein, and had no effect on the IL-1β level under unstimulated control conditions (Figure 7B). Triamcinolone acetonide decreased statistically significantly ($p<0.05$) the cytosolic level of the mature IL-1β protein under the control and hyperosmotic conditions (Figure 7B). The data may suggest that COX2 activity stimulates the hyperosmotic expression of angiogenic and inflammatory factor genes, and the priming and activation of the NLRP3 inflammasome in RPE cells.

DISCUSSION

AMD is a chronic inflammatory disease associated with systemic and local inflammation [3–5]. Retinal inflammation in AMD is indicated, for example, by the finding that the NLRP3 inflammasome is expressed in the RPE of eyes affected by geographic atrophy or choroidal neovascularization [6]. It has been shown that deletion of *COX2* or inhibition of COX2 activity reduces the development of choroidal neovascularization in animal models [24,25]; these findings suggest that COX activity may play a role in promoting outer retinal neovascularization, the hallmark of wet AMD. In this study, we investigated the regulation of *COX* genes in cultured human RPE cells. Hypoxia, a pathogenic condition of AMD [41], stimulated the expression of the *COX2* gene in RPE cells (Figure 1G). The expression of *COX* genes was also increased in response to an elevation of the extracellular osmolarity (Figure 1B). The increase in extracellular osmolarity after intake of dietary salt is the main condition that induces acute hypertension in the elderly [14–16]. Hypertension is a risk factor of AMD [11–13]. In addition, expression of the *COX2* gene is induced by various growth factors and IL-1β (Figure

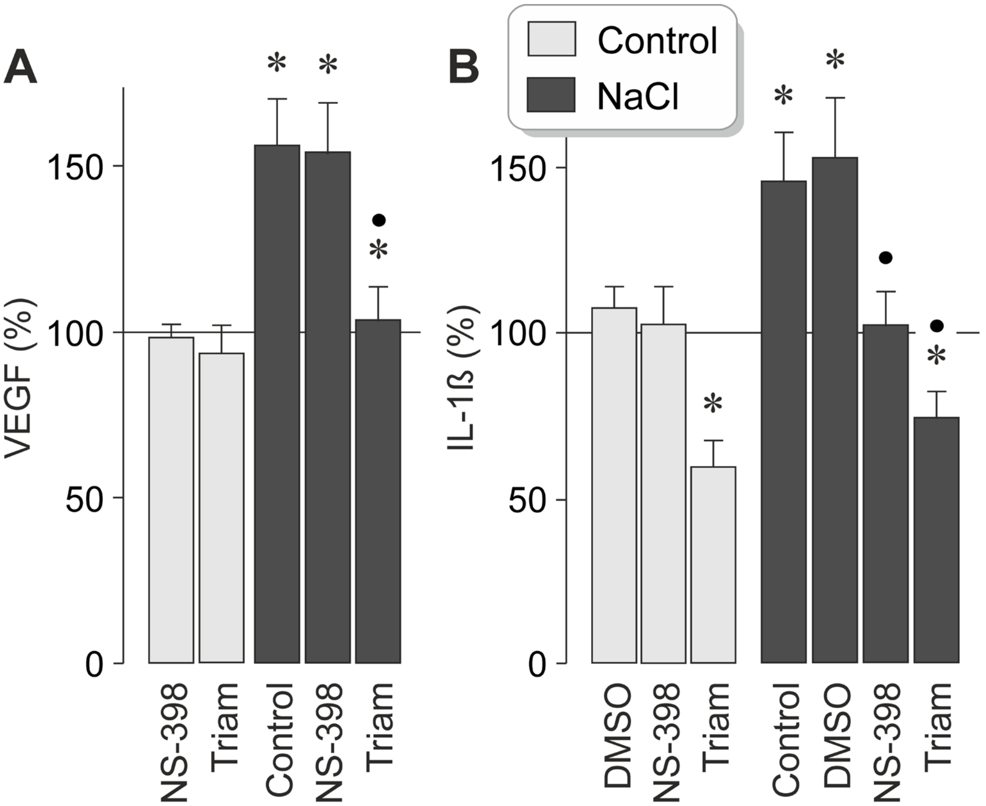

Figure 7. Effects of cyclooxygenase-2 (COX2) inhibition on NaCl-induced vascular endothelial growth factor A_{165} (VEGF-A_{165}) and mature interleukin-1ß (IL-1ß) protein levels. **A**: Effects of COX2 inhibition on NaCl-induced increases of the extracellular level of VEGF-A_{165} protein and (**B**) the cytosolic IL-1ß protein level. The cells were stimulated for 24 (**A**) and 6 h (**B**) with high (+ 100 mM) NaCl in the absence and presence of the COX2 antagonist NS-398 (50 µM) and the anti-inflammatory glucocorticoid triamcinolone acetonide (Triam; 50 µM), respectively. Vehicle control was made with dimethyl sulfoxide (DMSO; 1:1,000). The protein levels in the cultured media (**A**) and in the cell lysates (**B**) were determined with enzyme-linked immunosorbent assay (ELISA). Each bar represents data obtained in three to five independent experiments with cell lines from different donors. Each experiment was performed with cell lines from four to five donors. Statistically significant difference versus unstimulated control (100%): *$p<0.05$. Statistically significant difference versus NaCl control: ●$p<0.05$.

1H). This finding agrees with previous studies that showed that expression of the *COX2* gene in RPE cells is induced by growth factors, like PDGF, bFGF, and TGF-β [22], as well as by proinflammatory cytokines, like IL-1β [32].

Expression of the *COX2* gene in RPE cells depends on extracellular osmolarity; the expression is increased under hyperosmotic conditions (Figure 1B–D) and decreased under hypoosmotic conditions (Figure 1E). Extracellular hyperosmolarity also increased the level of the COX2 protein in RPE cells (Figure 2A, B). However, the expression of the *COX1* gene is apparently less dependent on extracellular osmolarity. This assumption is based on the findings that NaCl-induced expression of the *COX1* gene occurred after a longer time period (Figure 1B), and at higher NaCl concentrations, than the expression of the *COX2* gene (Figure 1C, D).

The intracellular signal transduction pathways implicated in mediating NaCl-induced *COX2* gene expression are summarized in Figure 8. It was shown that high extracellular NaCl induces phosphorylation of various key intracellular signal transduction molecules, including p38 MAPK and ERK1/2, in RPE cells [17]. We found that activation of several signal transduction pathways (p38 MAPK, ERK1/2, and PI3K pathways) contributes to *COX2* gene expression in RPE cells in response to high extracellular NaCl (Figure 3). Hyperosmotic expression of the *COX2* gene also depends upon intracellular calcium signaling which involves activation of PLCγ and PKCα/β (Figure 3). It is likely that intracellular calcium signaling is also implicated in the activation of PLA_2; extracellular hyperosmolarity was shown to induce expression of calcium-dependent PLA_2 genes (*PLA2G4A*, Gene ID 5321; OMIM 600522; *PLA2G5* Gene ID 5322; OMIM 601192) in RPE cells [20]. It was described that lipopolysaccharide-induced expression of *COX2* in an RPE cell line depends on the activity of PLD [42]. However, NaCl-induced expression of *COX2* is apparently not dependent on PLD activity (Figure 3).

We found that certain pharmacological blockers, that is, the ERK1/2 inhibitor PD98059 and the PLCγ inhibitor U73122, have contrary effects on *COX2* gene expression under control and hyperosmotic conditions (Figure 3). Similar contrary results were described previously for the effect of U73122 on the expression of the c-Fos gene [36] and the effect of PD98059 on the expression of the *NLRP3* gene [18]. The reason for the different effects of certain pharmacological blockers under control and hyperosmotic conditions is unclear. Several signal transduction pathways are involved in mediating NaCl-induced *COX2* gene expression in RPE cells (Figure 3). It was shown that different signal transduction pathways may have synergistic, redundant, additive, opposite, and competitive relationships [43]. It is conceivable that the interactions between various signal transduction pathways in RPE cells alter under diverse conditions, resulting in different regulation of gene expression. In addition, diverse signal transduction molecules are differently regulated in their activation state under different conditions; therefore, the same signaling molecule may produce different effects on gene expression under diverse conditions. These alterations may explain, in part, the different effects of certain pharmacological blockers under control and NaCl-stimulated conditions. It was shown that different calcium-binding proteins are expressed under various conditions in RPE cells (e.g., high extracellular NaCl induces expression of calcium-dependent PLA_2 genes [20]); therefore, calcium that is released from intracellular stores may have different, in part, contrary effects under control and NaCl-stimulated conditions that may explain somewhat the contrary effects of U73122 under the two conditions (Figure 3). Further research is required to determine the effects of different signal transduction pathways on the expression of the *COX2* gene in RPE cells under various conditions.

RPE cells were shown to express the epithelial sodium channel ($E_{Na}C$) [44] which is a major determinant of the cellular sodium homeostasis in various cell systems. We found that early NaCl-induced expression of the *COX2* gene depends upon the elevation of extracellular osmolarity, while delayed expression mainly depends on alteration of the transmembrane NaCl gradient (Figure 1B, F). We did not find an alteration in the gene expression of the pore-forming α subunit of $E_{Na}C$ (*SCNN1A*; Gene ID 6337; OMIM 600228) in RPE cells in response to high (+ 100 mM) NaCl within 24 h of stimulation (fold changes to unstimulated control: 2 h: 1.54±0.43; 6 h: 1.00±0.45 and 24 h: 1.13±0.47; p>0.05). In addition, the high (+ 100 mM) NaCl-induced expression of the *COX2* gene in RPE cells (measured after 6 h of stimulation) was not altered in the presence of the $E_{Na}C$ blocker amiloride (100 µM; 93.58±11.29% of NaCl effect compared to control, 100%; p>0.05). Further research is required to evaluate the involvement of $E_{Na}C$ in the regulation of gene expression in RPE cells in response to high extracellular NaCl.

Hyperosmotic expression of the *COX2* gene in RPE cells also depends on receptor-mediated signaling mechanisms. It was shown that high extracellular NaCl induces the release of various growth factors, like VEGF, bFGF, and TGF-β1, from RPE cells [17,35]. Exogenous bFGF and TGF-β1 induced expression of the *COX2* gene (Figure 1H), and pharmacological inhibition of FGF and TGF-β receptor signaling decreased NaCl-induced expression of the *COX2* gene (Figure 4). However, exogenous VEGF induced a small elevation in

COX2 gene expression (Figure 1H), and inhibition of VEGF receptor-2 signaling did not decrease hyperosmotic expression of the *COX2* gene (Figure 4); these findings may suggest that autocrine or paracrine VEGF signaling induced by high extracellular NaCl [17] does not contribute to NaCl-induced expression of the *COX2* gene in RPE cells. In addition to FGF and TGF-β receptor signaling, autocrine or paracrine activation of IL-1 receptors likely mediated by IL-1β released from the cells contributes to the full NaCl-induced expression of the *COX2* gene (Figure 4). Hyperosmotic activation of the *COX2* gene is mediated (at least in part) by the activity of NFAT5 (Figure 5B), whereas various other transcription factors (HIF-1, STAT3, NF-κB, AP-1, and CREB) are likely not involved (Figure 5A). However, it cannot be ruled out that additional transcription factors and intracellular signaling molecules not investigated in the present study contribute to hyperosmotic expression of the *COX2* gene in RPE cells.

It was suggested that ocular neovascularization is mediated by dual interdependent gene expression pathways that involve VEGF and COX2 [45]. In various cell systems, prostaglandins induce expression of VEGF [21]. Prostaglandins are also known to induce a breakdown of the blood–retinal barrier [46], and thus, may contribute to the development of subretinal edema in patients with wet AMD in situ. Inhibition of COX2 activity is a well-known therapeutic strategy of antiangiogenesis [47]. Deletion or inhibition of COX2 reduces the extent of experimental choroidal neovascularization and subretinal fibrosis; these effects were explained with retinal downregulation of VEGF, IL-1β, and TGF-β2 [24,25]. It was also described that inhibition of COX2 activity decreases secretion of VEGF and TGF-β2 from mouse RPE cells [25]. In the present study, we showed that inhibition of COX2 activity decreases NaCl-induced expression of the *VEGFA*, *IL1B*, and *NLRP3* genes in human RPE cells, while it had no effect on NaCl-induced expression of the *TGFB2* gene

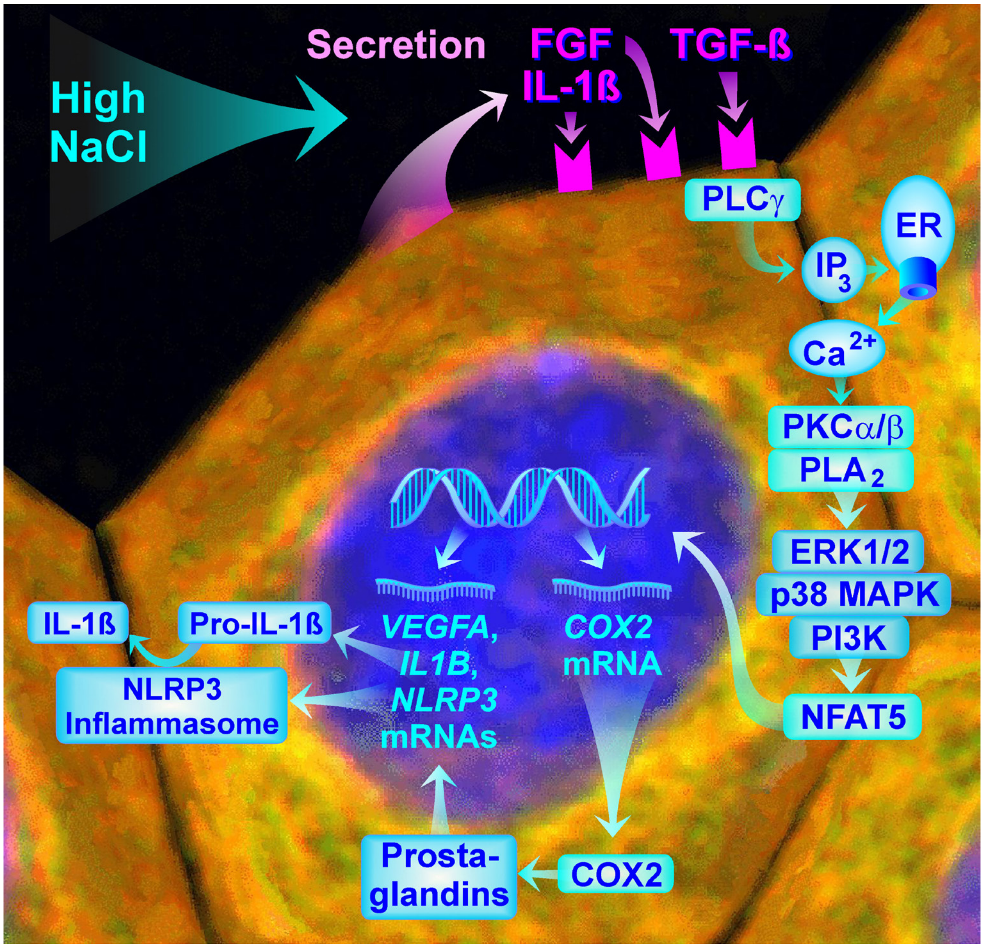

Figure 8. Schematic summary of the intracellular signal transduction pathways implicated in mediating NaCl-induced cyclooxygenase-2 (*COX2*) gene expression and the effects of COX2 activity in retinal pigment epithelial (RPE) cells. High extracellular NaCl induces release of inflammatory and growth factors from RPE cells; autocrine or paracrine activation of fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), and interleukin-1 (IL-1) receptors increases NaCl-induced expression of the *COX2* gene. NaCl-induced expression of the *COX2* gene is dependent on intracellular calcium signaling mediated by phospholipase Cγ (PLCγ) and IP_3-induced release of calcium from the endoplasmic reticulum (ER), as well as phospholipase A_2 (PLA_2) activity. These events are likely implicated in activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 mitogen-activated protein kinase (p38 MAPK), and phosphatidylinositol-3 kinase (PI3K) signal transduction pathways that contribute to the expression of the osmosensitive transcription factor nuclear factor of activated T cell 5 (NFAT5). NFAT5 activity is involved in mediating NaCl-induced *COX2* gene expression. COX2 activity stimulates the hyperosmotic expression of angiogenic (*VEGFA*) and inflammatory factor (*NLRP3* and *IL1B*) genes, and IL-1β production by the activated NLRP3 inflammasome.

(Figure 6). We also found that inhibition of COX2 activity abrogates the NaCl-induced increase in the cellular level of mature IL-1β (Figure 7B), suggesting that COX2 activity stimulates (in addition to the priming; Figure 6) activation of the NLRP3 inflammasome in RPE cells. However, we did not find an effect of a COX2 inhibitor on NaCl-induced secretion of VEGF from human RPE cells (Figure 7A). The conflicting findings regarding the effect of COX2 inhibition on the secretion of VEGF from mouse and human RPE cells might be explained with species-dependent differences of RPE cells, and the different stimulation conditions used.

The anti-inflammatory effect of corticosteroids is, in part, mediated by inhibition of PLA_2 activity [48]. The finding that triamcinolone acetonide decreased NaCl-induced expression of *COX2* (Figure 3) is in line with a previous study which showed that triamcinolone induces downregulation of basal expression of COX2 and VEGF in rat RPE cells; triamcinolone also inhibits induction of COX2 (but not of VEGF) in response to treatment with photoreceptor outer segments [49]. In human RPE cells, triamcinolone was shown to inhibit NaCl-induced expression of *VEGFA* and secretion of VEGF [17]. In this study, we showed that triamcinolone also inhibits activation of the inflammasome; this is indicated by the decreased cytosolic level of the mature IL-1β protein under control and NaCl-stimulated conditions (Figure 7B). Considering that activation of the NLRP3 inflammasome may play a central role in mediating the degeneration of the RPE in AMD [6,7,9], inhibition of inflammasome activation should delay or prevent age-related degeneration of the RPE. We also found that COX2 inhibitors block NaCl-induced priming (Figure 6), and activation of the inflammasome (Figure 7B) in RPE cells, suggesting that COX inhibitors might have protective effects in AMD. It was shown that deletion or inhibition of COX2 reduces the development of choroidal neovascularization in animal models [24,25]. However, there are low or no established effects of COX inhibitors in the therapy of AMD; it is suggested that COX inhibitors may be helpful as cotherapy of steroid therapy [50]. Because pharmacological COX2 inhibitors increase expression of *COX2* under control conditions (Figure 3), preventive use of such inhibitors is not recommended.

The present data showed that high extracellular NaCl and osmolarity have direct effects on RPE cells which, include, for example, increased expression of COX2. COX2-derived prostaglandins may stimulate retinal inflammation and angiogenesis. Whether the present results obtained in cell culture experiments have relevance for in vivo conditions remains to be determined in future investigations. We found statistically significant effects of high NaCl on expression of the *COX2* gene when more than 10 mM NaCl were added to the culture medium (Figure 1C, D). In the experiments, the addition of 30 mM NaCl to the culture medium, which induced statistically significant upregulation of *COX2* (Figure 1C, D), increased extracellular osmolarity from 287.5 ± 1.6 to 346.9±2.3 mosm/kg H_2O. Pathological blood osmolarity in human subjects may reach 360 mosm/kg H_2O [26,27], and the local extracellular NaCl concentration in the interstitium may be up to 110 mM higher than the plasma concentration of NaCl [28,29]. Because the basolateral membranes of RPE cells in situ have contact with the blood of fenestrated choroidal vessels, the present results may have relevance for in vivo conditions.

The plasma osmolarity and the salt sensitivity of blood pressure increase with age [51,52]. Therefore, high dietary salt may have detrimental effects particularly in aged salt-sensitive individuals. We found that high NaCl induced transient upregulation of the *COX2* gene (Figure 1C,D). Similar transient upregulation was described previously with respect to NaCl-induced expression of angiogenic factors in RPE cells [17,35]. It is suggested that repetitive salt-induced increases in plasma osmolality during postprandial phases have greater effects than a persistent elevation of the plasma salt level [16]. Restriction of dietary salt intake or increased intake of NaCl-lowering minerals may be helpful to decelerate the progression of AMD [16].

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