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Cyclosporin-A Induced Toxicity in Rat Renal Collecting Duct Cells: Interference with Enhanced Hypertonicity Induced Apoptosis

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Key Words

Cyclosporin-A• Nephrotoxicity • TNFα • Death receptor • Hyperosmolality

Abstract

Background/ Aims: Rat renal inner medullary collecting duct (IMCD) cells are physiologically exposed to a wide range of ambient tonicity. To maintain their function upon changes in osmolality, IMCD cells induce expression of osmoprotective and antiapoptotic genes, mainly mediated by the transcription factor Tonicity Enhancer Binding Protein (TonEBP). Some drugs like Cyclosporin-A (CsA) are discussed to interfere with the activity of TonEBP and thereby mediate their nephrotoxic effects. The aim of our study was to further understand CsA toxicity during elevation of ambient osmolality. Methods: First we examined cytotoxicity of CsA in IMCD exposed to elevated tonicity. Employing microarray analysis of gene expression, real-time PCR and immunoassays, we scrutinized pathways contributing to this effect. Results: We show that in IMCD cells CsA but not FK506 increases apoptosis upon an increase in tonicity. This effect is independent of cellular TonEBP localization or activity and reactive oxygen species. Microarray studies revealed marked quantitative differences in gene expression. Functional analysis showed overrepresentation of genes associated with cell death in presence of CsA. This correlated with increased mRNA expression of genes associated with the death receptor pathway and detection of TNFα in culture medium of cells treated with CsA. Conclusion: Our results show that CsA cytotoxicity is induced under elevated ambient osmolality and that death receptor signaling probably contributes to CsA cytotoxicity.

Introduction

Cyclosporin-A (CsA) is a frequently used drug that significantly improves survival of solid organ transplants and ameliorates severe autoimmune diseases [1]. Despite recent advances in immunosuppressive therapy such as the use of mTOR-inhibitors or monoclonal antibodies, therapeutic regimens including calcineurin inhibitors like CsA or FK506 are still of clinical relevance for renal transplant recipients [2]. A common adverse effect is

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calcineurin inhibitor induced nephrotoxicity. Chronic CsA nephrotoxicity is characterized by tubular atrophy and interstitial fibrosis with progressive renal impairment [3], which can lead to progressive kidney injury and allograft loss.

Physiologically, kidney function regulates salt and water homeostasis and maintains systemic plasma osmolality at a constant level. During this process, inner medullary kidney cells are physiologically exposed to an interstitial osmolality up to four times plasma osmolality [4].

Hypertonicity itself alters several intracellular processes by decrease of cell volume and changes in intracellular ion concentrations. In this process, protein function as well as DNA stability are modified [5]. A variety of compensatory mechanisms are essential to prevent cell death. Regulatory volume increase contributes to osmoadaptation as well as expression of heat shock proteins and solute carriers and induction of signaling pathways involved in DNA repair and cell cycle delay [6]. Many of these adaptations are mediated by the transcription factor Tonicity Enhancer Binding Protein (TonEBP) [6].

Interestingly, several different aspects of crosstalk between the cellular responses to both CsA and elevated extracellular osmolality have been observed [7, 8]. In a rat model of CsA nephrotoxicity, TonEBP, solute carriers, urea transporters [9] and aquaporin-2 (AQP2) [7] were found to be downregulated. In a cell culture model, calcineurin signaling was shown to be involved in the hyperosmolality-triggered activation of AQP2 gene expression [10].

In addition, CsA is known to activate several stress pathways. The influence of CsA on cell viability largely depends on cell type and experimental conditions: CsA is regarded as a trigger for the intrinsic mitochondrial pathway of apoptosis as well as a promoter of the extrinsic pathway [11], however, also protective effects on apoptosis and mitochondrial membrane permeability were observed [12, 13]. Upon induction of endoplasmatic reticulum stress, CsA treatment leads to phenotypic changes, de-differentiation and cell death [14].

Taking into account the physiological conditions in the renal medulla, an interaction between adaptation to hypertonicity and drug-induced apoptosis might be a tissue specific predisposition leading to CsA induced nephrotoxicity.

The aim of this study was to investigate the effects of CsA on parameters such as cell viability, TonEBP activity and cellular redox status (reactive oxygen species [ROS]) during exposure to increased ambient tonicity in a primary cell culture model of rat inner medullary collecting duct (IMCD) cells. In addition, we performed microarray analysis, evaluated several functional pathways and scrutinized the role of identified candidate proteins from the death receptor pathway at the protein level.

Medium	Medium formulation compared to basal medium (300 mOsm/kg)		
	+ NaCl [mM]	*+ Urea [mM]*	*+ Mannitol [mM]*
600	100	100	-
600NM	100	-	100
600 UM	-	100	200
900	200	200	-
900NM	200	100	100
900UM	100	200	200

Table 1. Medium formulations

Materials and Methods

Cell culture

Primary cultured IMCD cells were prepared as previously described [15]. Female Wistar rats (age 2-3 months) were sacrificed by decapitation. Kidneys were removed and inner medulla, including papilla, was isolated, chopped into small pieces and digested in PBS (Biochrom, Berlin, Germany) containing 0.2% hyaluronidase (Sigma, Deisenhofen, Germany) and 0.2% collagenase type CLS-II (Sigma) at 37° C for 90 min. Cells were seeded in 24 well plates (Falcon, Germany) or on glass cover slips, coated with collagen type IV (Becton-Dickinson, Heidelberg, Germany) at a density of approximately 10^5 cells/cm^2 and cultivated in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (100 IU/ml) and streptomycin (100 µg/ml), 0.2% glutamine, 1% non essential amino acids and 1% ultroser (BioSepra Inc., Marlborough, MA, USA). During the initial 24 h cells were grown at a medium osmolality of 600 mOsm/kg before proceeding with the experimental procedures described in the next paragraph. Cells were grown to confluency. The high initial medium osmolality was chosen in order to obtain growth conditions with preferential selectivity for IMCD cells [16].

Medium compositions and treatment protocol

In this study media of different osmolalities and solute compositions were employed. Medium of an osmolality of 600 mOsm/kg was obtained by addition of 100 mM NaCl and 100 mM urea, medium of an osmolality of 900 mOsm/kg by addition of 200 mM NaCl and 200 mM urea to the DMEM medium. In parts of our study, the elevation of medium osmolality was obtained with equiosmolar mannitol instead of urea, indicated as 600NM or 900NM, or instead of NaCl, indicated as 600UM or 900UM (Tab. 1). Osmolality was checked using an osmometer (Knauer, Berlin, Germany).

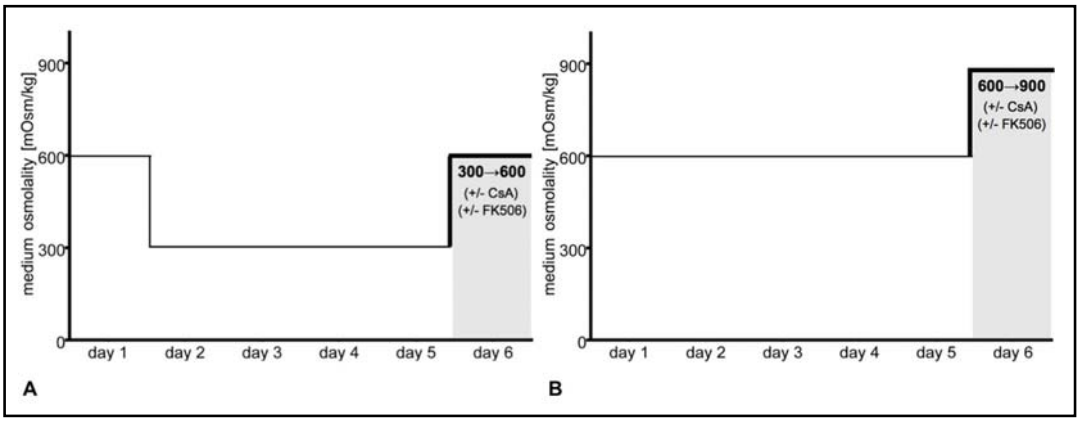


Fig. 1. Cell culture protocols. The graph depicts time course of media changes and calcineurin inhibitor addition. For composition of media with different osmolalities see Tab. 1.

Table 2. Gene names, gene symbols, accession numbers and primer sequences of genes used for the validation by real-time PCR.

Gene description and acc. no.	Primer sequences
Aldose reductase (AR) NM_012498	Sense- GTG CTG ATC CGG TTC CCC ATC Antisense- CTC ATC AAG GCG CAC ACC CTC
Aquaporin-2 (AQP2) NM_012909	Sense- TGC ATC TTT GCC TCC ACC GAC GAG Antisense- CAT GGA GCA ACC GGT GAA ATA G
Betaine/Gaba Transporter 1 (BGT-1) NM_017335.1	Sense- CCT CCA TGG CCT GTG TAC CGC Antisense- GA GTT cTT GCT TGA CTG GAG AG
Glycerinaldehyd-3-phosphat Dehydrogenase (GAPDH) NM_017008	Sense- CAT CAA CGA CCC CTT CAT T Antisense- ACT CCA CGA CAT ACT CAG CAC
Heat shock protein 4 (HSP70) NM_153629.1	Sense- GCA CCA ATG AGG CGA TGG AGT G Antisense- GGA CTG CAA ATA TTT GTC AGC TC
Nuclear factor of activated T-cells 5 (NFAT5/ TonEBP) NM_001107425	Sense- TCC TGG CTC ATC TCA GCA GAC Antisense- ACC GAG GAG GCC AGT GGG GC
Solute carrier family 14 (urea transporter), member 1 (UT-B) NM_019346.2	Sense- ATG GCA CTC ACC TGG CAG ACC Antisense- GGC CAA GCA GAA GGA CCA GG
Solute carrier family 14 (urea transporter), member 2 (UT-A) NM_019347.1	Sense- GTG ACA GAA GAG AGG TCT GCC a Antisense- TGC CAG GGT TGT TGG TTG TGA G
Tumor necrosis factor (ligand) superfamily, member 15 (TL1A) NM_145765.1a	Sense- TCT ACG TCA TCA CCT GGC AGA C Antisense- GGT TCT TGG TGA AGG CCA TCC
TNF superfamily, member 2 (TNF α) NM_012675	Sense- GTG ACA GAA GAG AGG TCT GCC Antisense- GCT CCT CCG CTT GGT GGT TTG

The experimental procedures used for the tonicity studies are visualized in Fig. 1. On day two defined basal media osmolalilty of 300 mOsm/kg or 600 mOsm/kg was applied. Culture medium was exchanged every 48 h.

On day 5 culture medium osmolality was elevated in presence or absence of CsA (5 µM, Novartis, Basel, Switzerland) or FK506 (80 ng/ml, Fujisawa, Osaka, Japan) as indicated. Measurements were performed on day 6 after 24 h of incubation if not differently indicated.

The experiments with the TNFα blocking antibody certolizumab (UCB, Brussels, Belgium) were carried out according to the same protocol: 10mM or 20mM certolizumab was additionally applied during the elevation of medium osmolality in presence or absence of CsA.

Apoptosis assay

Apoptosis was determined by detecting annexin-V binding using flow cytometry as described previously [17]. IMCD cells, treated as described above, were removed from the plates after incubation (45 min) with Accutase™ (Sigma) and labeled with annexin-V-fluorescein isothiocyanate and propidium iodide (PI; 5 µg/ml) in staining buffer (containing 1% bovine serum albumin in 50 mM 2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethansulfonic acid buffer (HEPES), pH 7.4) for 25 min at 4° C. After staining cells were washed in phosphate buffer and applied to FACScan flow cytometry (Becton Dickinson, Mountain View, CA, USA).

Caspase 3/7 activity

Caspase 3/7 activity was determined using the caspase 3/7 detecting component of a commercial Triplex assay (Promega, Madison, USA). Cells were grown in a black 96 well microtiter plate and incubated under indicated experimental conditions (Fig. 1). The test protocol supplied by the manufacturer was followed strictly. To assess caspase 3/7 activity, luminescence of the indicator product (cleaved from the DEVD tetrapeptide containing product) was measured over a period of 500 ms [18] using a plate reader (Tecan, Infinite 200-Pro, Crailsheim, Germany).

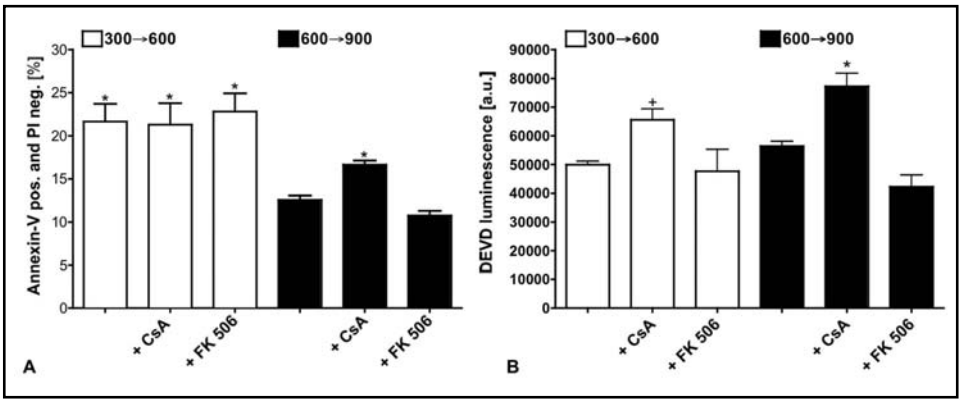


Fig. 2. CsA elevates hypertonicity-induced apoptosis and caspase activation. (A) Hypertonicity-induced apoptosis was detected by annexin-V and PI staining with subsequent flow sorting. Cells that were annexin-V positive and PI negative were declared apoptotic. (B) Caspase activity was assessed by luminescence measurement of a caspase 3/7 cleaved reagent (DEVD tetrapeptide). Measurements in 300→600 (white columns) and 600→900 (black columns) were performed in presence of CsA (5 μM) or FK506 (80 ng/ml) and in cells without additional treatment. Mean values ± SEM of 7 (A) or 4 (B) monolayers. * denotes statistical difference to 600→900, + denotes statistical difference to 300→600 (one way ANOVA, Tukey's post test p<0,05).

Immunofluorescence

The cellular localization of TonEBP was determined via immunofluorescence using a commercially available polyclonal TonEBP/NFAT5 antibody (Abcam, Cambridge, UK). First, IMCD cells were fixed in PBS containing 4% paraformaldehyde (20 min), washed with PBS (3 times, 10 min), permeabilized in PBS containing 0.1% triton X100 (5 min), again washed with PBS (3 times, 10 min) and, to block unspecific binding sites, incubated in blocking solution (0.35% fish skin gelatine in PBS, 20 min, 37° C). Then the cover slips were incubated with specific antibody against TonEBP (90 min, in a humified chamber, 37° C), washed with PBS (3 times, 10 min) and incubated for 90 min with an Alexa 488 labelled anti-rabbit IgG antibody and DAPI (4',6-diamidino-2-phenylindole, dichloride; both Molecular Probes, Leiden, Netherlands). Last, cells were washed with PBS and mounted with Crystalmount (Biomeda, Foster City, CA, USA). Images were taken with a fluorescence microscope (Axiovert, Zeiss, Oberkochen, Germany). Quantification of TonEBP immunostaining in nucleus and cytosol was done by optical densitometry. 10 cells per group were randomly chosen for analysis by the fact that they met the diameter of the representatively taken picture. The ratio of nuclear and cytosolic density was calculated.

ROS detection

The cell-permeable fluorogenic substrate 2',7'-dichlorofluorescein diacetate (DCFDA, Molecular Probes, Eugene, USA) is oxidized to highly fluorescent 2',7'-dichlorofluorescein by H_2O_2, and thus, can be utilized to examine intracellular generation of ROS. For these measurements IMCD were grown on 96-well plates as described above. Confluent cells were incubated with 5 μM DCFDA diluted in culture medium (30 min, 37° C), then in dye free culture medium (30 min, 37° C). Medium was changed to dye and serum free medium and baseline fluorescence was assessed before indicated experimental inducements were applied. Fluorescence was measured at excitation and emission wavelengths of 485 and 525 nm, respectively, by a fluorescence plate reader (Tecan) in intervals of 5 min. Background fluorescence of unloaded cells was subtracted.

Microarray analysis

Total RNA was isolated using an RNeasy-kit (Qiagen, Hilden, Germany) and used to prepare biotinylated target cDNA. Target cDNAs were processed according to manufacturer's instructions (array type Rat Gene 1.0ST; http://www.affymetrix.com). Data were analyzed using Affymetrix GCOS array analysis software. Signal intensities were quantile normalized using XRAY software (Biotiquesystems, Reno NV, USA). The data discussed in this publication have been deposited in NCBIs Gene Expression Omnibus. The data can be accessed through the accession number GSE18945.

Identification of differentially expressed genes

Significant changes in the gene expression were identified using class comparison with the BRB ArrayTools developed by R. Simon and A. Peng (http://linus.nci.nih.gov/BRB-ArrayTools.html). Nominal significance level of each univariate test was set to 0.001. Confidence level of false discovery rate assessment used was 90% and the maximum allowed numbers of false-positive genes were set to 10.

Functional annotation

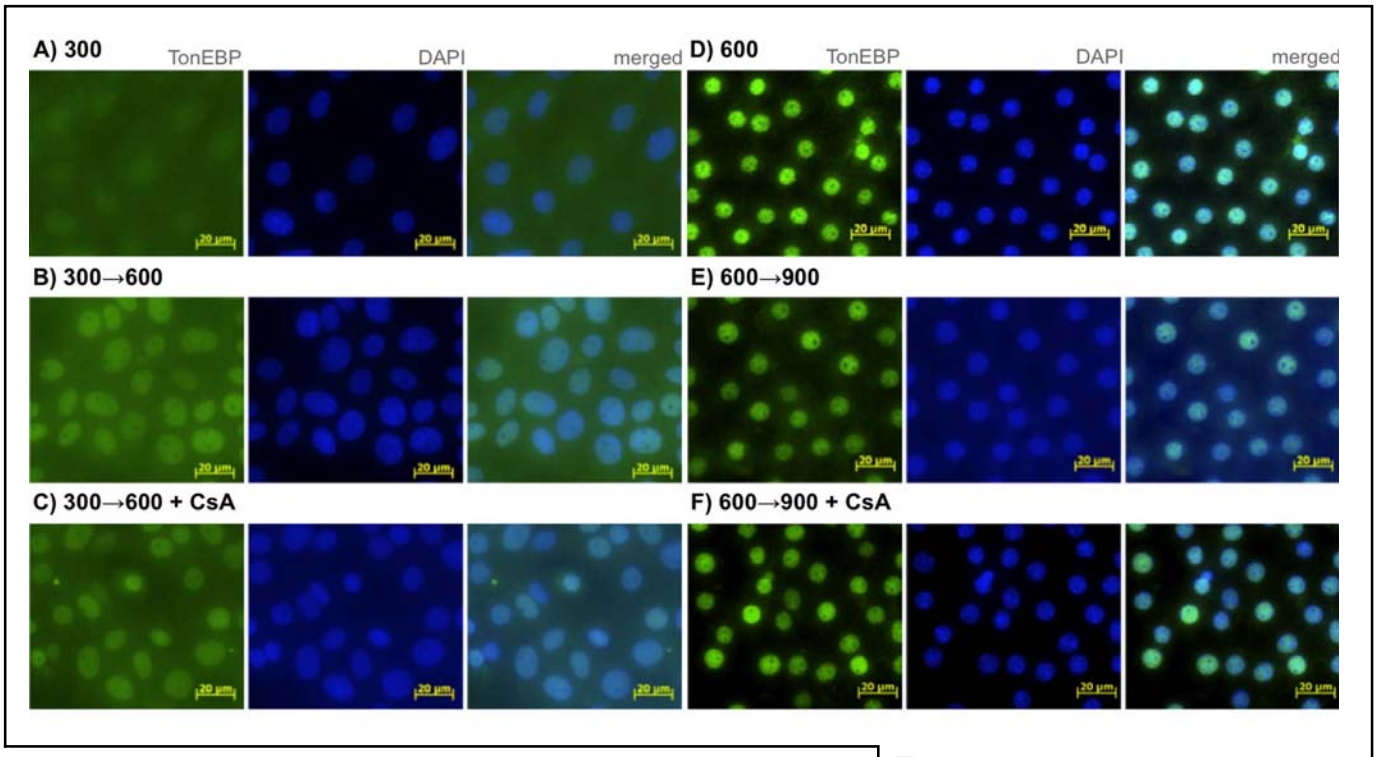
The Database for Annotation, Visualization, and Integrated Discovery (DAVID) was used for functional classification [19]. Gene enrichment analysis was performed for the lists of up- or down-regulated genes to identify gene ontology (GO) terms [20] overrepresented within a given candidate list (p<0.05, Fishers exact test).

Pathway analysis and graphical representation

Data were also visualized using Ingenuity Pathways Analysis (IPA, Ingenuity® Systems, details at www.ingenuity.com). The lists with significantly regulated genes were uploaded in IPA and expression values were mapped to IPA canonical pathways library.

Real-time PCR

Real-time PCR was performed using the SYBR Green PCR Master Mix with the ABI PRISM 7900 Sequence Detection System. All instruments and reagents were purchased from



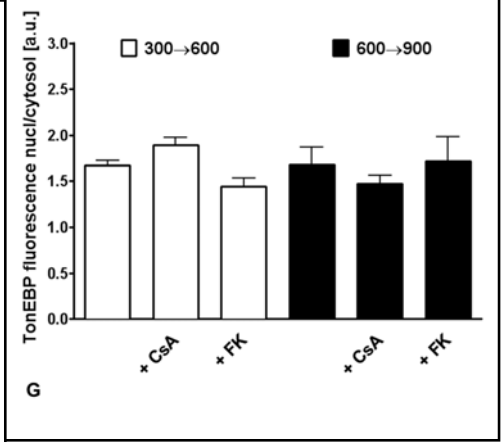


Fig. 3. Nuclear localization of TonEBP is increased after hypertonic challenge independent of CsA. TonEBP localization was observed by immunofluorescence using a TonEBP antibody and DAPI nucleic acid staining. Cells were cultivated at continuous osmolality or were exposed to increased environmental tonicity in presence or absence of CsA 5 µM (Fig. 1). (A) continuous cultivation at 300 mOsm/kg, (B) 300→600, (C) 300→600 + CsA, (D) continuous cultivation at 600 mOsm/kg, (E) 600→900, (F) 600→900 + CsA. Representative graphs from 2 independent observations were chosen. (G) Quantification of TonEBP immunostaining in nucleus and cytosol was performed using optical densitometry. The ratio of nuclear and cytosolic density was determined. Measurements in 300→600 (white columns) were normalized to the ratio during continuous cultivation at 300 mOsm/kg, those in 600→900 (black columns) were normalized to the ratio during continuous cultivation at 600 mOsm/kg. Mean values ± SEM of 10 graphs from 2 monolayers.

Applied Biosystems (Darmstadt, Germany). Relative gene expression values were evaluated with the $2^{-\Delta\Delta Ct}$ method using GAPDH as reference gene [21]. Specific primer pairs were used and a list with the gene names, accession numbers and gene symbols is provided in Tab. 2.

TNFα measurement

TNFα levels in culture medium were measured using a commercial ELISA kit (R&D Systems, Wiesbaden, Germany). Cells were grown to confluence in a 24 well microtiter plate and incubated 24 h in similar volume of medium (composition as indicated). TNFα concentration in the cell free medium supernatant was measured in duplicate according to the manufacturer's instructions. The optical density was measured using a microplate reader (Tecan). The TNFα concentrations were calculated using WinFitting Software (Tecan).

Statistics

Data are presented as mean values ± SEM with the indicated number of measurements. Data were tested with unpaired t-test or one way ANOVA and Tukey's post test using GraphPad-Prism 4.0 (San Diego, CA, USA). A p-value ≤ 0.05 was accepted to indicate statistically significant differences.

Results

CsA interferes with elevated tonicity increased cell viability

High concentrations of NaCl or urea impair viability of renal cells [22]. To analyze if CsA toxicity is influenced by tonicity, IMCD cells were treated with 5 µM CsA for

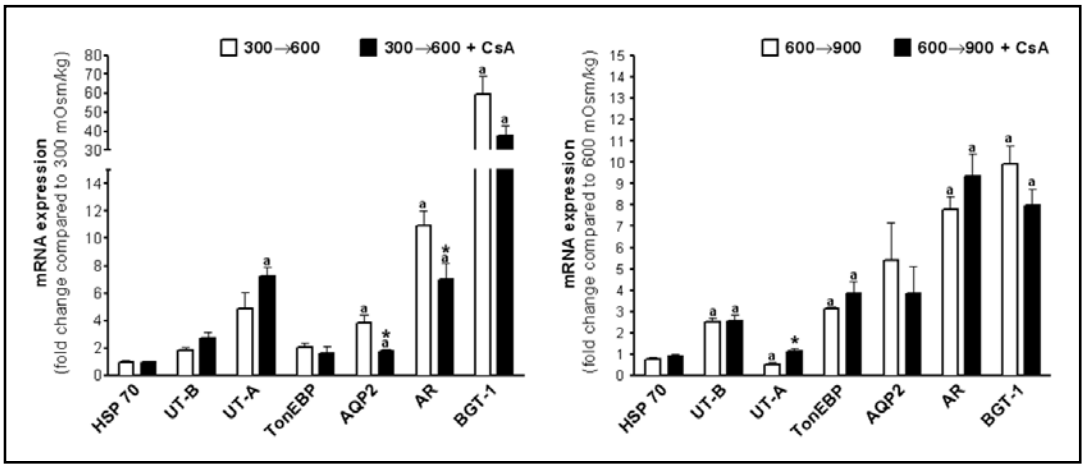


Fig. 4. No consistent change in TonEBP target gene expression during hypertonic challenge and CsA treatment. Changes in gene expression for selected TonEBP targets were analyzed by real-time PCR using specific primer pairs. Relative changes were evaluated using the $2^{-\Delta\Delta ct}$ method. (A) Gene expression in 300→600 compared to continuous cultivation at 300 mOsm/kg. (B) Gene expression in 600→900 compared to continuous cultivation at 600 mOsm/kg. Data is presented for untreated cells (white columns) and in presence of CsA 5μM (black columns) for the following genes: Heat shock protein 70 (HSP70), urea transporter A (UT-A), urea transporter B (UT-B), tonicity enhancer binding protein (TonEBP), aquaporin 2 (AQP2), aldose reductase, betaine/ GABA transporter 1 (BGT-1). Mean values ± SEM from 3 monolayers of 3 independent cell cultures. * denotes statistical difference to 300→600 (A) or 600→900 (B) [a] denotes statistical difference to 300 mOsm/kg (A) or 600 mOsm/kg (B) (two-tailed t-test, $p < 0.05$).

24 h. No changes in the number of apoptotic cells were detectable in IMCD cells continuously cultivated at 300 mOsm/kg, 600 mOsm/kg or 900 mOsm/kg in presence of CsA compared to untreated cells (data not shown). The crucial processes of adaptation to hyperosmolality occur within minutes and last for a few hours after the elevation of ambient osmolality [6]. At the same time, tubular cell viability is already altered after CsA application of 24 h or less [8, 14]. Thus we examined the ability of IMCD cells to adapt to an increased osmolality for 24 h in the presence of CsA. We elevated the osmolality by 300 mOsm/kg (for details see methods): in the first group the osmolality was increased from 300 to 600 mOsm/kg (300→600) and in the second group from 600 to 900 mOsm/kg (600→900), both in the presence or absence of CsA (Fig. 1). Within the 300→600 group, ~ 21% and in 600→900 ~ 12% of cells were apoptotic referring to annexin-V/PI staining (Fig. 2A).

The rate of appearance of apoptotic cells was not different between the groups 300→600, 300→600 + CsA and 300→600 + FK506. In 600→900 + CsA, the rate of apoptotic cells was significantly elevated compared to 600→900. However, both in the 600→900 and also in the 300→600 group caspase 3/7 activity was significantly higher in the presence of CsA (Fig. 2B). CsA enhanced both the rate of annexin-V positive cells and caspase 3/7 activity, while treatment with another calcineurin inhibitor, FK506, did not.

TonEBP localization and TonEBP target gene expression are heterogeneously affected by CsA

TonEBP is endogenously expressed in primary cultured rat renal IMCD cells. Renal CsA toxicity was attributed to decreased nuclear localization of TonEBP during 16 h of CsA treatment in MDCK cells [8]. In addition, a variety of other mechanisms have been proposed to contribute to CsA nephrotoxicity, including modifications of cell cycle progression, alterations in expression of heat shock proteins and alterations of cytoskeleton proteins [8, 22]. To test whether the pro-apoptotic effects of CsA under hypertonic conditions were due to alterations of TonEBP activity, we used immunofluorescence analysis to evaluate TonEBP localization (Fig. 3) and real-time PCR (Fig. 4) to measure the expression of its target genes.

While IMCD cells cultivated at 300 mOsm/kg showed weak nuclear TonEBP staining (Fig. 3A), cells cultivated at 600 mOsm/kg showed a strong nuclear localization of TonEBP (Fig. 3D). Nuclear localization of TonEBP was elevated in 300→600 (Fig. 3B) compared to the cells continuously cultivated at 300 mOsm/kg. In 600→900 (Fig. 3E), nuclear TonEBP staining was approximately as strong as during continuous cultivation at 600 mOsm/kg. There was no difference in nuclear localization between CsA-treated (Figs. 3C and 3F) and control cells (Figs. 3B and 3E). Quantification of TonEBP fluorescence signal (nuclear: cytoplasmatic ratio) is shown

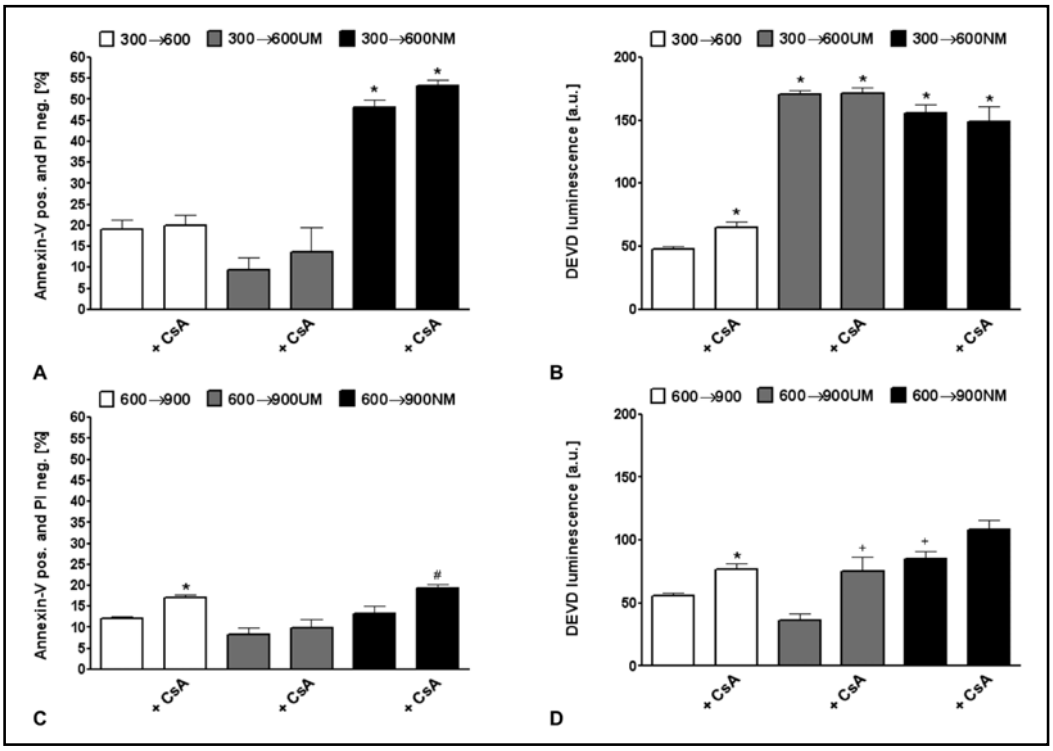


Fig. 5. CsA and medium solutes influence apoptosis and caspase activity. Hypertonicity-induced apoptosis was detected by annexin-V and PI staining with subsequent flow sorting. Cells that were annexin-V positive and PI negative were declared apoptotic (A; C). Caspase activity was assessed by luminescence measurement of a caspase 3/7 cleaved reagent (DEVD tetrapeptide) (B; D). Measurements were performed 24 h after increasing osmolality via addition of different solutes from 300 to 600 mOsm/kg (A; B) respectively 600 to 900 mOsm/kg (C; D) as described in Tab. 1. Values are presented as mean ± SEM of 5 (A; C) or 4 (B; D) monolayers. * denotes statistical difference to 300→600 (A; B) respectively 600→900 (C; D), # denotes statistical difference to 600→900NM, + denotes statistical difference to 600→900UM (one way ANOVA, Tukey's post test $p<0,05$).

in Fig. 3G. These results suggest that CsA does not interfere with nuclear TonEBP translocation or localization.

Within a 24 h increase in tonicity, the expression of typical TonEBP target genes was induced in IMCD cells (Figs. 4A and 4B). In 300→600, the expression of AQP2, betaine/GABA-transporter 1 (BGT-1) and aldose reductase (AR) was upregulated compared to continuous cultivation at 300 mOsm/ kg (Fig. 4A). Expression of other target genes like urea transporters A and B (UT-A and UT-B), heat shock protein 70 (HSP 70) and TonEBP itself was not affected. Consistent with other studies, the upregulation of AQP2 and AR expression was decreased in the presence of CsA [10].

In 600→900, the expression of TonEBP, AR, UT-B and BGT-1 was upregulated compared to continuous cultivation at 600 mOsm/kg (Fig. 4B). The expression of these genes was not affected by CsA. In contrast, UT-A expression was downregulated in 600→900 and this effect was reversed in the presence of CsA.

Due to low transfection rates, the implementation of luciferase assays of TonEBP activity was limited in our primary IMCD cells. However, the heterogeneous effect of CsA on the expression of TonEBP target genes during exposure to enhanced ambient tonicity suggests that the observed toxicity of CsA is not mediated by alterations of TonEBP action.

Neither NaCl nor urea solely promotes hypertonicity-dependent CsA cytotoxicity

As different medium components such as urea or NaCl in high concentrations cause particular cellular alterations [6], we evaluated the influence of medium composition on CsA toxicity.

Thus, we observed cells treated with medium in which either NaCl or urea was equiosmolaly substituted by mannitol (see methods and Tab. 1: 600NM or 600UM for cells cultured at 600 mOsm/kg and 900NM or 900UM for cells cultured at 900 mOsm/kg). In 300→600NM the rate of annexin-V positive cells was two-fold higher compared to 300→600. CsA treatment did not lead to changes in the rate of annexin-V positive cells. Caspase 3/7 activity was assessed under the same experimental conditions. Compared to 300→600, caspase 3/7 activity was significantly enhanced in 300→600 + CsA. Also in 300→600UM and 300→600NM caspase 3/7 activity was elevated, but CsA had no additional effect (Fig. 5B). As already shown in Fig. 2, CsA enhanced the rate of

annexin-V positive cells in 600→900. This effect was not present in 600→900UM but in 600→900NM. The rate of annexin-V positive cells was not significantly different between 600→900, 600→900UM and 600→900NM (Fig. 5C). Higher caspase 3/7 activity compared to 600→900 was detectable in the group 600→900NM (Fig. 5D). In 600→900UM + CsA, caspase 3/7 activity was higher than in the comparable group without CsA. Thus, we observed an apoptosis-enhancing effect of CsA treatment in 600→900 using annexin-V staining and caspase 3/7 assay.

Concentration of ROS is not affected by CsA

Next we tested whether the concentration of intracellular ROS (measured as 2',7'-dichlorodi-hydrofluorescence diacetate [DCFDA] fluorescence) is changed in cells exposed to elevated ambient tonicity in presence of CsA. However, no significant differences were observed after 24 h (data not shown) or during short term dynamic measurements in the first 2 h after exposition (Fig. 6). As a positive control, H_2O_2 led to the expected increase in ROS. Therefore we conclude that, the observed enhancement of CsA cytotoxicity under hypertonic conditions is not due to alterations in ROS signaling in this cell model.

Microarray analysis show large quantitative differences in gene expression induced by CsA

To elucidate potential mechanisms of toxicity, we used microarrays to compare gene expression 24 h after the elevation of extracellular osmolality in the presence or absence of CsA.

A quantitative analysis of three biological replicates in each group revealed 1317 genes differentially expressed in 300→600 compared to 300 mOsm/kg control (Tab. 3). The expression of 1174 genes in 300→600 + CsA was significantly changed compared to cells continuously cultivated at 300 mOsm/kg. In the group 600→900, 1229 and in 600→900 + CsA 1339 genes were differentially expressed in comparison to cells continuously cultivated at 600 mOsm/kg. In all cases approximately one third of these genes were upregulated and two thirds were downregulated (Tab. 3).

A more detailed analysis of these up- and downregulated genes showed that 675 genes were common in 300→600 with or without CsA (Fig. 7A). 642 genes were unique to the 300→600 group and 499 genes unique to the 300→600 + CsA group. In 600→900 with or without CsA, 725 genes were found differentially expressed in both groups, expression of 504 genes was

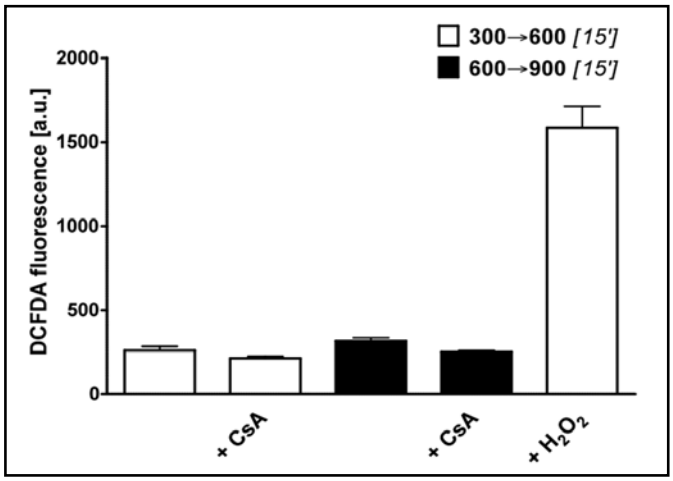


Fig. 6. No alterations of ROS during hypertonic challenge and CsA treatment. ROS was quantified as 2',7'-dichlorodihydrofluorescence diacetate (DCFDA) flourescence 15' after elevating osmolality from 300 to 600 mOsm/kg (300→600 *[15']*, white columns) or from 600 to 900 mOsm/kg (600→900 *[15']*, black columns) in presence or absence of CsA (5 µM). H_2O_2 (250 µM) was used as a positive control. Values are presented as mean ± SEM of 6 monolayers.

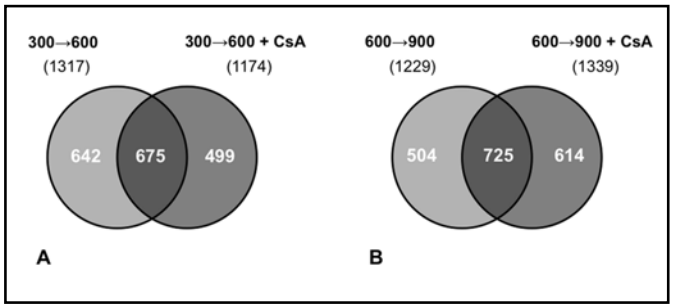


Fig. 7. Differences in gene expression during hypertonic challenge and CsA treatment. The venn diagram illustrates the common and unique genes. The total number of differentially expressed genes is given in brackets. (A) Number of common and unique genes between the CsA- treated and untreated group in 300→600. (B) Number of common and unique genes between the CsA- treated and untreated group in 600→900.

	300→600		600→900	
	-	+ CsA 5 µM	-	+ CsA 5 µM
downregulated genes	894	787	713	739
upregulated genes	423	387	516	600
Σ differentially expressed genes	1317	1174	1229	1339

Table 3. Alterations in gene expression due to hypertonic challenge. Differentially expressed genes in 300→600 +/- CsA compared to cells continuously cultivated at 300 mOsm/kg and in 600→900 +/- CsA compared to continuous cultivation at 600 mOsm/kg.

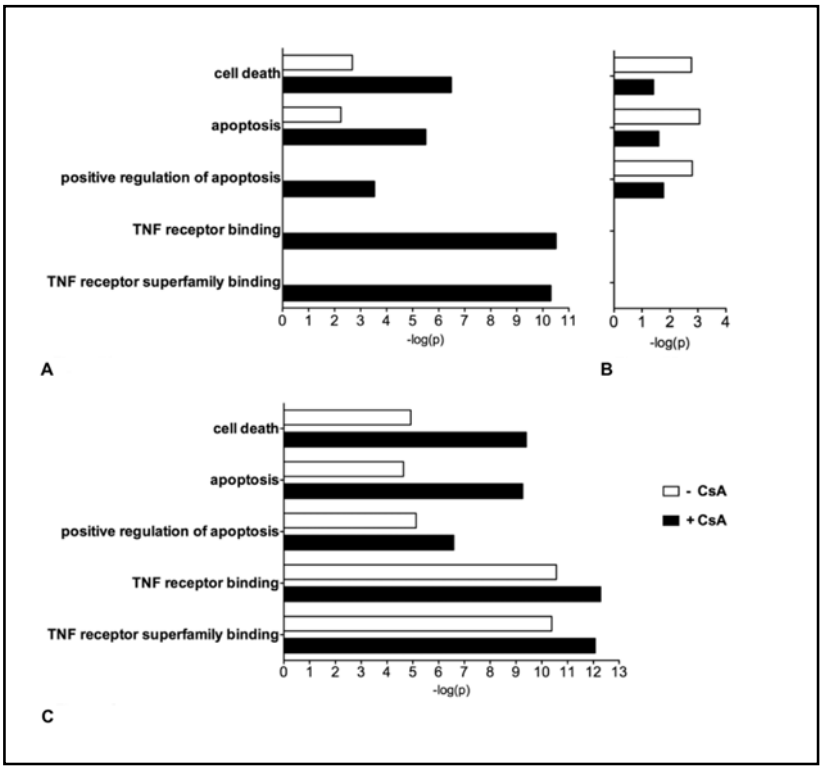


Fig. 8. Overrepresentation of genes promoting cell death during hypertonic challenge and CsA treatment. Selected gene ontology (GO) terms associated with cell death were statistically overrepresented among the regulated genes. (A) Overrepresentation of selected GO terms among the downregulated genes in 300→600. (B) Overrepresentation of selected GO terms among the upregulated genes in 300→600. (C) Overrepresentation of selected GO terms among the upregulated genes in 600→900. Analysis was performed for data obtained in the absence (- CsA, white bars) or in the presence of CsA (5 μM) (+ CsA, black bars) during the elevation of tonicity. The negative logarithmically plotted data represent the p-values from Fisher's exact test. A lower p (higher -log(p)) indicates more genes associated with a specific GO term ($p < 0.05$).

only altered in absence, 614 in presence of CsA (Fig. 7B). Thus, approximately 50 % of the genes differentially expressed during adaptation to hypertonicity in presence or absence of CsA were congruent.

Pathways involved in cell death are affected by CsA

Functional relevance of these changes in gene expression can be approached by Gene Ontology (GO) term examination. We analyzed overrepresentation of certain GO terms (biological processes, molecular function and cellular components) among the differentially expressed genes and compared data between the groups with and without CsA. Here we focus on five processes and cellular components associated with cell death, apoptosis and the death receptor pathway (cell death, apoptosis, positive regulation of apoptosis, TNF receptor binding, TNF receptor superfamily binding; Fig. 8).

In the 300→600 group, genes associated with cell death and apoptosis were slightly overrepresented among the downregulated genes (Fig. 8A). Treatment with CsA promoted this effect. Interestingly, the representation of three of these five functional categories was also eminent among the upregulated genes (Fig. 8B).

Scrutinizing the 600→900 group, genes associated with GO terms such as cell death, apoptosis, positive regulation of apoptosis, TNF- receptor binding and TNF receptor superfamily binding were overrepresented among the upregulated genes (Fig. 8C). The CsA-treated group exhibited even more genes associated with these terms, indicated by the lower p-value.

Obviously, transcription of a large number of cellular genes is regulated during the pro-apoptotic effect of CsA in hypertonic environment. The canonical death receptor pathway was additionally visualized using the Ingenuity Pathway Knowledge Base. Compared to continuous cultivation at 600 mOsm/kg, there are numerous changes in the canonical death receptor signaling in 600→900 (Fig. 9A) and 600→900 + CsA (Fig. 9B). Several genes within this pathway were affected in the presence (600→900 + CsA) but not in the absence (600→900) of CsA. Among these were the upregulated extracellular messenger TNFα, TL1A, TRAF2, cIAP, IkappaB and HSP 27 and the downregulated Jun Kinase, caspase 7 and caspase 2. Only few genes within this pathway were affected in the 300→600 comparison (Figs. 9C and 9D).

Real time PCR and immunoassays show increased TNFα expression

Next, we validated the expression of TNFα and TL1A by real-time PCR. Consistent with the microarray data, TNFα and TL1A were upregulated in IMCD cells exposed to a hypertonic medium for 24 h (Fig. 10). The hypertonicity-induced upregulation of TNFα in 300→600 was not affected in presence of CsA. While in 600→900, expression of TNFα and TL1A mRNA was similar as in

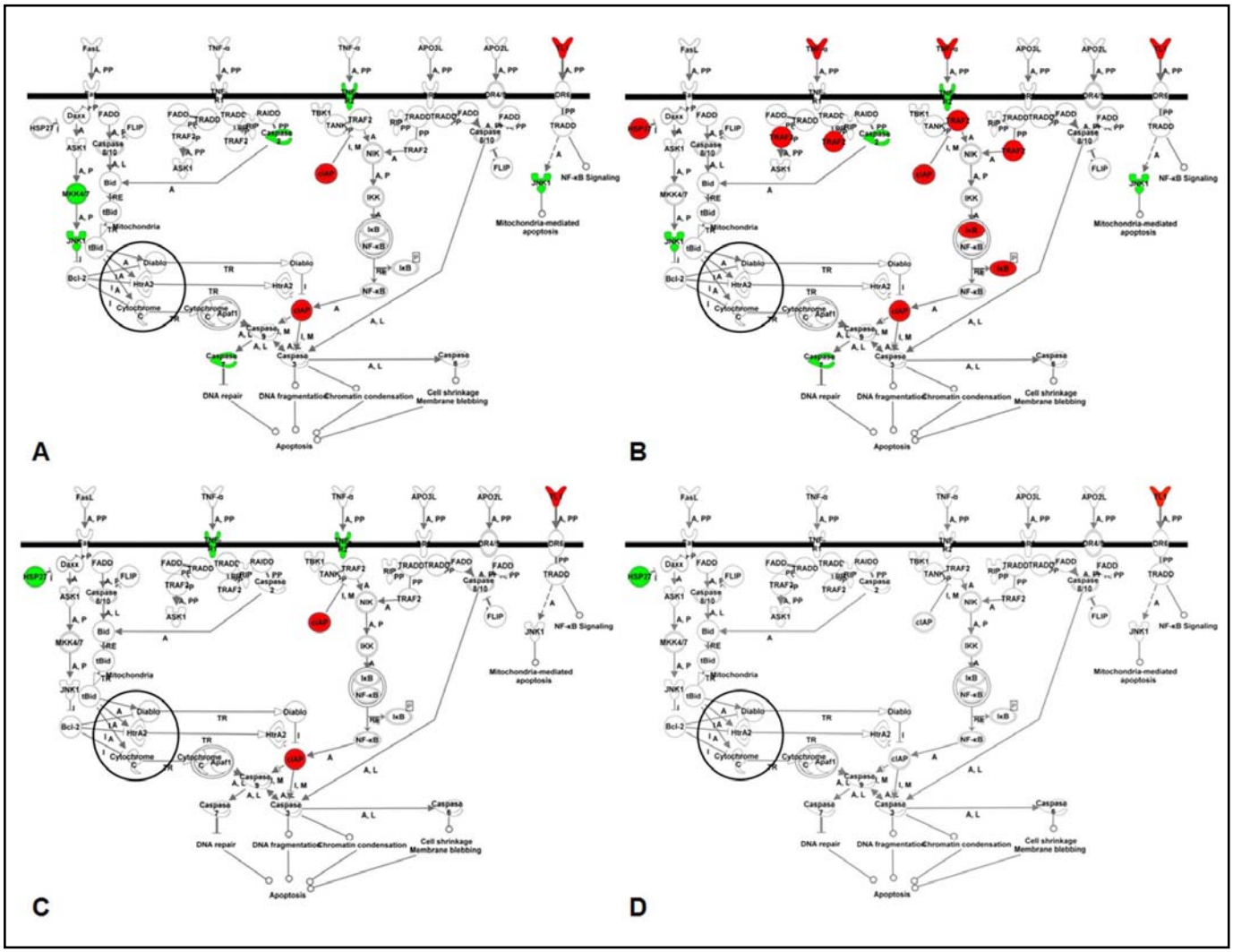


Fig. 9. Altered expression of genes from death receptor pathway during hypertonic challenge and CsA treatment. Analysis of canonical pathways revealed the presence of up- and downregulated genes of the death receptor pathway in 600→900 (A) and 600→900 + CsA (B), the representation of up- and downregulated genes of the death receptor pathway in 300→600 (C) and 300→600 + CsA (D). The datasets were analyzed using the Ingenuity Pathways Analysis software (Ingenuity® Systems, http:/www.ingenuity.com). The node colour indicates the expression level of the genes (upregulated, red and downregulated, green). Nodes and edges are displayed with various shapes and labels that present the functional class of genes and the nature of the relationship between the nodes, respectively.

300→600, in 600→900 + CsA these two TNF-receptor family ligands were significantly upregulated. However, no change on expression levels was observed for TL1A and TNFα expression in western blot analysis of whole cell lysates (data not shown). TNFα could be detected in cell supernatants using an immunoassay (ELISA; Fig. 11). However, simultaneous application of the TNFα blocking antibody certolizumab did not inhibit caspase 3/7 activation during CsA and hypertonic challenge.

Discussion

Addressing the mechanisms of CsA toxicity in the IMCD, we observed that in this cellular model apoptosis was increased during an elevation of medium osmolality in the presence of CsA (Fig. 2). This corresponds to other studies describing effects after 24 h of CsA treatment in cell culture [8, 14, 23]. Accelerated apoptosis contributes to CsA associated interstitial fibrosis, an important feature

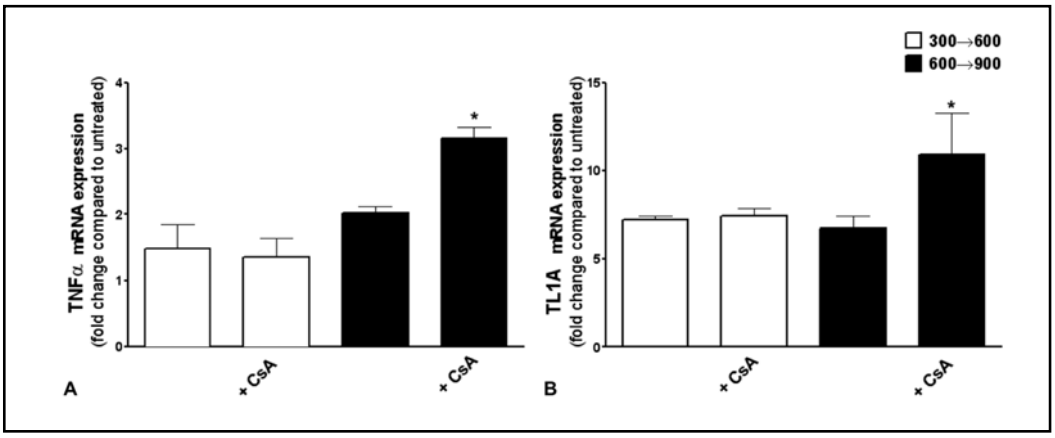


Fig. 10. TNFα and TL1A mRNA upregulation during hypertonic challenge and CsA treatment. Changes in gene expression of TNFα (A) and TL1A (B) were validated by real-time PCR using specific primer pairs. Relative changes were evaluated using the $2^{-\Delta\Delta ct}$ method. Changes are shown for 300→600 compared to continuous cultivation at 300 mOsm/kg (white columns) and 600→900 compared to continuous cultivation at 600 mOsm/kg (black columns) for untreated and + CsA (5μM) treated cells. Values are presented as mean ± SEM of 3 monolayers. * denotes statistical difference to untreated cells (two-tailed t-test, $p < 0.05$).

of chronic CsA nephrotoxicity [24]. Possibly, studying the effect of CsA on IMCD cell survival can help to understand the severe side effects of long term treatment.

Transferred to the *in vivo* situation, the current results might implicate that the risk of tubular damage is influenced by increased medullary concentration of solutes and urea in antidiuresis. The same treatment with FK506, another calcineurin inhibitor, did not elevate the rate of apoptosis in this model, suggesting a calcineurin-independent mechanism. This is consistent with well characterized calcineurin- independent side effects of CsA on cell viability in other models [12-14] and reflects the common observation that FK506 is less nephrotoxic compared to CsA [25].

As a key finding in this study, we demonstrate that CsA but not FK506 toxicity correlates with medium osmolality (Fig. 5). This leads to the question if CsA hampers adaptation to hypertonicity in inner medullary kidney cells, and if so, which mechanisms are involved in this process. Since the transcription factor TonEBP plays a key role in cellular osmoadaptation, a likely mechanism would be either direct interaction with this protein or downstream effects on its target proteins. Endogenous TonEBP expression and regulatory signaling pathways are well conserved in this cell model (Fig. 3).

In contrast to other transcription factors from the NFAT family, TonEBP activity is not controlled via dephosphorylation by calcineurin [26]. The key mechanism to control TonEBP expression and activity is interstitial tonicity [27].

At 300 mOsm/kg there was only weak nuclear TonEBP localization, but nuclear localization increased in correlation with tonicity (Fig. 3), illustrating its function in

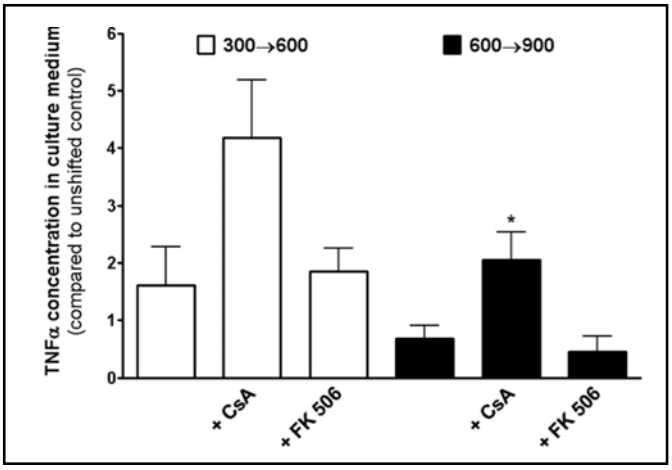


Fig. 11. Detection of TNFα in culture medium after 24h of hyperosmolal challange. TNFα protein concentration in the culture medium was assessed performing a specific ELISA immunoassay. Changes are shown for 300→600 compared to continuous cultivation at 300 mOsm/kg (white columns) and 600→900 compared to continuous cultivation at 600 mOsm/kg (black columns) for untreated and + CsA (5 μM) treated cells. Values are presented as mean ± SEM of 4 monolayers. * denotes statistical difference to 600→900 (one way ANOVA, Tukey's post test $p<0,05$).

gene expression under such conditions (Fig. 4). Some authors attributed increased apoptosis in HepG2 cells exposed to elevated ambient tonicity and CsA for 16 h to interference with nuclear localization of TonEBP [8]. However, the immunofluorescence data (Fig. 3) suggest that nuclear shuttling of TonEBP is not significantly inhibited by CsA treatment in our model. When culturing

the cells at 600 mOsm/kg, there is already a higher nuclear TonEBP abundance compared to cultivation at 300 mOsm/kg. Thus, predominant interference of CsA with nuclear trafficking would most likely exert its pro-apoptotic effect in 300→600 + CsA. Yet, CsA-attributable cytotoxicity was highest in 600→900, but not in 300→600 (Fig. 2). Furthermore, there was no consistent change in the expression of typical target genes of TonEBP in the presence of CsA (Fig. 4). Alterations in transcriptional regulators other than TonEBP or changes in mRNA stability might account for this effect. In conclusion, we suggest that significant interference with TonEBP localization is not the prominent mechanism promoting CsA cytotoxicity in this particular cell model.

In different models, CsA did not directly inhibit TonEBP expression or activity. CsA treatment of up to 60 min did not alter TonEBP localization or phosphorylation [26]. *In vivo*, TonEBP protein abundance and nuclear localization was not affected after 7 days of CsA treatment in a rat model [7]. The observation that these parameters decreased after 28 days was attributed to the reduced expression of sodium transporters leading to decreased tonicity of the renal medulla.

However, CsA can hamper osmoadaptation other than via direct interference with TonEBP localization.

Several mechanisms might add to the fact that CsA toxicity correlates with enhanced medium osmolality. One may be interference with osmoadaptation downstream or independent of TonEBP activation, e.g. via posttranscriptional protein modification. For example, CsA but not FK506 reduced the hypertonicity-induced activity of the sodium/ myo-inositol transporter [28]. Other mechanisms may be that CsA influences the cytoskeleton of the cells or pathways regulating cell death.

As elevation of tonicity and increasing extracellular osmolality by a membran-permeant solute (i.e. urea) have distinct intracellular effects, specific mechanisms provide adaptations to either increased NaCl or urea concentrations [6, 29]. Our data support the idea of crosstalk between adaptation to elevated urea and elevated NaCl (Fig. 5, [30]), but seemingly CsA toxicity does not depend on these factors. However, as previously shown, the relation of different measurable apoptotic features is influenced by the exposure time and on the specific pro-apoptotic stimulus (Fig. 5, [31]). This can be due to differences in mechanism of apoptosis and status of the cell in apoptosis. For example, caspase activation could precede annexin-V externalization and in addition, other mechanisms can influence cell membrane integrity.

ROS are an intracellular messaging entity which are involved in regulation of renal cell function [32]. ROS have been shown to regulate multiple signaling pathways, including TonEBP activation [33]. Hypertonicity as well as CsA is associated with elevated levels of intracellular ROS [34]. As high ROS promote cell damage, the interference of CsA with the cellular redox status might account for increased cell death [11]. The influence of CsA on ROS appears just as heterogeneous as the effect of ROS on cell viability. Some authors describe increase of ROS and lipid peroxidation in a rat model after 7 days of CsA treatment and observe beneficial effects of parallel administration of antioxidants [35], but in other animal models serum parameters of antioxidant status were not changed after 7 weeks of CsA treatment [36]. In our study we provide evidence that during elevation of extracellular osmolality ROS are not altered by additional treatment with CsA (Fig. 6). This could be explained by the generally elevated level of ROS during an increase of osmolality or by immediate efficacy of compensatory mechanisms.

As mentioned above, extracellular challenges such as hypertonicity require adaptive alterations of gene expression and drugs such as CsA further interfere with gene expression (Tab. 3). Systematic gene expression profiling using microarray analysis showed that the absolute number of differentially expressed genes was relatively constant among our experimental protocols (Fig. 7), but detailed qualitative analysis of altered genes revealed large differences. The qualitative differences suggest that distinct mechanisms are required for a balanced compensation of the hyperosmolal challenge in presence or absence of CsA.

Hypertonicity is also known to upregulate TNFα [37] and it has been described that TNFα is differentially controlled and upregulated during CsA treatment [38]. Microarray analysis showed significant overrepresentation of genes involved in the death receptor pathway among the upregulated genes and an overrepresentation of death promoting genes 600→900 + CsA group (Fig. 8C). Furthermore, we confirmed upregulation of TNF receptor ligands like TNFα and TL1A in presence of CsA by real time PCR (Fig. 10) and found TNFα in medium supernatants of cells treated with CsA for 24h (Fig. 11).

TNF receptor signaling activates survival, proliferation and apoptotic pathways. Death receptor substrates TNFα and TL1A promote an extrinsic pathway of apoptosis. Interestingly, in kidney allograft recipients CsA toxicity was associated with elevated soluble TNF receptors [39]. Our results indicate that under hypertonic conditions the death receptor pathway might be a crucial

factor in CsA induced apoptosis involving TNFα release (Figs. 8-11). These findings are consistent with other studies postulating that autocrine TNFα contributes to endoplasmatic reticulum (ER) stress induced apoptosis [40] and that prolonged ER stress accounts for apoptotic cell death in chronic CsA nephropathy [41]. However, antibody mediated blockade of extracellular TNFα did not decrease caspase 3/7 activation under cell culture conditions (data not shown; [42]). Although TNFα release occurs, autocrine TNFα mediated caspase 3/7 activation does not seem to be the predominant mechanism responsible for CsA cytotoxicity under hypertonic conditions. The downstream effect of TL1A on caspase 3 [43, 44] might contribute to apoptosis as well as the quantitative changes in regulation of a variety of proteins involved in death receptor signaling (Figs. 8 and 9).

To conclude, CsA induced cytotoxicity is aggravated by hypertonic conditions in primary cultured IMCD. This effect is independent of calcineurin. We also provide an analysis of transcriptomic changes in IMCD cells undergoing hypertonic challenges and compared the findings to cells undergoing the same protocol with additional CsA treatment. We identified genes upregulated explicitly in the death receptor pathway correlating with the CsA specific cytotoxic effect. Further studies are necessary to understand the role of death receptor signaling and the molecular mechanisms of osmolality- and CsA-induced nephrotoxicity, to foster the understanding of immunosuppressant side effects and to advance immunosuppressant therapy.

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