P2Y₁ Receptor Signaling Contributes to High Salt-Induced Priming of the NLRP3 Inflammasome in Retinal Pigment Epithelial Cells

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Abstract

Background

Systemic hypertension is a risk factor of age-related macular degeneration (AMD), a chronic inflammatory disease. Acute hypertension is caused by increased extracellular osmolarity after intake of dietary salt (NaCl). We determined in cultured human retinal pigment epithelial (RPE) cells whether high extracellular NaCl alters the gene expression of inflammasome-associated proteins, and whether autocrine/paracrine purinergic (P2) receptor signaling contributes to the NaCl-induced NLRP3 gene expression.

Methodology/Principal Findings

Hyperosmolarity was induced by the addition of 100 mM NaCl or sucrose to the culture medium. Gene and protein expression levels were determined with real-time RT-PCR and Western blot analysis, respectively. IL-1β and IL-18 levels were evaluated with ELISA. Nuclear factor of activated T cell 5 (NFAT5) expression was knocked down with siRNA. High extracellular NaCl induced NLRP3 and pro-IL-1β gene expression, while the gene expression of further inflammasome-associated proteins (NLRP1, NLRP2, NLRP6, NLRP7, NLRP12, NLRC4, AIM2, ASC, procaspase-1, pro-IL-18) was not altered or below the detection threshold. The NaCl-induced NLRP3 gene expression was partially dependent on the activities of phospholipase C, IP₃ receptors, protein kinase C, the serum and glucocorticoid-regulated kinase, p38 MAPK, ERK1/2, JNK, PI3K, and the transcription factors HIF-1 and NFAT5. Pannexin-dependent ATP release and P2Y₁ receptor activation is required for the full induction of NLRP3 gene expression. High NaCl induced a transient increase of the NLRP3 protein level and a moderate NLRP3 inflammasome activation, as indicated by the transient increase of the cytosolic level of mature IL-1β. High NaCl also induced secretion of IL-18.
Conclusion

High extracellular NaCl induces priming of the NLRP3 inflammasome in RPE cells, in part via P2Y<sub>1</sub> receptor signaling. The inflammasome priming effect of NaCl suggests that high intake of dietary salt may promote local retinal inflammation implicated in the development of AMD.

Introduction

Age-related macular degeneration (AMD) is the most common cause of irreversible blindness in the elderly of developed countries [1]. The majority of patients suffer from the dry form of AMD which is, in the late stage, characterized by geographic atrophy, i.e., degeneration of the outer retina including the retinal pigment epithelium (RPE). The remaining patients suffer from the neovascular form characterized by choroidal neovascularization. AMD is associated with systemic and local inflammation [2–4]. Generally, inflammatory processes are activated by cytosolic protein-signaling complexes, termed inflammasomes. Inflammasomes drive the proteolytic activation of caspase-1 and maturation of the inflammatory cytokines interleukin (IL)-1β and IL-18 [5, 6]. Inflammasomes are a group of protein complexes that consist of at least three components, (i) a receptor molecule that recognizes pathogen- and damage-associated molecular patterns, e.g., NOD (nucleotide-binding oligomerization domain receptors)-like receptors (NLRs), (ii) the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase-activating recruitment domain), and (iii) the cysteine protease caspase-1 [6]. Various lines of evidence indicate that the NLRP3 inflammasome is expressed in the RPE of eyes affected by geographic atrophy or neovascular AMD [7], that NLRP3 inflammasome activation in RPE cells is implicated in mediating RPE cell degeneration in geographic atrophy [8, 9], and that NLRP3 inflammasome activation in the RPE may promote neovascular AMD pathologies like RPE barrier breakdown and choroidal neovascularization [10]. The NLRP3 inflammasome in RPE cells can be activated by various factors and conditions that are suggested to be implicated in the pathogenesis of AMD such as complement factors, Alu RNA accumulation, lipofuscin-mediated photooxidative damage, peroxidized lipids, lysosomal destabilization, and overexpression of vascular endothelial growth factor (VEGF) [7–15].

In addition to advanced age, race, and genetic factors, lifestyle factors such as sun light exposure, cigarette smoking, and nutrition influence the risk of AMD. Furthermore, systemic hypertension is a risk factor of AMD [16–18]. The main condition that causes acute hypertension is increased extracellular osmolarity following intake of dietary salt (NaCl) [19, 20]. High extracellular NaCl and extracellular hyperosmolarity are known causes of systemic immune activation [21, 22]. In murine macrophages, inflammasomes are sensors of hyperosmotic stress [23]. Because elevated extracellular osmolarity and high extracellular NaCl induce the production and secretion of angiogenic factors like VEGF and basic fibroblast growth factor (bFGF) in RPE cells [24, 25], high salt intake may contribute to the progression of AMD towards the neovascular stage. However, it is not known whether high extracellular NaCl also induces priming and activation of inflammasomes in RPE cells. Therefore, we determined the effects of high extracellular NaCl on the gene expression of inflammasome-associated proteins in human RPE cells. We found that high NaCl induces priming and transient activation of the NLRP3 inflammasome in RPE cells. The NaCl-induced priming of the NLRP3 inflammasome is in part dependent on autocrine/paracrine P2Y<sub>1</sub> receptor signaling and the transcriptional activities of hypoxia-inducible transcription factor 1 (HIF-1) and nuclear factor of activated T cell 5 (NFAT5).
Materials and Methods

Ethics Statement

The study followed the tenets of Declaration of Helsinki 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). Tissues were obtained with the written informed consent from relatives of all donors.

Materials

Cell culture components and solutions were purchased from Gibco BRL (Paisley, UK). The recombinant human IL-1 receptor antagonist was from R&D Systems (Abingdon, UK). AG1478, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), HIF inhibitor, LY294002, PD98059, SP600125, and SU1498 were obtained from Calbiochem (Bad Soden, Germany). A-438079, AR-C 118925XX, ARL-67156, 8-(3-chlorostyryl) caffeine (CSC), GSK650394, caffeic acid phenethyl ester (CAPE), MRS2179, the pannexin-blocking peptide 10panx, the scrambled control peptide 10panxScr, and SB203580 were from Tocris (Ellisville, MO). Ac-YVAD-CHO was obtained from Santa Cruz Biotechnology (Heidelberg, Germany), and A-438079, 2-aminoethoxydiphenyl borate (2-APB), apyrase, Gö6976, H-89, N-nitrobenzylthioinosine (NBTI), PP2, SB431542, U73122, and all other agents used were from Sigma-Aldrich (Taufkirchen, Germany), unless stated otherwise. The following antibodies were used: a mouse anti-human NLRP3 (1:750; Enzo, Lausen, Switzerland) a rabbit anti-human β-actin (1:1000; Cell Signaling, Frankfurt/M., Germany), anti-rabbit IgG conjugated with alkaline phosphatase (1:2000; Cell Signaling), and anti-mouse IgG conjugated with alkaline phosphatase (1:2000; Cell Signaling).

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 (#745, 07/25/2011). Post-mortem 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 and cultured as described [26]. Cell lines derived from different donors were used in passages 3 to 5. In most experiments, near-confluent cultures (confluency ~90%) were growth arrested in medium without serum for 16 h, and subsequently, serum-free media with and without test substances were added. To determine whether the confluency degree of cultured RPE cells influences the high NaCl-induced gene expression of inflammasome-associated proteins and to examine the effect of NFAT5 siRNA, we used confluent cultures. The confluency of the cultures was evaluated microscopically. The isoosmotic control medium contained 127.6 mM NaCl and had an osmolarity of 281 mOsm/kg H₂O. Hyperosmotic media were made up by addition of NaCl or sucrose. Addition of 50 and 100 mM NaCl to the culture medium resulted in osmolarities of the media of 381 and 457 mOsm/kg H₂O, respectively. The hypoosmotic medium (60% osmolarity) was made up by adding distilled water. The cells were preincubated with pharmacological inhibitors for 30 min before osmotic challenge.
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 by agarose gel electrophoresis. The $A_{260}/A_{280}$ 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 total RNA using the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas, St. Leon-Roth, Germany).

Real-time RT-PCR

Real-time 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 cDNA, specific primer set (0.2 µM each), and 7.5 µl of a 2x 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; melting curve, 55°C with the temperature gradually (0.5°C) increased up to 95°C. The amplified samples were analyzed by standard agarose gel electrophoresis. The mRNA expression was normalized to the level of ß-actin mRNA. The changes in mRNA expression were calculated according to the $2^{-\Delta\Delta CT}$ method (CT, cycle threshold), with $\Delta CT = CT_{\text{target gene}} - CT_{\text{actb}}$ and $\Delta\Delta CT = \Delta CT_{\text{treatment}} - \Delta CT_{\text{control}}$.

Western blot analysis

The cells were washed twice with prechilled phosphate-buffered saline (pH 7.4; Invitrogen, Paisley, UK), 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 x g for 10 min, and the supernatants were analyzed by immunoblotting. Equal amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis; immunoreactive bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

ELISA

Cells were cultured at 3 x 10³ cells per well in 12-well plates. At a confluency of ~90%, the cells were cultured in serum-free medium for 16 h. Subsequently, the culture medium was changed, and the cells were stimulated with a hyperosmotic medium (+ 100 mM NaCl). Culture supernatants (1 ml) and cell lysates (150 µl) were collected after 3 and 6 h, and the levels of mature IL-1β and IL-18 were determined with ELISA (HSLB00C; R&D Systems; detection threshold: 0.067 pg/ml IL-1β; IL-18 ELISA Kit; RayBiotech, Norcross, GA).

siRNA transfection

Cells were seeded at 7 x 10⁴ cells per well in 12-well culture plates and were allowed to growth up to confluency of 60–80%. Thereafter, the cells were transfected with NFAT5 siRNA and nontargeted siRNA, respectively (10 nM each), using HiPerfect reagent (Qiagen) in F-10 medium containing 10% fetal bovine serum (Invitrogen) according to the manufacturer's instructions. After 24 or 48 h, serum-free iso- or hyperosmotic medium (+ 100 mM NaCl) was added for 6 h. Total RNA was extracted, and the NLRP3 mRNA level was determined with real-time RT-PCR analysis.
Cell viability

Cell viability was determined by trypan blue exclusion. Cells were seeded at 5 x 10^4 cells per well in 6-well plates. After reaching a confluency of ~90%, the cells were cultured in serum-free medium for 16 h and then in serum-free iso- or hyperosmotic medium (+ 100 mM NaCl) for 2 and 6 h. After trypsinization, the cells were stained with trypan blue (0.4%), and the number of viable (non-stained) and dead (stained) cells were determined using a hemocytometer.

Statistics

For each test, at least three independent experiments were performed using cell lines from different donors. Data are expressed as means ± SEM. Statistical analysis was made using Prism (Graphpad Software, San Diego, CA). Significance was determined by one-way ANOVA followed by Bonferroni’s multiple comparison test and by Mann-Whitney U test, respectively, and was accepted at P<0.05.

Results

Gene expression of inflammasome-associated proteins

Activation of inflammasomes requires an initial priming signal and a subsequent activation signal [5, 6]. The priming event is triggered by signals which induce expression of inflammasome receptor and pro-IL-1β genes [5, 6]. To determine whether high extracellular NaCl
induces expression of inflammasome-associated genes in RPE cells, we carried out real-time RT-PCR analysis using RNA extracted from cultured human RPE cell lines from different donors. Stimulation of the cells with high (+ 100 mM) extracellular NaCl induced a significant (P < 0.05) increase in the cellular level of NLRP3 mRNA while the expression levels of other inflammasome receptor genes (NLRP2, AIM2) were not significantly (P > 0.05) altered (Fig 1A). NLRP1, NLRP6, NLRP7, NLRP12, and NLRC4 mRNAs were not detected in RNAs extracted from different cell lines cultured under control and high NaCl conditions (not shown). Stimulation of RPE cells with high extracellular NaCl did also not alter the mRNA levels of the adaptor protein ASC and the inflammatory caspases 1, 4, and 5 (Fig 1A). High NaCl induced expression of the pro-IL-1β gene but not of the pro-IL-18 gene (Fig 1B). Similar results with upregulation of NLRP3 and pro-IL-1β gene expression, and no alterations in the expression of NLRP2, ASC, caspase 1, and pro-IL-18 genes, were found in confluent RPE cells stimulated with high NaCl (Fig 1C). The stimulatory effects on the expression of NLRP3 and pro-IL-

![Fig 1. Osmotic regulation of the gene expression of inflammasome-associated proteins in human RPE cells.](image)

The mRNA levels were determined with real-time RT-PCR analysis in cells cultured 2, 6, and 24 h (as indicated by the panels of the bars) under hyper- and hypoosmotic conditions, respectively, and are expressed as folds of unstimulated control. Hyperosmolarity was induced by addition of NaCl (100 mM) or sucrose (200 mM) to the culture medium. Hypoosmolarity (60% osmolality) was induced by addition of distilled water. A. Effects of high NaCl on the gene expression of inflammasome-associated proteins. Casp, caspase. B. Effects of high NaCl on the expression of pro-IL-1β and pro-IL-18 genes. C. Dose-dependent effects of high NaCl on the gene expression of inflammasome-associated proteins in confluent RPE cell cultures. 10, 50, and 100 mM NaCl were added to the culture medium, as indicated above the bars. D. Dose-dependent effect of high extracellular NaCl on the NLRP3 mRNA level in near-confluent cultures. 10 to 100 mM NaCl were added to the culture medium. E. Effects of sucrose-induced hyperosmolarity and extracellular hypoosmolarity on the expression of the NLRP3 gene. Each bar represents data obtained in 3–7 independent experiments using cell lines from different donors. Significant difference vs. unstimulated control: *P < 0.05.

doi:10.1371/journal.pone.0165653.g001
The β genes suggest that high extracellular NaCl is a priming signal for the NLRP3 inflammasome in RPE cells.

The effect of high extracellular NaCl on the NLRP3 mRNA level was dose-dependent (Fig 1C and 1D). The NLRP3 mRNA level was also increased in cells cultured in media that were made up hyperosmotic by addition of 200 mM sucrose (Fig 1E) which induced an equal elevation of the extracellular osmolarity (by 200 mOsm/kg H$_2$O) like 100 mM NaCl. The NaCl- (Fig 1A) and sucrose-induced expression of the NLRP3 gene (Fig 1E) displayed similar amplitudes and time dependencies, suggesting that the NaCl-induced NLRP3 gene expression was predominantly mediated by the elevation of the extracellular osmolarity. A hypoosmotic medium (60% osmolarity) induced a small transient increase of the NLRP3 gene expression (Fig 1E). The data indicate that the NLRP3 gene is transcriptionally activated in RPE cells by changes of the extracellular osmolarity.

**Intracellular signaling involved in NaCl-induced NLRP3 gene expression**

To determine the intracellular signaling involved in mediating the NaCl-induced expression of the NLRP3 gene in RPE cells, we tested pharmacological blockers of key intracellular signal transduction molecules. The NaCl-induced NLRP3 gene expression was significantly ($P < 0.05$) decreased, but not abrogated, by pharmacological inhibitors of the p38 mitogen-activated protein kinase (p38 MAPK; SB203580), extracellular signal-regulated kinases 1 and 2 (ERK1/2; PD98059), c-Jun NH$_2$-terminal kinase (JNK; SP600125), and phosphatidylinositol-3 kinases (PI3K; LY294002) signal transduction pathways (Fig 2). The NaCl-induced expression of the NLRP3 gene was also decreased by inhibitors of phospholipase C$_γ$ (PLC$_γ$; U73122), calcium-binding proteins (ruthenium red), protein kinases C (PKC) α/β (Gö6976), and the serum and glucocorticoid-regulated kinase (SGK; GSK650394) (Fig 2). 2-APB, an inhibitor of store-operated calcium entry channels, inositol trisphosphate (IP$_3$) receptors, and transient receptor potential (TRP) channels, fully prevented the NaCl-induced expression of the NLRP3 gene (Fig 2). The protein kinase A inhibitor H-89 and the inhibitor of Src tyrosine kinases PP2 had no effects (Fig 2). The cell-permeable reducing agent dithiothreitol and the reactive oxygen species (ROS) inhibitor N-acetyl-L-cysteine (NAC) did not inhibit the hyperosmotic expression of the NLRP3 gene (Fig 2). Furthermore, the inhibitor of mitochondrial permeability transition, cyclosporin A, was without effect (Fig 2). In addition, the cyclooxygenase inhibitor indomethacin and the antiinflammatory glucocorticoid triamcinolone acetonide did not inhibit the NaCl-induced NLRP3 gene expression (Fig 2). The data suggest that activation of various intracellular signal transduction cascades, as well as of PLC, IP$_3$ receptors, PKC, and SGK are involved in mediating the stimulatory effect of high extracellular NaCl on the expression of the NLRP3 gene in RPE cells.

**Receptor-dependent signaling involved in NaCl-induced NLRP3 gene expression**

It has been shown that extracellular hyperosmolarity induces a release of growth factors like VEGF, bFGF, and transforming growth factor (TGF)-β1 from RPE cells [24, 25]. To determine whether growth factor receptor signaling is involved in mediating the hyperosmotic expression of the NLRP3 gene in RPE cells, we tested pharmacological inhibitors of the following receptor kinases: VEGF receptor-2 (SU1498; 10 μM), platelet-derived growth factor receptor tyrosine kinase (AG1296; 10 μM), epidermal growth factor receptor tyrosine kinase (AG1478; 600 nM), TGF-β1 superfamily activin receptor-like kinase receptors (SB431542; 10 μM), and FGF receptor kinase (PD173074; 500 nM). Neither of these agents inhibited the increase of the NLRP3 gene expression in cells stimulated with high (+ 100 mM) NaCl for 6 h (data not shown).
Fig 2. Intracellular signaling involved in the NaCl-induced expression of the NLRP3 gene in RPE cells. The level of NLRP3 mRNA was determined with real-time RT-PCR analysis in cells cultured 6 h in iso- (control) and hyperosmotic (+ 100 mM NaCl) media. The following agents were tested: the inhibitor of p38 MAPK activation, SB203580 (10 μM), the inhibitor of ERK1/2 activation, PD98059 (20 μM), the JNK inhibitor SP600125 (10 μM), the inhibitor of PI3K-related kinases, LY294002 (5 μM), the PLCγ inhibitor U73122 (4 μM), the inhibitor of calcium-binding proteins, ruthenium red (Ru Red; 30 μM), the inhibitor
Adenosine 5′-triphosphate (ATP) released from stressed cells is a danger signal which activates the NLRP3 inflammasome in various cell systems [27]. Extracellular ATP acts at purinergic metabotropic (P2Y) and ionotropic (P2X) receptors. RPE cells were shown to express multiple purinergic receptor subtypes including P2Y, P2X, and adenosine receptors [8, 28]. By using RT-PCR analysis, we found that both acutely isolated and cultured RPE cells contain transcripts of P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2X<sub>7</sub>, and adenosine A<sub>1</sub> and A<sub>2B</sub> receptor genes (Fig 3A). The expression levels of P2X<sub>7</sub>, A<sub>1</sub>, and A<sub>2B</sub> receptor genes were similar in acutely isolated and cultured cells while the expression levels of P2Y<sub>1</sub> and P2Y<sub>2</sub> receptor genes were lower in cultured cells compared to acutely isolated cells, as indicated by the significantly (P<0.05) increased cycle numbers necessary for the detection of the transcripts (Fig 3B). The expression levels of P2X<sub>7</sub> transcripts in acutely isolated and cultured cells were very low, as indicated by the high cycle threshold numbers (Fig 3B).

To determine whether purinergic receptor signaling is involved in mediating the NaCl-induced NLRP3 gene expression in RPE cells, we used pharmacological receptor antagonists. We found that addition of the ATP-hydrolyzing enzyme apyrase and the P2Y<sub>1</sub> receptor antagonist MRS2179, respectively, to the culture medium decreased significantly (P<0.05) the NLRP3 mRNA level in RPE cells under unstimulated control conditions (Fig 3C). Both agents also decreased (by 40–50%) the NaCl-induced NLRP3 gene expression (Fig 3C). On the other hand, antagonists of P2Y<sub>2</sub> (AR-C 118925XX), P2X<sub>7</sub> (A-438079), adenosine A<sub>1</sub> (DPCPX), and adenosine A<sub>2A</sub> receptors (CSC), as well as the ecto-ATPase inhibitor ARL-67156 and the antagonist of nucleoside transporters, NBTI, had no effects on the cellular level of NLRP3 transcripts under control and NaCl-stimulated conditions (Fig 3C). The data suggest that the NLRP3 gene expression under normal and high NaCl conditions is in part regulated by autocrine/paracrine purinergic signaling that involves a release of ATP and P2Y<sub>1</sub> receptor activation. It has been shown that hyperosmotic stress induces a pannexin-1 mediated release of ATP from T cells [29]. We found that the pannexin-blocking peptide<sup>10</sup>panx decreased the NaCl-induced expression of the NLRP3 gene in RPE cells to a similar extent like apyrase while a scrambled control peptide had no effect (Fig 3C). The data suggest that high extracellular NaCl induces a pannexin-dependent release of ATP from RPE cells.

Role of transcription factors in NaCl-induced NLRP3 gene expression

It has been shown that hyperosmotic stress induces expression of various transcription factors in RPE cells like HIF-1α, nuclear factor (NF)-κB, and NFAT5 [24]. To determine which transcription factors mediate the NaCl-induced NLRP3 gene expression in RPE cells, we used pharmacological blockers. The NaCl-induced NLRP3 gene expression was not altered in the presence of the inhibitor of signal transducer and activator of transcription 3 (STAT3), Stattic [30], and the NF-κB inhibitor CAPE [31], while a HIF inhibitor [32] partially inhibited the hyperosmotic expression of the NLRP3 gene (Fig 4A).

In various cell systems, cellular survival in hyperosmotic stress depends on the transcriptional activity of NFAT5 [33]. To determine whether NFAT5 activity is involved in mediating the NaCl-induced expression of the NLRP3 gene in RPE cells, we knocked down NFAT5.
Fig 3. Involvement of P2Y<sub>1</sub>, receptor signaling in mediating the NaCl-induced expression of the NLRP3 gene in RPE cells. A. Presence of P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2X<sub>7</sub>, adenosine A<sub>1</sub> receptor, and adenosine A<sub>2B</sub> receptor gene transcripts in the cells. To confirm the correct lengths of PCR products, agarose gel electrophoresis was carried out using products obtained from cultured cells of the 4th passage (1) and from cells that were acutely isolated from eyes of two post-mortem donors without apparent eye diseases (2, 3). Negative controls (0) were done by adding double-distilled water instead of cDNA as

B. Acutely isolated cells
Cultured cells

Δ cycle threshold number

P2Y<sub>1</sub> P2Y<sub>2</sub> P2X<sub>7</sub> A<sub>1</sub> A<sub>2B</sub>

C

Control NaCl

NLRP3 mRNA (fold of control)

Apyrase MRS2179 AR-C A-438079 DPCPX CSC ARL-67156 NBTI 10 Panx Scr

Control Apyrase MRS2179 AR-C A-438079 DPCPX CSC ARL-67156 NBTI 10 Panx Scr

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expression by transfection of the cells with NFAT5 siRNA. As negative control, nontargeted scrambled siRNA was used. As shown in Fig 4B, high NaCl induced a significantly ($P < 0.05$) smaller increase of the NLRP3 mRNA level in cells transfected with NFAT5 siRNA compared to cells transfected with nontargeted siRNA. The data suggest that the NaCl-induced expression of the NLRP3 gene in RPE cells is in part dependent on the activities of HIF-1 and NFAT5.

High NaCl induces transient NLRP3 inflammasome activation

After the initial priming event, inflammasome activation is triggered by signals which induce assembly of NLRP3, ASC, and procaspase-1 into the inflammasome protein complex; activated inflammasomes drive the processing of procaspase-1 into the active form and the maturation of IL-1$\beta$ [5, 6]. Western blot analysis showed that RPE cells contained NLRP3 protein at low level, and that the level of NLRP3 protein increased time-dependently under unstimulated control conditions (Fig 5A) (while the level of NLRP3 mRNA did not increase under control conditions; not shown). The data suggest that NLRP3 protein is continuously produced in unstimulated RPE cells. Stimulation with high extracellular NaCl induced a significant ($P < 0.05$) increase of the cytosolic NLRP3 protein level after 3 h of stimulation, and a decrease of the NLRP3 protein level after 6 h of stimulation (Fig 5A and 5B).

In order to determine whether high extracellular NaCl induces activation of the NLRP3 inflammasome, we determined with ELISA the levels of mature IL-1$\beta$ in RPE cell lysates and culture supernatants. As shown in Fig 5C, the cytosolic level of mature IL-1$\beta$ increased time-dependently under unstimulated control conditions (while the level of pro-IL-1$\beta$ mRNA did not increase under control conditions; not shown). The data suggest that NLRP3 protein is continuously produced in unstimulated RPE cells. Stimulation with high extracellular NaCl induced a significant ($P < 0.05$) increase (to 199.9 ± 39.5% of control, 100%) of the cytosolic IL-1$\beta$ level after 3 h of stimulation (Fig 5C). On the other hand, the cytosolic IL-1$\beta$ level in cells cultured 6 h under control and NaCl-stimulated conditions were not significantly ($P > 0.05$) different (Fig 5C).

As positive control, lysosomes were destabilized using the lysosomotropic agent L-leucyl-L-leucine methyl ester which is known to induce NLRP3 inflammasome activation in RPE cells [7]. As shown in Fig 5C, lysosomal destabilization resulted in a time-dependent increase of the cytosolic level of mature IL-1$\beta$ in RPE cells, in the mean to 555.6 ± 203.6% and 1205.8 ± 315.7% of control (100%), respectively, after 3 and 6 h of stimulation. However, we did not detect IL-1$\beta$ with ELISA in the conditioned media of cells cultured 3 or 6 h under isoosmotic control and hyperosmotic (+ 100 mM NaCl) conditions, or in the presence of L-leucyl-L-leucine methyl ester (1 mM; data not shown). On the other hand, we found a time-dependent increase of the IL-18 protein level in the cultured media during stimulation of the cells with high extracellular NaCl (Fig 5D), suggesting that high NaCl induces a secretion of IL-18 from RPE cells. The data suggest that high NaCl induces a transient activation of the NLRP3 inflammasome resulting in maturation of IL-1$\beta$ and secretion of IL-18.
Involvement of P2Y<sub>1</sub> receptor activation in NaCl-induced angiogenic factor expression

It has been shown that high extracellular NaCl induces gene expression of angiogenic factors like VEGF and bFGF in RPE cells [24, 25]. The caspase-1 inhibitor Ac-YVAD-CHO and a recombinant human IL-1 receptor antagonist did not inhibit the NaCl-induced expression of VEGF and bFGF genes in RPE cells (Fig 6A and 6B), suggesting that the hyperosmotic expression of angiogenic factor genes is independent on inflammasome activation. However, the NaCl-induced expression of VEGF and bFGF genes was significantly (<i>P</i>&lt;0.05) decreased by the P2Y<sub>1</sub> receptor antagonist MRS2179 while the P2X<sub>7</sub> receptor antagonist A-438079 had no effect (Fig 6A and 6B).

RPE cell viability

To determine whether osmotic stress alters RPE cell viability, we measured the viability of cells cultured in the presence of different NaCl concentrations. As shown in Fig 7A, increases of the...
extracellular NaCl level by more than 10 mM induced a moderate, dose-dependent decrease in the viability of RPE cells which was significant (P < 0.05) after 6 h of stimulation. Induction of NLRP3 inflammasome activation by lysosomal destabilization with L-leucyl-L-leucine methyl ester resulted in a significant (P < 0.05) decrease of the cell viability in the presence but not in the absence of high NaCl (Fig 7B).

**Discussion**

Chronic innate immune activation contributes to the dysfunction and progressive degeneration of the RPE underlying AMD [2, 4]. Activation of the NLRP3 inflammasome was implicated in mediating the degeneration of the RPE in geographic atrophy [8, 9], the late stage of dry AMD, and the development of choroidal neovascularization [7, 10], the hallmark of neovascular AMD. Systemic hypertension is a risk factor of AMD [16–18]. The main condition that induces acute hypertension is the increase of the extracellular osmolarity following intake of dietary salt [19, 20]. Elevated extracellular osmolarity and high extracellular NaCl are known to induce systemic immune activation [21, 22]. In the present study, we investigated whether high extracellular osmolarity and NaCl induce priming and activation of inflammasomes in RPE cells. We found that high extracellular NaCl induces expression of the NLRP3 gene in RPE cells (Fig 1A) while the expression of other inflammasome receptor genes remained unaltered (NLRP2, AIM2) or was below the detection threshold (NLRP1, NLRP6, NLRP7, NLRP12, NLRC4). High NaCl also induced expression of the pro-IL-1β gene and had no effect on the pro-IL-18 gene expression (Fig 1B). The data are consistent with recent studies which showed that IL-18, but not IL-1β, is constitutively expressed in RPE cells [14], and that NLRP3 inflammasome activation in a RPE cell line results in production of IL-1β, but not IL-18 [34].
The findings that high NaCl did not induce expression of ASC and pro-caspase-1 genes (Fig 1A) are in line with a study in macrophages which showed that lipopolysaccharide-induced priming of the cells involves NLRP3 and pro-IL-1β gene expression, but not alterations of ASC and pro-caspase-1 gene expression [35]. The stimulatory effects on the expression of NLRP3 and pro-IL-1β genes suggest that high extracellular NaCl induces priming of the NLRP3 inflammasome in RPE cells.

The increases of the levels of NLRP3 and mature IL-1β proteins (Fig 5A–5C) indicate that the NLRP3 inflammasome is continuously activated in cultured RPE cells under unstimulated control conditions (Fig 5A–5C). Priming of the NLRP3 inflammasome under unstimulated control conditions is also suggested by the effects of apyrase and the P2Y_{1} receptor antagonist which decreased the control level of NLRP3 gene expression (Fig 3C). High extracellular NaCl induced a transient increase of the NLRP3 protein level in RPE cells (Fig 5A and 5B) and a transient activation of the NLRP3 inflammasome, as indicated by the increase of the cytosolic

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**Fig 6.** The NaCl-induced expression of VEGF (A) and bFGF (B) genes depends in part on P2Y_{1} receptor signaling. The mRNA levels were determined with real-time RT-PCR analysis in cells cultured 6 h in iso- (control) and hyperosmotic (+ 100 mM NaCl) media, and are expressed as folds of control. The following pharmacological agents were tested: the caspase-1 inhibitor Ac-YVAD-CHO (Ac-Y; 500 nM), a recombinant human IL-1 receptor antagonist (IL-1RA; 1 μg/ml), the P2Y_{1} receptor antagonist MRS2179 (30 μM), and the P2X_{7} receptor antagonist A-438079 (50 nM). Means ± SEM of 3–7 independent experiments using cell lines from different donors. Significant difference vs. unstimulated control: *P<0.05. Significant difference vs. NaCl control: ●P<0.05.

doi:10.1371/journal.pone.0165653.g006
level of mature IL-1β which was observed after 3 h of stimulation (Fig 5C). After 6 h of stimulation with high NaCl, the NLRP3 protein level returned to the control level, and the cytosolic IL-1β level was not different between cells cultured under control and high-NaCl conditions (Fig 5B and 5C). On the other hand, continuous activation of the NLRP3 inflammasome induced by lysosomal destabilization with L-leucyl-L-leucine methyl ester resulted in a higher level of cytosolic IL-1β after 6 h compared to 3 h of stimulation (Fig 5C). We did not detect IL-1β in the conditioned media of cells cultured in the presence of high NaCl or L-leucyl-L-leucine methyl ester (not shown). However, we found that high NaCl induces a secretion of IL-18 from RPE cells (Fig 5D). The present data are in agreement with a recent study which showed that RPE cells (in contrast to bone marrow-derived cells) release IL-18 rather than IL-1β in response to various triggers of inflammasome activation [14]. We found that the activation of the NLRP3 inflammasome is transient under high-NaCl conditions. It can not be ruled out that the time-dependent deactivation of the NLRP3 inflammasome and the failure of IL-1β secretion result (at least in part) from the reduction of the cell viability under high NaCl conditions (Fig 7A). However, the fact that activation of the NLRP3 inflammasome by lysosomal destabilization decreases the RPE cell viability (Fig 7B) may suggest that the time-dependent...
deactivation of the NLRP3 inflammasome supports the survival of RPE cells under high NaCl conditions.

We found that both extracellular hyper- and hypoosmolarity induced NLRP3 gene expression (Fig 1A and 1E). This is consistent with previous studies which showed that various activators of the NLRP3 inflammasome produce cell shrinking or swelling which can be also induced by extracellular hyper- and hypoosmolarity, respectively [36, 37]. However, the hyper- and hypoosmotic expression of the NLRP3 gene in RPE cells is likely mediated by different signal transduction mechanisms. We found that the activity of NFAT5 is involved in mediating the hyperosmotic induction of the NLRP3 gene expression (Fig 4B). It has been shown that the expression of NFAT5 in RPE cells is increased by extracellular hyperosmolarity and decreased by extracellular hypoosmolarity [24]. The lack of NFAT5 activation under hypoosmotic conditions may (at least in part) explain the relatively small activation of the NLRP3 gene under these conditions (Fig 1E). The data also suggest that different intracellular signaling mechanisms may contribute independently to the NaCl-induced expression of the NLRP3 gene.

We found evidence that multiple signal transduction pathways mediate the NaCl-induced expression of the NLRP3 gene in RPE cells. The NaCl-induced NLRP3 gene expression is in part dependent on activation of p38 MAPK, ERK1/2, JNK, PI3K signal transduction pathways, and the activities of phospholipase C, IP\textsubscript{3} receptors, protein kinase C, and SGK. SGK, a main mediator of cellular sodium homeostasis, is induced by high extracellular NaCl in various cell systems and increases the protein abundance and activity of ion channels, carriers, and the sodium/potassium-ATPase in response to osmotic stress [38, 39]. We also found evidence that the NaCl-induced expression of the NLRP3 gene depends in part on the transcriptional activities of HIF-1 and NFAT5. However, it remains to be determined whether the NLRP3 gene is directly or indirectly activated by HIF-1 and NFAT5. It is likely that the transcriptional activities of HIF-1 and NFAT5 mediate the expression of protein kinases like SGK [40] which are involved in the regulation of the RPE cell response to osmotic stress. The present data are in agreement with previous studies which showed in various cell systems that HIF-1 activity is implicated in the induction of NLRP3 inflammasome activation [41, 42].

It has been shown that the NLRP3 inflammasome can be activated by ROS-dependent and -independent mechanisms [43]. We found that the reducing agent dithiothreitol and the ROS inhibitor NAC did not alter the NaCl-induced expression of the NLRP3 gene (Fig 2). The data suggest that oxidative stress does not play a role in the NaCl-induced priming of the NLRP3 inflammasome in RPE cells. In addition, the inhibitor of mitochondrial destabilization, cyclosporin A, did not reduce the NaCl-induced expression of the NLRP3 gene (Fig 2), suggesting that mitochondrial dysfunction, a main cause of cellular oxidative stress, plays no role. The data are consistent with our observations that high NaCl does not induce gene expression of the nuclear factor E2-related factor-2 (NRF2) in RPE cells (not shown), a key transcription factor that regulates the cellular antioxidative defense, and that the high NaCl-induced expression of the NFAT5 gene is also not dependent on oxidative stress and mitochondrial dysfunction (not shown).

We found evidence that autocrine/paracrine purinergic signaling is required for the full expression of the NLRP3 gene in response to osmotic stress. This signaling involves a pan-nexin-dependent release of ATP and P2Y\textsubscript{1} receptor activation (Fig 3C). Activation of P2Y\textsubscript{1} receptors may trigger a PLC- and IP\textsubscript{3}-mediated calcium mobilization from internal stores and activation of PKC; inhibition of these intracellular pathways decreased the NaCl-induced expression of the NLRP3 gene (Fig 2) to a similar extent as apyrase and the P2Y\textsubscript{1} receptor blocker MRS2179 (Fig 3C). PLC- and IP\textsubscript{3}-mediated calcium mobilization was described to be critical for the activation of the NLRP3 inflammasome in macrophages after stimulation with extracellular ATP [44]. We also found that the NaCl-induced expression of VEGF and bFGF
genes depends in part on P2Y$_1$ receptor signaling (Fig 6A and 6B). However, because the NaCl-induced expression of VEGF and bFGF genes was independent on inflammasome activation (Fig 6A and 6B), activation of P2Y$_1$ receptors may independently trigger angiogenic factor expression and priming of the inflammasome in RPE cells. The finding that the NaCl-induced expression of VEGF and bFGF genes is independent on inflammasome activation is in line with a recent study which showed that inflammasome activation in RPE cells reduces, but not increases, the constitutive secretion of VEGF [45].

We found significant effects of high extracellular NaCl on the expression of the NLRP3 gene when more than 10 mM NaCl were added to the culture medium (Fig 1D). It is generally accepted that the highest pathological blood osmolarity in human subjects is around 360 mOsm/kg which can be achieved by addition of 40 mM NaCl to the culture medium [39, 46]. However, less well appreciated is that the local extracellular NaCl concentration in the interstitium may be considerably higher (160–250 mM) than the plasma concentration of NaCl (~140 mM) [47, 48]. Therefore, the present results may have relevance for in-vivo conditions.

In the developed world, the intake of dietary salt rapidly increased in the past along with the consumption of processed foods which often contain salt contents more than 100 times higher compared to home-made meals [49]. Increased intake of dietary salt may represent an environmental risk factor for the progression of AMD [24]. Here, we show that high extracellular NaCl induces priming of the NLRP3 inflammasome in RPE cells. In addition to the stimulatory effects of high salt on systemic immune processes [21, 22], salt-induced inflammasome priming in RPE cells may contribute to retinal inflammation in AMD. High salt may render RPE cells more susceptible to inflammatory stimuli and pathogenic factors involved in mediating degeneration of the RPE. This assumption is supported by the fact that lysosomal destabilization induces RPE cell death under high NaCl conditions but not under control conditions (Fig 7B). This mechanism may link dietary salt intake as the main cause of acute hypertension and innate immune activation in RPE pathology. Because the NLRP3 inflammasome is time-dependently deactivated in response to long-term NaCl stimulation (Fig 5A–5C), repetitive increases of the plasma NaCl level in postprandial phases will have greater effects than a persistent elevation of the NaCl level. We found that autocrine/paracrine P2Y$_1$ receptor signaling is involved in mediating the salt-induced priming of the NLRP3 inflammasome in RPE cells and the expression of angiogenic factor genes. Because both effects may contribute to the development and progression of AMD, P2Y$_1$ receptors may represent a target for the development of pharmacological approaches to treat age-related retinal inflammation and degeneration.

**Acknowledgments**

The authors thank Ute Weinbrecht for excellent technical assistance.

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**Funding acquisition:** PW LK.

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References

1. Klein R, Klein BE, Knudtson MD, Meuer SM, Swift M, Gangnon RE (2007) Fifteen-year cumulative incidence of age-related macular degeneration: the Beaver Dam Eye Study. Ophthalmology 114: 253–262. doi: 10.1016/j.ophtha.2006.10.040 PMID: 17270675

2. Xu H, Chen M, Forrester JV (2009) Para-inflammation in the aging retina. Prog Retin Eye Res 28: 349–368. doi: 10.1016/j.preteyeres.2009.06.001 PMID: 19560552

3. Cheung CM, Wong TY (2014) Is age-related macular degeneration a manifestation of systemic disease? New prospects for early intervention and treatment. J Intern Med 276: 140–153. doi: 10.1111/joim.12227 PMID: 24581182

4. Nita M, Grzybowski A, Ascaso FJ, Huerva V (2014) Age-related macular degeneration in the aspect of chronic low-grade inflammation (pathophysiological para-inflammation). Mediators Inflamm 2014: 930671. doi: 10.1155/2014/930671 PMID: 25214719

5. Gross O, Thomas CJ, Guarda G, Tschopp J (2011) The inflammasome: an integrated view. Immuno logical Rev 243: 136–151.

6. Latz E, Xiao TS, Stutz A (2013) Activation and regulation of the inflammasomes. Nat Rev Immunol 13: 397–411. doi: 10.1038/nri3452 PMID: 23702978

7. Tseng WA, Thein T, Kinnunen K, Lashkari K, Gregory MS, D’Amore PA, et al. (2013) NLRP3 inflammasome activation in retinal pigment epithelial cells by lysosomal destabilization: implications for age-related macular degeneration. Invest Ophthal Vis Sci 54: 110–120. doi: 10.1167/iovs.12-10655 PMID: 23221073

8. Kerur N, Hirano Y, Tarallo V, Fowler BJ, Bastos-Carvalho A, Yasuma T, et al. (2013) TLR-independent and P2X7-dependent signaling mediate Alu RNA-induced NLRP3 inflammasome activation in geographic atrophy. Invest Ophthal Vis Sci 54; 7395–7401. doi: 10.1167/iovs.13-12500 PMID: 24114355

9. Fowler BJ, Gelfand BD, Kim Y, Kerur N, Taralovo V, Hirano Y, et al. (2014) Nucleoside reverse transcriptase inhibitors possess intrinsic anti-inflammatory activity. Science 346: 1000–1003. doi: 10.1126/ science.1261754 PMID: 25141314

10. Marneros AG (2013) NLRP3 inflammasome blockade inhibits VEGF-A-induced age-related macular degeneration. Cell Rep 4: 945–958. doi: 10.1016/j.celrep.2013.08.002 PMID: 24012762

11. Doyle SL, Campbell M, Ozaki E, Salomon RG, Mori A, Kenna PF, et al. (2012) NLRP3 has a protective role in age-related macular degeneration through the induction of IL-18 by drusen components. Nat Med 18: 791–798. doi: 10.1038/nm.2717 PMID: 22484808

12. Kauppinen A, Niskanen H, Suuronen T, Kinnunen K, Salminen A, Kaarmiranta K (2012) Oxidative stress activates NLRP3 inflammasomes in ARPE-19 cells—implications for age-related macular degeneration (AMD). Immunol Lett 147: 29–33. doi: 10.1016/j.imlet.2012.05.005 PMID: 22698861

13. Anderson OA, Finkelstein A, Shima DT (2013) A2E induces IL-1β production in retinal pigment epithelial cells via the NLRP3 inflammasome. PLoS One 8: e67263. doi: 10.1371/journal.pone.0067263 PMID: 24364044

14. Shi G, Chen S, Wandu WS, Ogbeitun O, Nugent LF, Maminishkis A, et al. (2015) Inflammasomes induced by 7-ketocholesterol and other stimuli in RPE and in bone marrow-derived cells differ markedly in their production of IL-1β and IL-18. Invest Ophthal Vis Sci 56: 1658–1664. doi: 10.1167/iovs.14-14557 PMID: 25678688

15. Brandstetter C, Mohr LK, Latz E, Holz FG, Krohne TU (2015) Light induces NLRP3 inflammasome activation in retinal pigment epithelial cells via lipofuscin-mediated photooxidative damage. J Mol Med (Berl) 93: 905–916.

16. Sperduto RD, Hiller R (1986) Systemic hypertension and age-related maculopathy in the Framingham Study. Arch Ophthalmol 104: 216–219. PMID: 3947296
17. Klein R, Klein BE, Tomany SC, Cruickshanks KJ (2003) The association of cardiovascular disease with the long-term incidence of age-related maculopathy: the Beaver Dam Eye Study. Ophthalmology 110: 1273–1280. doi: 10.1016/S0161-6420(03)00599-2 PMID: 12799274

18. Van Leeuwen R, Ikram MK, Vingerling JR, Witteman JC, Hofman A, de Jong PT (2003) Blood pressure, atherosclerosis, and the incidence of age-related maculopathy: the Rotterdam Study. Invest Ophthalmol Vis Sci 44: 3771–3777. PMID: 12939290

19. Lifton RP, Gharavi AG, Geller DS (2001) Molecular mechanisms of human hypertension. Cell 104: 545–556. PMID: 11557392

20. He FJ, Markandu ND, Sagnella GA, de Wardener HE, MacGregor GA (2005) Plasma sodium: ignored and underestimated. Hypertension 45: 98–102. doi: 10.1161/01.HYP.0000149341.79450.a2 PMID: 15557392

21. Junger WG, Liu FC, Loomis WH, Hoyt DB (1994) Hypertonic saline enhances cellular immune function. Circ Shock 42: 190–196. PMID: 8055665

22. Yi B, Titze J, Rykova M, Feuerrecker M, Vassilieva G, Nichiporuk I, et al. (2015) Effects of dietary salt levels on monocyte cells and immune responses in healthy human subjects: a longitudinal study. Transl Res 166: 103–110. doi: 10.1016/j.trsl.2014.11.007 PMID: 25497276

23. Ip WKE, Medzhitov R (2015) Macrophages monitor tissue osmolarity and induce inflammatory response through NLRP3 and NLRC4 inflammasome activation. Nat Commun 6: 6931. doi: 10.1038/ncomms7931 PMID: 25959047

24. Hollborn M, Vogler S, Reichenbach A, Wiedemann P, Bringmann A, Kohen L (2015) Regulation of the hyperosmotic induction of aquaporin 5 and VEGF in retinal pigment epithelial cells: Involvement of NFAT5. Mol Vis 21: 360–377. PMID: 25878490

25. Veltmann M, Hollborn M, Reichenbach A, Wiedemann P, Kohen L, Bringmann A (2016) Osmotic induction of angiogenic growth factor expression in human retinal pigment epithelial cells. PLoS One 11:e0147312. doi: 10.1371/journal.pone.0147312 PMID: 26800359

26. Chen R, Hollborn M, Groche A, Reichenbach A, Wiedemann P, Bringmann A, et al. (2014) Effects of the vegetable polyphenols epigallocatechin-3-gallate, luteolin, apigenin, myricetin, quercetin, and cyanidin in retinal pigment epithelial cells. Mol Vis 20: 242–258.

27. Bours MJ, Dagnelie PC, Giuliani AL, Wesselius A, Di Virgilio F (2011) P2 receptors and extracellular ATP: a novel homeostatic pathway in inflammation. Front Biosci (Schol Ed) 3: 1443–1456.

28. Housley GD, Bringmann A, Reichenbach A (2009) Purinergic signaling in special senses. Trends Neurosci 32: 128–141. doi: 10.1016/j.tins.2009.01.001 PMID: 19232752

29. Woehrle T, Yip L, Manohar M, Sumi Y, Yao Y, Chen Y, et al. (2010) Hypertonic stress regulates T cell function via pannexin-1 hemichannels and P2X receptors. J Leukoc Biol 88: 1181–1189. doi: 10.1189/jlb.0410211 PMID: 20884646

30. Schust J, Sperl B, Hollis A, Mayer TU, Berg T (2006) Statin: a small-molecule inhibitor of STAT3 activation and dimerization. Chem Biol 13: 1235–1242. doi: 10.1016/j.chembiol.2006.09.018 PMID: 17114005

31. Natarajan K, Singh S, Burke TR Jr, Grunberger D, Aggarwal BB (1996) Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-κB. Proc Natl Acad Sci U S A 93: 9090–9095. PMID: 8799159

32. Lee K, Lee JH, Boovanahalli SK, Jin Y, Lee M, Jin X, et al. (2007) (Aryloxycarbonylamino)benzoic acid analogues: a new class of hypoxia-inducible factor-1 inhibitors. J Med Chem 50: 1675–1684. doi: 10.1021/jm0610292 PMID: 17328532

33. Cheung CY, Ko BC (2013) NFAT5 in cellular adaptation to hypertonic stress—regulations and functional significance. J Mol Signal 8: 5. doi: 10.1186/1750-2187-8-5 PMID: 23618372

34. Pippino N, Korkmaz A, Hyttl M, Kinnunen K, Salminen A, Atalay M, et al. (2014) Decline in cellular clear ance systems induces inflammasome signaling in human ARPE-19 cells. Biochim Biophys Acta 1843: 3038–3046. doi: 10.1016/j.bbamcr.2014.09.015 PMID: 25268952

35. Bauernfeind F, Bartok E, Rieger A, Franchi L, Nuñez G, Hornung V (2011) Cutting edge: reactive oxygen species inhibitors block priming, but not activation, of the NLRP3 inflammasome. J Immunol 187: 613–617. doi: 10.4049/jimmunol.1100613 PMID: 21677136

36. Taylor SR, Gonzalez-Begne M, Dewhurst S, Chimini G, Higgins CF, Melvin JE, et al. (2008) Sequential shrinkage and swelling underlie P2X7-stimulated lymphocyte phosphatidyserine exposure and death. J Immunol 180: 300–308. PMID: 18097031

37. Schorn C, Frey B, Lauber K, Janko C, Stryss M, Keppeler H, et al. (2011) Sodium overload and water influx activate the NALP3 inflammasome. J Biol Chem 286: 35–41. doi: 10.1074/jbc.M110.139048 PMID: 21051542
38. Lang F, Böhmer C, Palmada M, Seebohm G, Strutz-Seebohm N, Vallon V (2006) (Patho)physiological significance of the serum- and glucocorticoid-inducible kinase isoforms. Physiol Rev 86: 1151–1178. doi: 10.1152/physrev.00050.2005 PMID: 17015487

39. Wu C, Yosel N, Thalhamer T, Zhu C, Xiao S, Kishi Y, et al. (2013) Induction of pathogenic T\(\text{H}\)17 cells by inducible salt-sensing kinase SGK1. Nature 496: 513–517. doi: 10.1038/nature11984 PMID: 23467085

40. Chen S, Grigsby CL, Law CS, Ni X, Nekrep N, Olsen K, et al. (2009) Tonicity-dependent induction of Sgk1 expression has a potential role in dehydration-induced natriuresis in rodents. J Clin Invest 119: 1647–1658. doi: 10.1172/JCI35314 PMID: 19436108

41. Nicholas SA, Bubnov VV, Yasinska IM, Sumbayev VV (2011) Involvement of xanthine oxidase and hypoxia-inducible factor 1 in Toll-like receptor 7/8-mediated activation of caspase 1 and interleukin-1β. Cell Mol Life Sci 68: 151–158. doi: 10.1007/s00018-010-0450-3 PMID: 20632067

42. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGlinchey AF, Goel G, et al. (2013) Succinate is an inflammatory signal that induces IL-1β through HIF-1α. Nature 496: 238–242. doi: 10.1038/nature11986 PMID: 23535595

43. Iyer SS, He Q, Janczy JR, Elliott EI, Zhong Z, Olivier AK, et al. (2013) Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation. Immunity 39: 311–323. doi: 10.1016/j.immuni.2013.08.001 PMID: 23954133

44. Murakami T, Ockinger J, Yu J, Byles V, McColl A, Hofer AM, et al. (2012) Critical role for calcium mobilization in activation of the NLRP3 inflammasome. Proc Natl Acad Sci U S A 109: 11282–11287. doi: 10.1073/pnas.1117765109 PMID: 22733741

45. Mohr LK, Hoffmann AV, Brandstetter C, Holz FG, Krohne TU (2015) Effects of inflammasome activation on secretion of inflammatory cytokines and vascular endothelial growth factor by retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 56: 6404–6413. doi: 10.1167/iovs.15-16898 PMID: 26447271

46. Kleineviehsfeld M, Manzel A, Titze J, Kvakan H, Yosef N, Linker RA, Muller DN, et al. (2013) Sodium chloride drives autoimmune disease by the induction of pathogenic T\(\text{H}\)17 cells. Nature 496: 518–522. doi: 10.1038/nature11868 PMID: 23467095

47. Go WY, Liu X, Roti MA, Liu F, Ho SN (2004) NFAT5/TonEBP mutant mice define osmotic stress as a critical feature of the lymphoid microenvironment. Proc Natl Acad Sci U S A 101: 10673–10678. doi: 10.1073/pnas.0403139101 PMID: 15247420

48. Machnik A, Neuhofer W, Jantsch J, Dahlmann A, Tammela T, Machura K, et al. (2009) Macrophages regulate salt-dependent volume and blood pressure by a vascular endothelial growth factor-C-dependent buffering mechanism. Nat Med 15: 545–552. doi: 10.1038/nm.1960 PMID: 19412173

49. Appel Lj, Frohlich ED, Hall JE, Pearson TA, Sacco RL, Seals DR, et al. (2011) The importance of population-wide sodium reduction as a means to prevent cardiovascular disease and stroke: a call to action from the American Heart Association. Circulation 123: 1138–1143. doi: 10.1161/CIR.0b013e31820a0793 PMID: 21293238