Osmotic regulation of NFAT5 expression in RPE cells: The involvement of purinergic receptor signaling

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Purpose: Systemic hypertension is a risk factor for age-related neovascular retinal diseases. The major condition that induces hypertension is the intake of dietary salt (NaCl) resulting in increased extracellular osmolarity. High extracellular NaCl was has been shown to induce angiogenic factor production in RPE cells, in part via the transcriptional activity of nuclear factor of activated T cell 5 (NFAT5). Here, we determined the signaling pathways that mediate the osmotic expression of the NFAT5 gene in RPE cells.

Methods: Cultured human RPE cells were stimulated with high (+100 mM) NaCl. Alterations in gene and protein expression were determined with real-time reverse transcriptase (RT)-PCR and western blot analysis, respectively.

Results: NaCl-induced NFAT5 gene expression was fully inhibited by calcium chelation and blockers of inositol triphosphate (IP3) receptors and phospholipases C and A2. Blockers of phospholipases C and A2 also prevented the NaCl-induced increase of the cellular NFAT5 protein level. Inhibitors of multiple intracellular signaling transduction pathways and kinases, including p38 mitogen-activated protein kinase (MAPK), extracellular signal–regulated kinases 1 and 2 (ERK1/2), c-Jun NH2-terminal kinase (JNK), phosphatidylinositol-3 kinase (PI3K), protein kinases A and C, Src tyrosine kinases, and calpains, as well as cyclooxygenase inhibitors, decreased the NaCl-induced expression of the NFAT5 gene. In addition, autocrine purinergic signaling mediated by a release of ATP and a nucleoside transporter-mediated release of adenosine, activation of P2X7, P2Y6, P2Y13, and adenosine A2 receptors, but not adenine A1 receptors, is required for the full expression of the NFAT5 gene in normosmotic conditions. NaCl-induced NFAT5 gene expression is in part dependent on the activity of nuclear factor κB (NF-κB). The NaCl-induced expression of NFAT5 protein was prevented by inhibitors of phospholipases C and A2, and an inhibitor of NF-κB, but it was not prevented by a P2Y6 inhibitor.

Conclusions: The data suggest that in addition to calcium signaling and activation of inflammatory enzymes, autocrine/purinergic signaling contributes to the stimulatory effect of osmotic stress on the expression of the NFAT5 gene in RPE cells. It is suggested that high intake of dietary salt induces RPE cell responses, which may contribute to age-related retinal diseases.

Diabetic retinopathy is the leading cause of vision loss in working age adults, and age-related macular degeneration (AMD) is the most common cause of blindness in the elderly [1,2]. Most AMD patients suffer from the dry form of AMD; in the late stage, this is characterized by geographic atrophy, that is, degeneration of the outer retina, including the photoreceptors and RPE. The remaining patients suffer from the neovascular form, which is characterized by choroidal neovascularization [3]. Progression of diabetic retinopathy results in retinal degeneration, macular edema, and retinal neovascularization. Vascular endothelial growth factor (VEGF) is the most relevant angiogenic factor that promotes retinal and choroidal neovascularization [4]. It has been shown that the synergistic action of further angiogenic factors, such as basic fibroblast growth factor (bFGF), is required for the angiogenic effect of VEGF [5].

Hyperglycemia is the primary risk factor for diabetic retinopathy, while systemic hypertension is the main secondary risk factor [6,7]. Control of the blood pressure, even in the normotensive range, reduces the risk of diabetic retinopathy and prevents microvascular complications and vision loss from diabetic retinopathy independently of glycemia [8,9]. Systemic hypertension also increases the risk of AMD [10-12]. The main condition that causes acute hypertension is the increase of extracellular osmolarity following intake of dietary salt (NaCl) [13]. Hypernatremia causes systemic hyperosmolarity [14,15], which induces blood volume expansion and thus hypertension [16]. The extracellular osmolarity and blood pressure–raising effects of dietary salt increase with age [17,18]. In experimental diabetic retinopathy, high salt intake also aggravates diabetes-induced retinal
alterations independently of changes in blood pressure [19]. It has been described that elevated extracellular osmolarity and high extracellular NaCl induce the production of angiogenic factors like VEGF and bFGF in RPE cells [20,21]. The high NaCl–induced production of angiogenic factors in RPE cells may contribute to the pathogenesis of age-related neovascular retinal diseases.

Cells possess several adaptive mechanisms that allow them to survive under osmotic stress conditions through the restoration of osmotic balance. Cell survival under hypotonic conditions is initially maintained by the activation of ion transport systems, and thereafter, by intracellular accumulation of small organic osmolytes like sorbitol, myo-inositol, and taurine [22]. The classical transcription factor that activates expression of osmoprotective genes is the nuclear factor of activated T cell 5 (NFAT5), also known as toxicity-responsive enhancer binding protein (TonEBP/OEBP) [22,23]. It has been shown that elevated extracellular osmolarity and high extracellular NaCl increase the NFAT5 gene and protein expression and induce DNA binding of NFAT5 in RPE cells; furthermore, it has been found that the hyperosmotic production of angiogenic factors in RPE cells depends in part on the transcriptional activity of NFAT5 [20,21]. In experimental diabetic retinopathy, the retinal expression of NFAT5 is increased, and knockdown of NFAT5 has neuroprotective effects [24]. Although osmotic stress may influence key pathogenic events that lead to the progression of AMD and diabetic retinopathy, at least in part via upregulation of NFAT5, almost nothing is known regarding the regulation of the osmotic NFAT5 expression in retinal cells. Therefore, we determined the signaling pathways that are involved in mediating the osmotic expression of the NFAT5 gene in human RPE cells. We found that in addition to calcium signaling and activation of inflammatory enzymes, autocrine/paracrine purinergic signaling contributes to the stimulatory effect of hyperosmotic stress on the expression of the NFAT5 gene.

**METHODS**

**Materials:** Cell culture components and solutions were purchased from Gibco BRL (Paisley, UK). Inhibitors of hypoxia-inducible transcription factor (HIF)—AG1478, LY294002, PD98059, SP600125, and SU1498—were obtained from Calbiochem (Bad Soden, Germany). Moreover, A-438079, AR-C 118925XX, caffeic acid phenethyl ester (CAPE), GSK650394, and SB203580 were obtained from Tocris (Ellisville, MO). Stattic was purchased from Enzo Life Science (Plymouth Meeting, PA), and PD173034 was kindly provided by Pfizer (Karlsruhe, Germany). Hoechst 33,342 was purchased from Life Technologies (Gaithersburg, MD). Adenosine-5′-O-(α,β-methylene)diphosphate (AOPCP), 2-aminoethoxydiphenyl borate (2-APB), 8-(3-chlorostyryl) caffeine (CSC), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), N-acetyl-L-cysteine, N-nitrobenzylthioinosine (NBTI), 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-NFAT5 (1:750; Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-β-actin (1:1000; Cell Signaling, Frankfurt am Main, Germany), and alkaline phosphatase-coupled anti-rabbit immunoglobulin G (IgG; 1:2000; Cell Signaling). The following antibodies were used for immunocytochemistry: rabbit anti-NFAT5 (1:100; Novus, Littleton, CO), 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 study followed the tenets of the Declaration of Helsinki for research involving human subjects. The use of human material was approved by the Ethics Committee of the University of Leipzig (approval #745, 07/25/2011). Postmortem eyes from human cornea donors without reported eye disease were obtained within 48 h of death with the written informed consent from the relatives for the use of retinal tissue in basic science. RPE cells were prepared as follows: vitreous and neural retina were removed, RPE cells were mechanically harvested, separated by digestion with 0.05% trypsin and 0.02% EDTA for 5 min at 37 °C and washed twice with phosphate-buffered saline (PBS). The cells were suspended in complete Ham F-10 medium containing 10% fetal bovine serum, glutamax II, and gentamycin, and were cultured in tissue culture flasks (Greiner, Nürtingen, Germany) in 95% air/5% CO₂ at 37 °C [25]. Cell lines derived from different donors were used in passages 3 to 5. Near-confluent cultures were growth arrested in medium without serum for 16 h, and subsequently, serum-free media with and without test substances were added. The hyperosmotic medium was made up by the addition of 100 mM NaCl. The cells were preincubated with pharmacological inhibitors 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 quality of the RNA was analyzed via agarose gel electrophoresis. The A₂₆₀/A₂₈₀ ratio of the optical density was measured using the NanoDrop1000 device (peQLab, Erlangen, Germany) and was between 1.95 and 2.03 for all RNA samples, indicating...
sufficient quality. After treatment with DNase I (Roche, Mannheim, Germany), cDNA was synthesized from 1 µg of total RNA using the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas, St. Leon-Roth, Germany).

**Real-time reverse transcriptase–PCR analysis:** Real-time reverse transcriptase (RT)-PCR 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 of cDNA, the specific primer set (0.2 µM each), and 7.5 µl of a 2× mastermix (iQ SYBR Green Supermix; BioRad) in a final volume of 15 µl. The following conditions were used: initial denaturation and enzyme activation (one cycle at 95 °C for 3 min); denaturation, amplification, and quantification, 45 cycles at 95 °C for 30 s, 58 °C for 20 s, and 72 °C for 45 s; and melting curve, 55 °C with the temperature gradually (0.5 °C) increased up to 95 °C. The amplified samples were analyzed using standard agarose gel electrophoresis. The mRNA expression was normalized to the level of β-actin mRNA. The changes in mRNA expression were calculated according to the $2^{\Delta\Delta CT}$ method (cycle threshold, CT), with $\Delta CT = CT_{\text{target gene}} - CT_{\text{actb}}$ and $\Delta\Delta CT = \Delta CT_{\text{treatment}} - \Delta CT_{\text{control}}$.

**Immunocytochemistry:** The cultures were fixed with 4% paraformaldehyde for 20 min at room temperature. After several washing steps in prechilled PBS (KH$_2$PO$_4$: 1.54 mM, NaCl: 155.17 mM, and Na$_2$HPO$_4$ x 7 H$_2$O: 2.71 mM; Invitrogen, Paisley, UK), the cultures were incubated in PBS containing dimethyl sulfoxide (DMSO; 1%) and 0.3% Triton X-100 for 15 min at room temperature. 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 room temperature. 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 33,342 (1:1000) were applied for 2 h at room temperature. After several washing steps, the coverslips were mounted with Shandon Immu-Mount (Thermo Fisher Scientific, Waltham, MA). Images were taken with a confocal laser scanning microscope (LSM 510 Meta; Zeiss, Oberkochen, Germany).

**Western blot analysis:** The cells were washed twice with PBS, scraped into 80 µl of lysis buffer (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1% protease inhibitor cocktail), and agitated at 4 °C for 30 min. Thereafter, the cell lysates were centrifuged at 13,000 ×g for 10 min, and the supernatants were analyzed by immunoblotting. Equal amounts of protein (35 µg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After blocking of nonspecific binding with 5% (w/v) nonfat dry milk in Tris-buffered saline plus 1% Tween-20 for 1 h, the antibodies were applied. Immunoreactive bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

**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 carried out using

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### Table 1. Primer pairs used in PCR experiments.

| Gene and accession number | Primer sequence (5‘→3’) | Amplicon (bp) |
|---------------------------|--------------------------|---------------|
| ACTB; NM_001101           | F: ATGGCCACGCGTCGTCCTCCAGC  
                           | R: CATGGTGGGTGCCGCCAGACAG | 237         |
| COX1; NM_080591.2         | F: TTGACCGCTACAGTGACGACG  
                           | R: ACGGATAAGTGGACGCCACT | 221         |
| COX2; NM_000963           | F: TGAGCAGCTACAGTTGCCTG    
                           | R: TGCTTGCTTGAAACAACCTG  | 158         |
| NFAT5; NM_006599.3        | F: TCACCATCATCCTCCACCT     
                           | R: CTGCAAATATGTCATCGCTGT  | 174         |
| PLA2G1B; NM_000928.2      | F: TACACAAACTACGGCTGCTACT  
                           | R: GCACGATGTAATGGTGGG    | 168         |
| PLA2G2A; NM_000300.3      | F: ATGTGGCCACAAATTCATGCCAGC  
                           | R: GCTCCTCTCAGTGGTTTATG  | 187         |
| PLA2G4A; NM_024420.2      | F: TGTAGCTGGAGCCATGAGATGAG  
                           | R: CAGCATGATTGGTGAATCTTCC | 187         |
| PLA2G5; NM_000929.2       | F: AGGCTTGCGACCTAAAATACCA  
                           | R: GTAGGACTGTTGGCAGATGTTG | 199         |
To determine whether the activities of other transcription factors contribute to the hyperosmotic induction of NFAT5, we used pharmacological blockers. The hyperosmotic NFAT5 gene expression remained unaltered in the presence of an HIF inhibitor [26] and the signal transducer and activator of transcription 3 (STAT3) inhibitor Stattic [27] (Figure 2). However, the inhibitor of the proinflammatory transcription factor NF-κB, CAPE [28], significantly (p<0.05) decreased the level of NFAT5 transcripts under hyperosmotic conditions (Figure 2). The data may suggest that NF-κB-induced expression of inflammatory proteins is required for the full expression of the NFAT5 gene in osmotic stress.

Oxidative stress is not involved in the osmotic expression of NFAT5: It has been reported that high NaCl induces cellular oxidative stress and that reactive oxygen species (ROS), a component of oxidative stress, may contribute to the NaCl-induced activation of NFAT5 [29]. To determine whether oxidative stress is required for the hyperosmotic transcriptional activation of the NFAT5 gene in RPE cells, we tested the cell-permeable reducing agent dithiothreitol and the ROS inhibitor N-acetyl-L-cysteine. Neither agent altered the NaCl-induced expression of the NFAT5 gene (Figure 3). One consequence of oxidative stress is activation of the mitochondrial permeability transition, resulting in mitochondrial dysfunction, energy failure, and enhanced free radical production. The inhibitor of mitochondrial permeability transition, cyclosporin A [30], did not alter the hyperosmotic expression of the NFAT5 gene (Figure 3). The data support the assumption that ROS and mitochondrial dysfunction are not involved in mediating the hyperosmotic expression of the NFAT5 gene in RPE cells.

Inflammatory enzyme activity involved in the osmotic expression of NFAT5: To determine whether the activities of inflammatory enzymes are involved in mediating the effect of high extracellular osmolarity on the NFAT5 gene expression in RPE cells, we tested inhibitors of enzymes that produce inflammatory lipids. As shown in Figure 3, the inhibitor of phospholipases A2 (PLA2), 4-bromophenacyl bromide, fully prevented the NaCl-induced upregulation of the NFAT5 gene expression. Inhibitors of cyclooxygenases (COX), acetylsalicylic acid and ibuprofen, partly prevented the hyperosmotic induction of NFAT5 (Figure 3). The data suggest that arachidonic acid and arachidonic acid metabolites, such as prostaglandins, are crucially involved in mediating the effect of high extracellular osmolarity on the NFAT5 gene expression in RPE cells. RPE cells express various PLA2 and COX genes (Figure 4A). High extracellular NaCl induced expression of COX1 and COX2 genes (Figure 4B). In addition, extracellular hyperosmolarity induced the expression of calcium-dependent PLA2 genes (PLA2G4A, PLA2G5), while the expression of PLA2G1B and PLA2G2A was not altered (Figure 4B).

Receptor signaling involved in the osmotic expression of NFAT5: It has been shown that extracellular hyperosmolarity
Figure 1. Intracellular signaling involved in mediating the osmotic expression of the NFAT5 gene in RPE cells. The level of NFAT5 mRNA was determined with real-time reverse transcriptase (RT)-PCR analysis in cells cultured 6 h in iso- (control) and hyperosmotic (+100 mM NaCl) media. A: Involvement of intracellular signal transduction pathways. The following blocking agents were tested: the inhibitor of p38 mitogen-activated protein kinase (MAPK) activation, SB203580 (10 µM); the inhibitor of extracellular signal–regulated kinases 1 and 2 (ERK1/2) activation, PD98059 (20 µM); the c-Jun NH₂-terminal kinase (JNK), inhibitor SP600125 (10 µM); and the inhibitor of phosphatidylinositol-3 kinase (PI3K)-related kinases, LY294002 (5 µM). Vehicle controls were made with dimethyl sulfoxide (DMSO; 0.1%) and NaOH (1 M; 1:150). B: Involvement of protein kinase activities and calcium signaling. The following blocking agents were tested: the inhibitor of protein kinase C α/β (PKCa/β), Gö6976 (1 µM); the protein kinase A (PKA) inhibitor, H-89 (1 µM); the inhibitor of Src tyrosine kinases, PP2 (100 nM); the calpain inhibitor, PD150606 (100 µM); the serum and glucocorticoid-regulated kinase (SGK) inhibitor GSK650394 (GSK; 1 µM); the cell-permeable calcium chelator, BAPTA/AM (5 µM); the phospholipase Cγ (PLCγ) inhibitor, U73122 (4 µM); and the inositol triphosphate (IP₃) receptor inhibitor, 2-aminoethoxydiphenylborate (2-APB; 100 µM). Means ± standard error of the mean (SEM) of 3–7 independent experiments using cell lines from different donors. Significant difference versus unstimulated control: *p<0.05. Significant difference versus NaCl control: ●p<0.05.
induces a release of growth factors like VEGF, bFGF, and transforming growth factor β1 (TGF-β1) from RPE cells [20,21]. To determine whether autocrine/paracrine receptor activation is required for the hyperosmotic expression of the NFAT5 gene, we tested inhibitors of receptor kinases. As shown in Figure 5A, the hyperosmotic expression of the NFAT5 gene remained unaltered in the presence of inhibitors of the epidermal growth factor (EGF) receptor tyrosine kinase (AG1478), the platelet-derived growth factor (PDGF) receptor tyrosine kinase (AG1296), VEGF receptor-2 (SU1498), and TGF-β1 superfamily activin receptor-like kinase receptors (SB431542). The lack of an effect of the broad-spectrum metalloproteinase inhibitor 1,10-phenanthroline (Figure 5A) suggests that shedding of growth factors from the extracellular matrix is not required for the hyperosmotic expression of the NFAT5 gene. The FGF receptor kinase inhibitor PD173074 induced a slight decrease of the NFAT5 gene expression under hyperosmotic conditions (Figure 5A), suggesting that autocrine FGF receptor signaling may be required for the full expression of the NFAT5 gene upon osmotic challenge.

In many cell systems, such as retinal glial cells [31], autocrine/paracrine purinergic signaling mediated by the osmo-/mechanosensitive release of ATP and subsequent activation of purinergic receptors is involved in the early cellular response to osmotic stress. Purinergic receptor signaling induces calcium responses and leads to the opening of ion channels in the plasma membrane; the transmembrane ion flux compensates for the osmotic gradient across the plasma membrane, thereby preventing alterations in the cellular volume under aniso-osmotic conditions. In retinal glial cells, cell volume regulation is mediated by both P2Y and adenosine receptors [31]. It has been shown that RPE cells express multiple subtypes of ionotropic (P2X) and metabotropic (P2Y) purinergic receptors, including P2X\textsubscript{7}, P2Y\textsubscript{1}, and P2Y\textsubscript{2}, as well as adenosine receptors [32,33]. We found that the ATP/ADP-degrading enzyme apyrase decreased the NFAT5 mRNA level significantly (p<0.05) under hyperosmotic conditions (Figure 5B), suggesting that autocrine/paracrine purinergic signaling is required for the full expression of the NFAT5 gene in osmotic stress. The NaCl-induced upregulation of the NFAT5 mRNA level was also partially inhibited by antagonists of P2X\textsubscript{7} (A-438079), P2Y\textsubscript{1} (MRS2179), P2Y\textsubscript{2} (AR-C 118925XX), and adenosine A\textsubscript{1} receptors (DPCPX; Figure 5B). In contrast, the adenosine A\textsubscript{2A} receptor antagonist CSC had no effect (Figure 5B). The data suggest that osmotic stress induces a release of ATP from RPE cells that subsequently activates P2X\textsubscript{7}, P2Y\textsubscript{1}, and P2Y\textsubscript{2} receptors, resulting in the induction of NFAT5 gene expression.

In addition to P2X and P2Y receptor activation, autocrine/paracrine adenosine signaling contributes to the osmotic induction of the NFAT5 gene (Figure 5B). Adenosine may be liberated from cells via equilibrative nucleoside transporters and may be generated extracellularly by enzymatic dephosphorylation of ATP. Pharmacological inhibition of the nucleoside transporter activity by NBTI significantly (p<0.05) decreased the osmotic induction of the NFAT5 gene expression in RPE cells (Figure 5B). To determine whether
adenosine is also extracellularly formed by dephosphorylation of ATP, we tested ARL-67156 (an ecto-ATPase inhibitor that blocks the conversion of ATP to ADP/AMP) and AOPCP (an ectonucleotidase inhibitor that blocks the conversion of AMP to adenosine). The osmotic upregulation of the NFAT5 gene expression was significantly ($p<0.05$) decreased by ARL-67156 but remained unaltered in the presence of AOPCP (Figure 5B). The data suggest that osmotic stimulation induces a transporter-mediated liberation of adenosine from RPE cells but not an extracellular formation of adenosine by enzymatic dephosphorylation of AMP.

Regulation of the osmotic expression of NFAT5 protein: To determine the intracellular regulation of the osmotic expression of NFAT5 protein, we performed immunocytochemical and western blot analyses. Using both methods, we found that extracellular hyperosmolarity (induced by the addition of NaCl to the culture medium) caused an increase in the NFAT5 protein level in RPE cells (Figure 6A and Figure 7A,B), as previously described [20]. Extracellular hyperosmolarity also induced an upregulation of vimentin expression and an alteration of the cell shape, as recognizable at the shortening of the cells (Figure 6A). Upregulation of vimentin and alteration of the cell shape may represent regulatory responses that contribute to cell survival under osmotic stress conditions. The inhibitors of phospholipases C and A$_2$, U73122 and 4-bromophenacyl bromide, fully prevented the osmotic increase of the NFAT5 protein level in RPE cells (Figures 6B and Figure 7A,B). In addition, we found that the NF-κB inhibitor CAPE prevented the osmotic increase of the NFAT5 protein level (Figure 7A,B). In contrast, the P2Y$_1$ receptor antagonist MRS2179 had no significant effects on the NFAT5 protein expression in RPE cells under either iso- or hyperosmotic conditions (Figure 7A,B).
DISCUSSION

Systemic hypertension increases the risks of age-related retinal neovascular diseases, such as diabetic retinopathy and AMD [6,7,10-12]. The main factor that causes acute hypertension is high salt intake resulting in increased extracellular osmolarity [13]. It has been shown that elevated extracellular osmolarity and high extracellular NaCl induce the production of angiogenic factors like VEGF and bFGF in RPE cells; the production of angiogenic factors is partly mediated by the transcriptional activity of NFAT5 [20,21]. This suggests that the activity of NFAT5 may contribute to the pathogenesis of age-related neovascular retinal diseases. Indeed, it has been shown that knockdown of NFAT5 has protective effects in experimental diabetic retinopathy [24]. However, almost nothing is known regarding the regulation of the NFAT5 expression in retinal cells. In the present study, we determined the signaling pathways that are involved in mediating the NaCl-induced expression of the NFAT5 gene in RPE cells.

It has been shown that high extracellular NaCl induces phosphorylation of various key intracellular signal transduction molecules, including p38 MAPK and ERK1/2, in RPE cells [20]. In different cell systems, activation of p38 MAPK, ERK1/2, JNK, and PI3K signal transduction pathways is involved in mediating the stimulatory effects of high extracellular osmolarity on the expression of osmoregulatory target genes [22,34-40]. We found that activation of multiple signal transduction pathways contributes to the NFAT5 gene expression in RPE cells in response to high extracellular NaCl (Figure 8). The NaCl-induced expression of the NFAT5 gene is in part dependent on the activation of the p38 MAPK, ERK1/2, JNK, and PI3K signal transduction pathways (Figure 1A). In addition, the activities of PKC, PKA, Src tyrosine kinases, and calpains are involved in mediating the NaCl-induced NFAT5 gene expression (Figure 1B). Inhibition of each of the pathways and kinases only partially reduce the NaCl-induced NFAT5 gene expression (Figure 1A,B). The present data are consistent with previous studies that showed that activation of multiple intracellular signal transduction pathways and kinases, including PKC, PKA, and PI3K, is required in different cell systems for the full osmotic Figure 4. Osmotic induction of inflammatory enzyme genes in RPE cells. A: Expression of COX (COX1, COX2) and PLA genes (PLA2G1B, PLA2G2A, PLA2G4A, PLA2G5) in cultured RPE cells derived from two different donors (1, 2). To confirm the correct lengths of PCR products, agarose gel electrophoresis was performed using products obtained from two different donors (1, 2). Negative controls (0) were established by adding double-distilled water instead of cDNA as template. B: Effects of extracellular hyperosmolarity induced by addition of high (+100 mM) NaCl on the expression of COX and PLA genes. The mRNA levels were determined with real-time reverse transcriptase (RT)-PCR analysis after stimulation of the cells for 2, 6, and 24 h, respectively, and are expressed as folds of unstimulated control. Means ± standard error of the mean (SEM) of 4–6 independent experiments using cell lines from different donors. Significant difference versus unstimulated control: *p<0.05.
Figure 5. Receptor signaling involved in mediating the osmotic expression of the NFAT5 gene in RPE cells. The level of NFAT5 mRNA was determined with real-time reverse transcriptase (RT)-PCR analysis in cells cultured 6 h in iso- (control) and hyperosmotic (+100 mM NaCl) media.

A: Involvement of autocrine/paracrine growth factor receptor signaling. The following blocking agents were tested: the inhibitor of the epidermal growth factor (EGF) receptor tyrosine kinase, AG1478 (600 nM); the blocker of the platelet-derived growth factor (PDGF) receptor tyrosine kinase, AG1296 (10 µM); the inhibitor of vascular endothelial growth factor (VEGF) receptor 2, SU1498 (10 µM); the inhibitor of transforming growth factor β1 (TGF-β1) superfamily activin receptor-like kinase receptors, SB431542 (10 µM); the fibroblast growth factor (FGF) receptor kinase inhibitor, PD173074 (500 nM); and the broad-spectrum metalloproteinase inhibitor, 1,10-phenanthroline (1,10-Phen; 10 µM).

B: Involvement of autocrine/paracrine purinergic signaling. The following blocking agents were tested: the ATP/ADP phosphohydrolase apyrase (10 U/ml), the P2X receptor antagonist A-438079 (50 nM), the P2Y receptor antagonist MRS2179 (30 µM), the P2Y receptor antagonist AR-C 118925XX (AR-C; 10 µM), the adenosine A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 50 nM), the adenosine A2A receptor antagonist 8-(3-chlorostyryl) caffeine (CSC, 200 nM), the antagonist of nucleoside transporters N-acetyl-L-cysteine, N-nitrobenzylthioinosine (NBTI; 10 µM), the ecto-ATPase inhibitor ARL-67156 (50 µM), and the ectonucleotidase inhibitor adenosine-5′-O-(α,β-methylene)-diphosphate (AOPCP; 250 µM). Means ± standard error of the mean (SEM) of 3–7 independent experiments using cell lines from different donors. Significant difference versus unstimulated control: *p<0.05. Significant difference versus NaCl control: ●p<0.05.
induction and activation of NFAT5 and that the inhibition of one pathway alone is not sufficient to fully abrogate the hyperosmotic induction of NFAT5 \([22,39,41-43]\).

The activity of the proinflammatory transcription factor NF-κB, but not that of HIF of STAT3, is required for the full expression of the NFAT5 gene in osmotic stress (Figure 2). Inhibition of NF-κB activity also prevented the osmotic increase of the NFAT5 protein level (Figure 7A,B). The lack of an effect of the HIF inhibitor is consistent with the previous finding that hypoxia does not induce NFAT5 gene expression in RPE cells \([20]\). The activity of PLA\(_2\) is crucially involved in the NaCl-induced expression of the NFAT5 gene and protein expression (Figure 3 and Figure 7A,B), while inhibition of COX partly prevents the effect of high NaCl on the NFAT5 gene expression (Figure 3). The data suggest that arachidonic acid and arachidonic acid metabolites, such as prostaglandins, are involved in mediating the effect of high NaCl on the expression of the NFAT5 gene in RPE cells. We found that extracellular hyperosmolarity induced the gene expression of COX and calcium-dependent PLA\(_2\) (Figure 4B). The data are consistent with previous studies that showed that high extracellular NaCl induces COX2 expression in renal cells and that prostaglandin E\(_2\) stimulates the expression of osmoprotective genes \([44,45]\). The increases of COX and PLA\(_2\) gene expression may represent a regulatory response following stimulation of the enzyme activities.

We found evidence that the hyperosmotic expression of the NFAT5 gene in RPE cells depends on intracellular calcium signaling, including the activation of PLC\(_\gamma\) and IP\(_3\)-mediated calcium release (Figure 1B), resulting in activation of PKC, calpains, and PLA\(_2\) (Figure 1B, Figure 3, and Figure 8). PLC\(_\gamma\)-induced calcium signaling also plays a crucial role in mediating the osmotic increase of the NFAT5 protein level (Figure 7A,B). The data are consistent with a previous study that showed that PLC\(_\gamma1\) is involved in mediating the NaCl-induced activation of NFAT5 in various cell systems \([46]\).

![Figure 6](image_url) Extracellular hyperosmolarity increases the NFAT5 immunoreactivity in RPE cells. Cultured RPE cells were immunolabeled with antibodies against NFAT5 (green) and vimentin (red). Cell nuclei were stained with Hoechst 33,342 (blue). A: Cells were cultured 6 h in iso- (control; left) and hyperosmotic (+100 mM NaCl) medium (right), respectively. Note the increased NFAT5 immunoreactivity and the altered shape of cells cultured in hyperosmotic medium compared to control cells. B: In the presence of the phospholipase C\(_\gamma\) (PLC\(_\gamma\)) inhibitor U73122 (4 µM), hyperosmotic medium did not cause an increase of NFAT5 immunoreactivity compared to control. C: Negative control obtained by omitting of the primary antibodies. The cells were cultured 6 h in hyperosmotic (+100 mM NaCl) medium. No unspecific labeling was observed following cell culture in iso-osmotic medium (not shown). Bars, 20 µm.
A major mechanism of the induction of calcium responses in osmotic stress is autocrine/paracrine activation of purinergic signaling mediated by the osmo-/mechanosensitive release of ATP and subsequent activation of purinergic receptors. We found that activation of P2X₁, P2Y₁, P2Y₂, and adenosine A₁ receptors, but not adenosine A₂A receptors, is involved in inducing the hyperosmotic expression of the NFAT5 gene in RPE cells (Figure 5B). Activation of P2Y receptors may result in PLC-dependent intracellular calcium responses. However, we found no effect of a P2Y₁ inhibitor on the NFAT5 protein level (Figure 7A,B). Purinergic receptor signaling may relieve cellular osmotic stress by the opening of ion channels [47] and by activation of NFAT5, which induces long-term protection from osmotic stress via inducing the production of small organic osmolytes [22]. It has been shown that extracellular ATP induces calcium responses in RPE cells, at least in part via P2X receptor activation; abnormal calcium homeostasis may result in cellular dysfunction and apoptosis [48].

Osmotic/mechanical stimuli are the main inducers of cellular ATP release, for example, from retinal glial and RPE cells [49-51]. Adenosine may be liberated from cells via equilibrative nucleoside transporters and may be formed in the extracellular space by the enzymatic dephosphorylation of ATP. We found that a blocker of nucleoside transporters (NBPTI) and the ecto-ATPase inhibitor ARL-67156 (which blocks the conversion of ATP to ADP/AMP) decreased the hyperosmotic NFAT5 gene expression, while the ectonucleotidase inhibitor AOPCP (which inhibits the conversion of AMP to adenosine) had no effect (Figure 5B). The data suggest that ATP, which is osmotically released from RPE cells, is extracellularly converted to ADP but not extracellularly degraded to adenosine. ATP/ADP acts at P2 receptors; upon activation, these induce a release of adenosine via nucleoside transporters (Figure 8). A similar mechanism of ATP-induced adenosine release has been described for the purinergic inhibition of osmotic Müller cell swelling [50]. The failure of extracellular adenosine formation from ATP released following osmotic stimulation was explained with the retinal distribution of nucleoside triphosphate diphosphohydrolases (NTPDases). It has been shown that the retinal parenchyma lacks a significant expression of NTPDase1 (which hydrolyzes ATP/ADP to AMP), while it expresses NTPDase2 (which hydrolyzes ATP to ADP) and

Figure 7. Hyperosmotic expression of NFAT5 protein in RPE cells. A: The level of NFAT5 protein (170 kDa) was determined by western blot analysis of cytosolic extracts of cells which were cultured 6 h in iso- (control; Co) and hyperosmotic (+100 mM NaCl) media in the presence and absence of the following inhibitory agents: the phospholipase A2 (PLA₂) inhibitor 4-bromophenacyl bromide (Bromo; 30 µM), the phospholipase Cγ (PLCγ) inhibitor U73122 (4 µM), the P2Y₇ receptor antagonist MRS2179 (30 µM), and the nuclear factor κB (NF-κB) inhibitor caffeic acid phenethyl ester (CAPE; 5 µM). Equal amounts of total protein (35 µg) were used for separation. β-Actin (45 kDa) was used as control for equal protein loading. B: Cellular level of NFAT5 protein, as determined by densitometrical analysis of western blots. Vehicle control was made with dimethyl sulfoxide (DMSO; 0.1%). Means ± standard error of the mean (SEM) of 3–7 independent experiments using cell lines from different donors. Significant difference versus unstimulated control: *p<0.05. Significant difference versus NaCl control: ●p<0.05.
ectonucleotidase (which hydrolyzes AMP to adenosine) \[52\]. Due to the lack of NTPDase1, the substrate of adenosine formation (AMP) is not generated in sufficient quantity at the surface of retinal cells.

The present data show that high extracellular NaCl activates various signaling pathways, including calcium signaling, autocrine/paracrine activation of purinergic receptors, and increased activity of inflammatory enzymes, which contribute to the activation of the osmoregulatory transcription factor NFAT5 in RPE cells (Figure 8). The data suggest that high intake of dietary salt induces RPE cell responses, which may predispose the cells to damage in response to pathological conditions associated with age-related retinal degeneration, for example, in AMD and diabetic retinopathy. Arachidonic acid and arachidonic acid metabolites like prostaglandin \(E_2\) are mediators of retinal edema \[53,54\], the main cause of visual deterioration in nonproliferative diabetic retinopathy. The high salt–induced expression of NFAT5 may also contribute to diabetic retinal degeneration \[24\] and to the production of angiogenic factors \[20,21\], while the salt-induced release of ATP and autocrine/paracrine P2X, receptor signaling may accelerate the degeneration of the RPE in geographic atrophy \[33\]: the late stage of dry AMD; photoreceptor degeneration \[55\], a characteristic of AMD; and microvascular cell death \[56\], a hallmark of diabetic retinopathy. The data may support the assumption that reducing the ingestion of dietary salt may have protective effects in age-related retinal diseases. However, it remains to be determined in clinical studies whether strategies that decrease the plasma osmolarity via reducing the extracellular NaCl level, such as through the reduction of dietary salt intake, increase of water intake, and increased intake of blood

**Figure 8.** Schematic summary of signaling cascades which contribute to the osmotic expression of the NFAT5 gene in RPE cells. The NaCl-induced expression of the NFAT5 gene is fully dependent on intracellular calcium signaling mediated by phospholipase C (PLC) and inositol triphosphate (IP\(_3\))-induced release of calcium from the endoplasmic reticulum (ER), as well as on activation of phospholipase A2 (PLA\(_2\)). Autocrine/paracrine purinergic receptor signaling, as well as activation of further receptors like the fibroblast growth factor (FGF) receptor, may induce release of calcium from intracellular stores. High extracellular NaCl induces a release of ATP from the cells which activates calcium-permeable P2X, receptors and metabotropic P2Y, receptors. Extracellular ATP is converted to ADP by the ectonucleotidase nucleoside triphosphate diphosphohydrolase 2 (NTPDase2); ADP activates P2Y, receptors. P2 receptor signaling results in activation of nucleoside transporters, which mediate a release of adenosine that activates A\(_1\) receptors. Intracellular calcium signaling results in activation of calcium-dependent enzymes, such as protein kinase C (PKC), calpains, and PLA2. In addition to PKA and Src tyrosine kinases, these enzymes contribute to activation of multiple intracellular signal transduction pathways, including extracellular signal–regulated kinases 1 and 2 (ERK1/2), p38 mitogen-activated protein kinase (MAPK), c-Jun NH\(_2\)-terminal kinase (JNK), and phosphatidylinositol-3 kinase (PI3K) pathways. One of the transcription factors mediating activation of the NFAT5 gene is nuclear factor \(\kappa B\) (NF-\(\kappa B\)).
pressure–lowering minerals [57], have beneficial effects in preventing age-related retinal diseases. Pharmacological approaches to preventing the pathogenesis of age-related retinal diseases may include inhibitors of NFAT5 activity and inhibitors of enzymes that produce inflammatory lipids.

ACKNOWLEDGMENTS
The authors thank Ute Weinbrecht for excellent technical assistance. This work was supported by grants from the Deutsche Forschungsgemeinschaft (KO 1547/7–1 to L.K.) and the Geschwister Freter Stiftung (Hannover, Germany).

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