Heliyon

Received:
17 July 2018
Revised:
31 October 2018
Accepted:
14 December 2018

Cite as: Cecilia I. Casali, Luciana C. Erjavec, María del Carmen Fernández-Tome. Sequential and synchronized hypertonicity-induced activation of Rel-family transcription factors is required for osmoprotection in renal cells.
Heliyon 4 (2018) e01072.
doi: 10.1016/j.heliyon.2018.e01072

Sequential and synchronized hypertonicity-induced activation of Rel-family transcription factors is required for osmoprotection in renal cells

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Abstract

NF-κB and TonEBP belong to the Rel-superfamily of transcription factors. Several specific stimuli, including hypertonicity which is a key factor for renal physiology, are able to activate them. It has been reported that, after hypertonic challenge, NF-κB activity can be modulated by TonEBP, considered as the master regulator of transcriptional activity in the presence of changes in environmental tonicity. In the present work we evaluated whether hypertonicity-induced gene transcription mediated by p65/RelA and TonEBP occurs by an independent action of each transcription factor or by acting together. To do this, we evaluated the expression of their specific target genes and cyclooxygenase-2 (COX-2), a common target of both transcription factors, in the renal epithelial cell line Madin-Darby canine kidney (MDCK) subjected to hypertonic environment. The results herein indicate that hypertonicity activates the Rel-family transcription factors p65/RelA and

https://doi.org/10.1016/j.heliyon.2018.e01072

TonEBP in MDCK cells, and that both are required for hypertonic induction of COX-2 and of their specific target genes. In addition, present data show that p65/RelA modulates TonEBP expression and both colocalize in nuclei of hypertonic cultures of MDCK cells. Thus, a sequential and synchronized action p65/RelA → TonEBP would be necessary for the expression of hypertonicity-induced protective genes.

Keywords: Cell biology, Biochemistry, Molecular biology

1. Introduction

Renal medullary interstitium is characterized by its elevated osmolarity due to the presence of high concentrations of sodium and urea that contribute to the urinary concentrating mechanism. Depending on the hydric state of the body, sodium and urea concentrations can abruptly vary to modify urine composition; but such fluctuations can severely affect the structure and function of renal cells [1, 2, 3]. When cell environment suffers an osmotic shock, various defensive mechanisms are activated. Then, renal cells subjected to high osmolarity activate the transcription of several membrane proteins (cotransporters sodium/myo-inositol (SMIT), sodium/chloride/betaine (BGT1) and sodium/chloride/taurine (TauT) [4, 5, 6, 7]) involved in the transport of protective organic osmolytes that counteract the increase in cytoplasmic ionic strength, molecular chaperone Hsp70 [8], and the cytoprotective protein cyclooxygenase 2 (COX-2) [9, 10, 11], among others. The transcription of all these genes is mediated by the tonicity responsive-enhancer binding protein (TonEBP) which is the major regulator in response to tonicity changes in renal cell environment [12, 13, 14].

TonEBP belongs to the Rel superfamily of transcription factor and its activation in renal cells by hyperosmolarity has been widely demonstrated [2, 9, 15, 16]. NF-κB also belongs to Rel-protein family [17]. Like TonEBP, NF-κB forms butterfly-shape heterodimers of Rel proteins (p65/Rel A, p50/p105, p52/p100, Rel B and c-Rel) that encircle DNA upon binding their κB sites in promoters and enhancers of a variety of genes, and induce or repress transcription [18, 19]. NF-κB is activated by different stimuli and regulates the expression of different target genes involved in diverse physiological functions as immune and inflammatory response, cell survival, growth and proliferation, and apoptosis [20, 21]. It has been demonstrated that in renal medullary interstitial cells [22] and in the mouse kidney cortical collecting duct cell line mpkCCD$_{cl4}$ [23, 24], hypertonicity can activate NF-κB. In renal cortical collecting ducts cells hypertonicity-activated NF-κB modulates the transcription of aquaporin-2 [23]. Besides, in renal medullary interstitial cells subjected to hypertonic culture conditions it has been shown that NF-κB mediates COX-2 expression [22]. In the renal epithelial cell line Madin-Darby canine kidney

https://doi.org/10.1016/j.heliyon.2018.e01072

(MDCK), hypertonicity-induced COX-2 transcription; we have demonstrated that such an expression is mediated by TonEBP [9]. In this cell line, it has been shown that COX-2 can be up-regulated through NF-κB activation after proinflammatory stimulus but not by hypertonicity [25]. MDCK cells have been used in several experimental protocols to evaluate and project the results to inner medullary structures since they behave as medullary collecting duct cells subjected to high sodium hyperosmotic medium [26, 27, 28, 29, 30, 31].

Considering that NF-κB and TonEBP belong to the same protein family, are activated by hyperosmolarity and mediate the expression of COX-2 protein, we hypothesized that both transcription factors would be able to act together or independently on their transcriptional activity on target genes. Thus, in the present work we examined the transcriptional activity of p65/RelA and TonEBP on their target genes expression and the possible interaction between both Rel-family transcription factors in MDCK cells.

2. Materials and methods

2.1. Cell culture conditions

Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection, passages 45−50) were grown in a mixture containing Dulbecco's modified Eagle's medium and Ham's-F12 (1:1), 10 % fetal bovine serum (FBS) and 1 % antibiotic mixture (GIBCO®). After reaching 70−80 % of confluence, cells were placed in low serum medium (0.5 % FBS) for 24 h and then subjected to hyperosmolar media made by the addition of an aliquot of sterile 5 M NaCl to commercial medium to achieve desired final concentrations (250 mM, ~512 mosm/kg H_2O). Media osmolarities were assessed by using an Osmometer (μOSMETTE, Precision Systems; Sudsbury, MA). In some experiments, cells were cultured in hyperosmolar media for 0,1.5, 3, 6, 12 and 24 h. In other set of experiments cells were treated for 24 h. No changes in media final osmolarities were detected due to the different serum concentration. In those experiments where specific inhibitors were used (10 or 20 μM Parthenolide, 100 or 500 μM PDTC, Sigma-Aldrich), they were added 30 min before the addition of NaCl to the medium.

After treatments, cells were harvested with 0.25 % trypsin-EDTA (GIBCO®). Cells were counted in a hemocytometer chamber (Neubauer's chamber) in the presence of trypan blue to obtain the number of total and viable cells. The viability was calculated from these data as the percentage of non-trypan blue stained cells of total counted cells. Aliquots of cell suspensions containing an adequate number of cells were used for the different experimental protocols. Despite non-viable cells were included in cell population used in the experiments, the number of trypan blue-stained cells was lower than 5 %. When it was required cells were resuspended in

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lysis-buffer (0.089 % NaCl-phosphate buffer, pH 7.2, containing 0.05 % Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, 1 mM leupeptin and 1 mM sodium orthovanadate).

2.2. Transfections

TonEBP silencing was performed by using siRNA duplex designed by Na et al. (5′-AUGGGCGGUGCUUGCAGCUCCUU-3′/5′-GGAGCUGCAAGCACGCCCAUU U-3′) [32]. MDCK cells were transfected with the siRNA duplex by means of Lipofectamine™ 3000 (Invitrogen) according to the manufacturer's protocol. After 24 h of transfection, cells were subjected to hypertonic NaCl medium for other 16 h. Then, cells were either collected for RT-PCR.

2.3. Cell fractionation

MDCK cells were grown, treated and collected as described above. To obtain nuclear and cytoplasmic fractions we followed the protocol described by Roth et al. [24]. Briefly, cells were resuspended and homogenized in 0.25 M sucrose in lysis-buffer A (10 mM HEPES-KOH, pH 7.9, containing 0.04 % Triton X-100, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF, 10 μg/ml aprotinin, 1 mM leupeptin and 1 mM sodium orthovanadate) on ice. The cell extracts were spun at 13000 × *g*. The supernatants, corresponding to cytoplasmic fractions, were collected and kept at −80 °C. The pellets were resuspended in 0.25 M Sucrose in lysis buffer A and respun. The pellets, nuclear fractions, were washed once with lysis-buffer A and kept in the same buffer at −80 °C. The nuclear and cytoplasmic fractions were analyzed by western blot.

2.4. Western blot analysis

This assay was performed in whole lysates and in cell fractions. To obtain whole lysates, after treatments cells were collected and counted as described above and resuspended in lysis-buffer (0.089 % NaCl-phosphate buffer, pH 7.2, containing 0.05 % Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, 1 mM leupeptin and 1 mM sodium orthovanadate). When cell fractions were used, they were resuspended in lysis buffer. In both types of samples, protein content was determined by Lowry procedure [33]. Aliquots containing 50 μg of lysate- or cell fractions- protein were incubated with 4 X Laemmli buffer at 100 °C for 5 min and resolved in a 10 % SDS-polyacrylamide gel and blotted to PVDF membranes (GE Healthcare Life-science). Blots were blocked with 10 % non-fat milk in TBS-Tween and incubated overnight at 4 °C with primary antibodies: rabbit polyclonal COX-2 antibody, 1:250 (Cayman Chemical Co), mouse monoclonal p65/RelA antibody; 1:500 (Santa Cruz Biotechnology), rabbit polyclonal antibody TonEBP, 1:500 (Santa Cruz

https://doi.org/10.1016/j.heliyon.2018.e01072

Biotechnology). As loading control, mouse polyclonal lamin A antibody (nuclear marker) 1:500 (Milipore) and rabbit polyclonal β-tubulin antibody (cytoplasmic marker), 1:5000 (AbCam), were used. After washing, blots were incubated with secondary antibody: donkey anti-rabbit horseradish peroxidase conjugate 1:6000 or sheep anti-mouse horseradish peroxidase conjugate 1:10000 (GE, Healthcare Life-science), and bands evidenced by means of ECL Plus western blotting analysis system (GE, Healthcare Life-science). The intensity of each band was estimated by optical densitometric analysis using Gel-Pro Analyzer 3.1.

In cell fractionation experiments followed by western blot, the value of optical density of p65/Rel A band was divided by the value of the optical density of Lamin A band in nuclear lines. In cytoplasmic lines, the value of optical density of p65/Rel A band was divided by the value of the optical density of β-tubulin band. With these estimations nuclear to cytoplasmic ratios were calculated. Thus, bars represent the nuclear to cytoplasmic ratio calculated as the quotient between p65-RelA/Lamin A ratio and p65-RelA/β-tubulin ratio of the three independent experiments. Similar procedure was followed for cell fractionation-western blot experiments for TonEBP protein. In this case, the bars represent the nuclear to cytoplasmic ratio calculated as the quotient between TonEBP/Lamin A ratio and TonEBP/β-tubulin ratio of the three independent experiments.

2.5. Co-Immunoprecipitation

After treatments cells were collected and the co-immunoprecipitation was performed with Co-Immunoprecipitation kit (Pierce). Briefly, cells were harvested in IP Lysis buffer (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% Glycerol, pH 7.4), aliquots containing 850 μg of lysate-protein were transferred to the columns containing the antibody (TonEBP or NF-κB, p65/RelA)- coupled resin. After incubation with gentle mixing overnight at 4 °C, the columns were washed with PBS twice and then the complexes were eluted with the Elution buffer. The samples were finally analyzed by western blot to detect p65/RelA or TonEBP.

2.6. RT-PCR

After treatments, 2×10^6 cells were used for total RNA extraction by using SV total RNA isolation system (Promega) in accordance with the manufacturer's instructions. The first-strand cDNA was synthesized using the reverse transcription system (Promega), after that, PCRs for NF-κB, TonEBP, IκB, BGT1, AR, SMIT, COX-2 and MCP-1gene-expression products were performed. The primers used were: NF-κB (p65/RelA), For: 5′ TGGAAGCACGAARGACAGAG 3′; Rev: 5′ GGACGAACACA GAGGTTGGT 3′, TonEBP, For: 5′ AAGGCAACTCAAAAGCAGGA 3′, Rev: 5′ CCTGCAACACTACTGGCTCA 3′, IκB For: 5′ AGCAATTTCTGGTTGGTTGG 3′, Rev: 5′ AAGGACGAGGAGTACGAGCA 3′, BGT1, For: 5′ GAGGTGTCC

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CTAGTCCCACA 3′, Rev: 5′ CACCCACAAAGTCCAGAGGT 3′, AR, For: 5′ GAGGACCTCTTCGTCGTCAG 3′, Rev: 5′ CTATAGGCGGTCACCACGAT 3′, SMIT, For: 5′ GCTCATGCCAAAGGCTCTAC 3′, Rev: 5′ TCACCACCA TAAAAGCCACA 3′, COX-2, For: 5′ TCAGCCATACAGCAAATCCTT 3′, Rev: 5′ GRGCACTGTGTTTGGAGTGG 3′; β-actin, For: 5′ CAAAGCCAACCGTGA GAAG 3′; Rev: 5′ CAGAGTCCATGACAATACCAG 3′ and MCP-1, For: 5′ TCTCCAGTCACCTGCTGCTA 3′, Rev: 5′ GGAATCCTGGACCCACTTCT 3′. After amplification, mixes were separated using 2% agarose gel electrophoresis (GenBiotech). β-actin was used as loading control.

2.7. Microscopy

Cells were cultured as described above but on glass coverslips. After treatments, cells were washed twice with sterile PBS, fixed with 3.7 % paraformaldheyde in PBS for 30 min, and permeabilized with 0.1% Triton-X100 in PBS for 30 min. Fixed cells were incubated with a mixture of mouse monoclonal p65/RelA antibody; 1:100 (Becton-Dickinson), TonEBP, 1:75 (Santa Cruz Biotechnology) and 2.5 μM Hoechst 33258 (Sigma-Aldrich) for 60 min. After labeling, samples were washed with PBS and incubated with FITC-conjugated goat anti-mouse 1:200 (Vector Lab) and Alexa Fluor 546-conjugated donkey anti-rabbit 1:200 (Invitrogen) secondary antibodies for 60 min. Then, samples were washed with PBS and mounted with a drop of Vectashield Mounting Medium (Vector Laboratories). Specimens were examined with an Olympus FV300 Confocal Microscope (Model BX61), with an acquisition software FluoView version 3.3 provided by the manufacturer. Confocal images represent a single Z section. All images were obtained with a cooled CCD camera and processed for output purposes using Image J software.

2.8. Statistical analysis

The results were expressed as the mean ± SEM. Data from controls and different treatments were analyzed by ANOVA and significant differences were assessed by "a posteriori" Dunnet or Tukey test ($p < 0.05$).

3. Results

3.1. Hypertonicity activates Rel family transcription factors in MDCK cells

The activation of a transcription factor may involve the increase in the levels of its mRNA and protein and/or its redistribution from the cytosol to the nuclear compartment and/or the activation of the transcription of specific target genes. So, to assess

https://doi.org/10.1016/j.heliyon.2018.e01072

whether hypertonicity activates p65/RelA in MDCK cells, the effect of 125 mM NaCl (512 ± 20 mOsm/Kg H_2O) on NF-κB expression, nucleo-cytoplasm distribution and transcriptional activity was evaluated (Fig. 1). Hypertonicity increased p65/RelA mRNA expression after 6 and 12 h of treatment (Fig. 1A), before the significant increase in protein levels detected after 12 and 24 h of treatment (Fig. 1B). p65/RelA translocation to the nucleus, necessary for transcription, was observed by immunofluorescence and cell fractionation (Fig. 1C and D). After treatment, isotonic cells were mostly labeled in the cytoplasm while in hypertonic cells p65/RelA dots were evidenced in the nucleoplasm. Western blot images of subcellular fractions (Fig. 1D) show that high NaCl medium did not increase nuclear p65/RelA protein after short-period treatments (1.5 and 3 h), but protein translocation became evident after longer incubation times (6, 12 and 24 h). The effect of hypertonicity on p65/RelA redistribution from the cytoplasm to the nucleus was better appreciated when the ratio between protein level in nuclear compartment respect to cytoplasm was calculated (Fig. 1D, Bars). p65/RelA hypertonicity-induced activity was evidenced by the increased mRNA level of its target gene MCP-1 (monocyte chemotactic protein-1) after 12 h of treatment (Fig. 1E) that coincides with the presence of p65/RelA in the nucleus. Hypertonic activation of TonEBP has been widely demonstrated [2, 9, 15, 16]. We evaluated if hypertonicity activates TonEBP in our experimental conditions by determining the levels of the TonEBP mRNA and protein, its redistribution from the cytosol to the nuclear compartment and its transcriptional activity (Fig. 2). Hypertonicity significantly increased TonEBP mRNA levels after 6 h and 12 h of treatment and became equal to control after 24 h (Fig. 2A). Western blot analysis shows that protein level significantly increased after 12 and 24 h (Fig. 2B). The presence of TonEBP in the nucleus was observed by immunofluorescence and western blot analysis of cell fractions (Fig. 2C and D). In contrast to that observed for p65/RelA, short incubation periods (1.5, 3 and 6 h) caused the highest nuclear distribution of TonEBP. The fact that nuclear/cytoplasmic ratio decreased after 12 h and 24 h when compared to 6 h (Fig. 2D, bars) could be attributed to the hypertonic induction of TonEBP expression (Fig. 2B and D). At 6 h, the protein level was the lowest and most of the protein was present in the nuclear compartment. After 12 h, the levels of the protein increased as consequence of its translation. Despite a high level of TonEBP protein found in nuclear compartment, cytoplasmic protein level also increased. Thus, the nuclear/cytoplasmic ratio at 12 h decreases respect to 6 h. After 24 h a similar effect was observed. The 24 h-nuclear TonEBP level was more intense compared to 6 h-nuclear TonEBP, and 24 h-cytoplasmic protein was also more intense than 6 h-cytoplasmic TonEBP. This is the reason why the nuclear/cytoplasmic ratio at 24 h was smaller than the ratio at 6 h. If we compare 12 h and 24 h, the nuclear/cytoplasmic ratio at 24 h was higher than at 12 h; this occurs because cytoplasmic levels of TonEBP protein at 12 h and 24 h were similar between them, while nuclear level of this protein at 24 h was higher than observed at 12 h.

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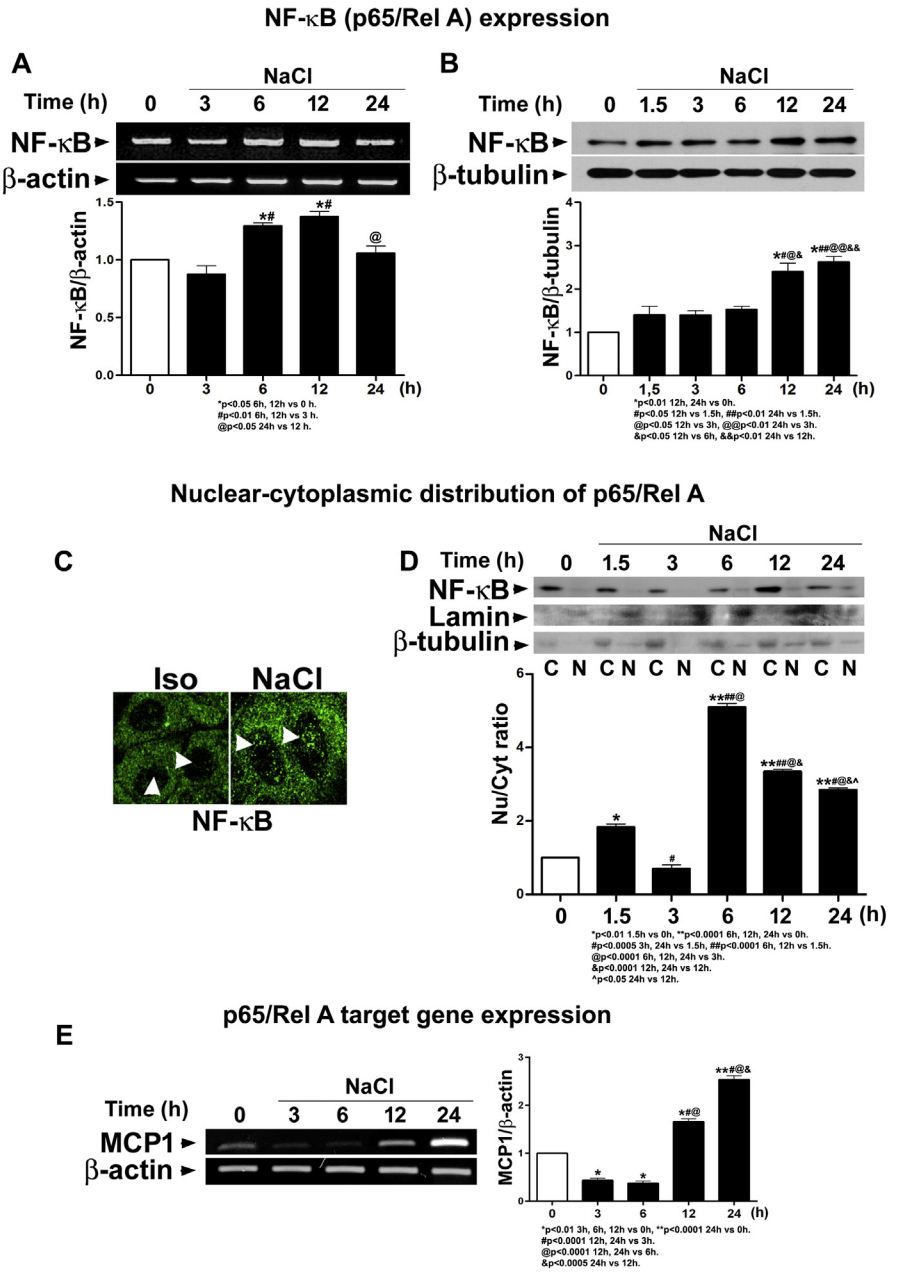

Fig. 1. Effect of hypertonicity on NF-κB expression, cellular distribution and activity in MDCK cells. MDCK cells were grown and subjected to 125 mM NaCl (~512 mosm/kg H_2O) for different periods of time (0, 1.5, 3, 6, 12 and 24 h) as described in Methods. After treatment, cells were collected and subjected to total RNA isolation followed by RT-PCR for NF-κB (p65/RelA) and β-actin (Panel A), or by western blot analysis probing the membrane with mouse monoclonal p65-RelA antibody (1:500) and rabbit polyclonal β-tubulin antibody (1:5000) (Panel B). Each image is representative of three independent experiments eliciting similar pattern. In Panel C, MDCK cells were grown on sterile coverslips and after 16 h of treatment, cells were fixed and stained with mouse monoclonal p65-RelA antibody (1:100) and revealed by using a FITC-conjugated secondary antibody (1:200). Samples were mounted with Vectashield Mounting Medium. Fluorescence images were obtained with a Nikon Eclipse T*i* with acquisition software Micrometrics SE Premium (Accu-Scope). The figure shows a representative

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The hypertonicity-induced transcriptional activation of TonEBP was determined by measuring the levels of its known target genes mRNA; SMIT (myo-inositol transporter), BGT1 (betaine/γ-aminobutyric acid transporter), AR (aldose reductase) and COX-2 (Fig. 2E). Three of the four target genes, SMIT, BGT1 and AR, increased their mRNA level at 6 h of treatment with a maximum at 12 h while COX-2 increase its mRNA after 12 h with a maximum at 24 h. Together these results indicate that hypertonicity activates the Rel family proteins p65/RelA and TonEBP in MDCK cells.

3.2. NF-κB - TonEBP coordinated transcriptional activity is required for target genes expression

COX-2 is considered a survival protein in renal cells. We and others demonstrated that hypertonicity up-regulates the expression of COX-2 in renal medullary cells [9, 11, 34, 35, 36]. Depending on the cell line studied, the transcriptional activation of COX-2 gene can be mediated by various transcription factors [9, 22, 35, 36]. We previously showed that hypertonicity-induced COX-2 expression is mediated by TonEBP; herein we evaluated whether hypertonicity-induced COX-2 expression also requires NF-κB activity in MDCK cells. To do this, before the addition of NaCl to the medium, MDCK cells were pre-incubated with specific inhibitors of NF-κB activity: PDTC and parthenolide (Parthe), that exert their effects through different mechanisms of action. In vitro experiments demonstrated that parthenolide binds to and inhibits the IκB kinase β (IKK) [37, 38, 39]. PDTC mechanism of action it is not well established yet. Some authors suggest that PDTC reversibly suppressed the release of the inhibitory subunit IκB from the latent cytoplasmic form of NF-κB at micromolar amounts of in cells cultures [40, 41]. Other authors suggest that PDTC inhibits the NF-κB pathway by an overall reduction of all NF-κB subunits and IKK complex (IKK-α, IKK-β and IKK-ɣ) expression [42]. In our experimental conditions, both PDTC and parthenolide strongly decreased MCP-1 mRNA levels, indicating that these inhibitors blocked NF-κB activity (Fig. 3C and E, right bar graph). Both inhibitors also decreased COX-2 protein levels (Fig. 3A and B), which was coincident with the decrease in mRNA expression (Fig. 3C and D, left bar graph). Thus, COX-2 can be considered a target gene for TonEBP and NF-κB.

It was shown that, in mouse cortical collecting duct (mCCD) cells, the activity of NF-κB after hypertonic challenge is modulated by TonEBP [24, 43]. In order to

image of three independent experiments. Panel D shows cell fractionation after hypertonic treatment followed by western blot analysis of nuclear and cytoplasmic fractions. Lamin A was used as nuclear marker while β-tubulin, as cytoplasmic marker. The nuclear fraction (p65-RelA/LaminA) to cytoplasmic fraction (p65-RelA/β-tubulin) ratios were calculated from the values obtained by the densitometry analysis of the western blot membranes from three independent experiments. The expression of MCP1, a target gene of NF-κB activity, was determined by RT-PCR (Panel E). The figure shows a representative image of three independent experiments. The results are expressed as the mean ± SEM of three independent experiments.

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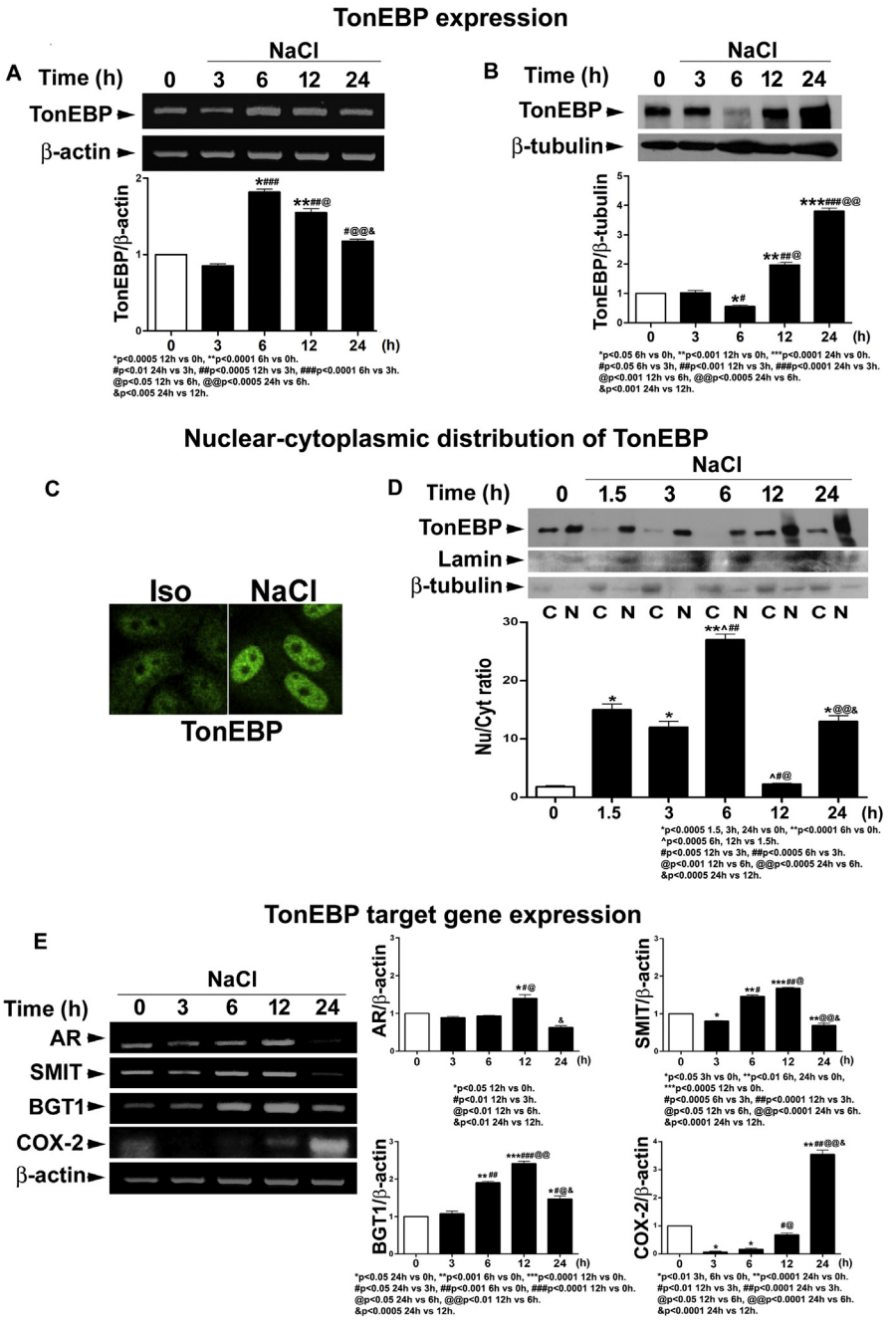

Fig. 2. Effect of hypertonicity on TonEBP expression, cellular distribution and activity in MDCK cells. MDCK cells were grown and subjected to 125 mM NaCl (~512 mosm/kg H_2O) for different periods of time (0, 1.5, 3, 6, 12 and 24 h) as described in Methods. After treatment, cells were collected and subjected to total RNA isolation followed by RT-PCR for TonEBP and β-actin (Panel A) or subjected to western blot analysis by probing the membrane with rabbit polyclonal TonEBP antibody (1:500) and rabbit polyclonal β-tubulin antibody (1:5000) (Panel B). Each image is representative of three independent experiments eliciting similar pattern. In Panel C, MDCK cells were grown on sterile coverslips and after 16 h of treatment, cells were fixed and stained with rabbit polyclonal TonEBP (1:75) antibody and revealed by using a FITC-conjugated secondary antibody (1:200). Samples were mounted with Vectashield Mounting Medium. Fluorescence images were obtained with a Nikon Eclipse T*i* with acquisition

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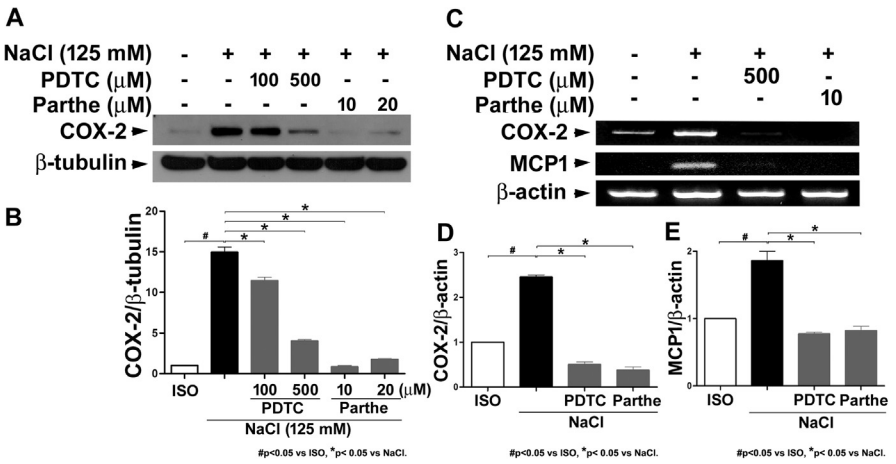

Fig. 3. Effect of NF-κB pathway inhibitors on COX-2 protein and mRNA expression. MDCK cells were grown as described in Methods. Before NaCl addition, cells were treated with different concentrations of PDTC or parthenolide (Parthe) for 30 min, and then an aliquot of 5 M NaCl was added to achieve 125 mM final concentration (~512 mosm/kg H_2O). After 24 h treatment, cell lysates were subjected to western blot analysis by probing PVDF membranes with rabbit polyclonal COX-2 antibody (1:250) and rabbit polyclonal β-tubulin antibody (1:5000) (Panel A shows a representative membrane and panel B shows bar graph representing the relative bands intensities ratios) or subjected to total RNA isolation followed by RT-PCR for COX-2, MCP1 and β-actin (Panel C shows a representative image and panels D and E show bar graphs representing the relative band intensities ratios). Each image is representative of three independent experiments eliciting similar pattern. The results are expressed as the mean ± SEM of three independent experiments.

evaluate such possibility in our experimental system, MDCK cells were incubated in isotonic and hypertonic conditions in the absence or the presence of NF-κB inhibitor and/or TonEBP siRNA. After treatments, the expression of their target genes was evaluated (Fig. 4). As showed above, hyperosmolarity up-regulated the expression of NF-κB target genes: MCP1, IκB and COX-2 (Fig. 4A and B). Parthenolide decreased the mRNA levels of all the genes evaluated, thus confirming the requirement of NF-κB activity for the expression of MCP1, IκB and COX-2. The absence of TonEBP caused the predictable decrease of its target genes SMIT, AR and BGT1 mRNA levels, compared with NaCl-induced expression (Fig. 4C and D), and the blockade of COX-2 expression, as it was also expected. TonEBP silencing did not affect IκB mRNA but, unexpectedly, impeded the expression of the NF-κB

software Micrometrics SE Premium (Accu-Scope). The figure shows a representative image of three independent experiments. Panel D shows cell fractionation after hypertonic treatment followed by western blot analysis of nuclear and cytoplasmic fractions. Lamin A was used as nuclear marker while β-tubulin, as cytoplasmic marker. The nuclear fraction (TonEBP/LaminA) to cytoplasmic fraction (TonEBP/β-tubulin) ratios were calculated from the values obtained by the densitometry analysis of the western blot membranes from three independent experiments. The expression of AR, BGT1, SMIT and COX-2, TonEBP target genes, was determined by RT-PCR (Panel E). The figure shows a representative image of three independent experiments. The results are expressed as the mean ± SEM of three independent experiments.

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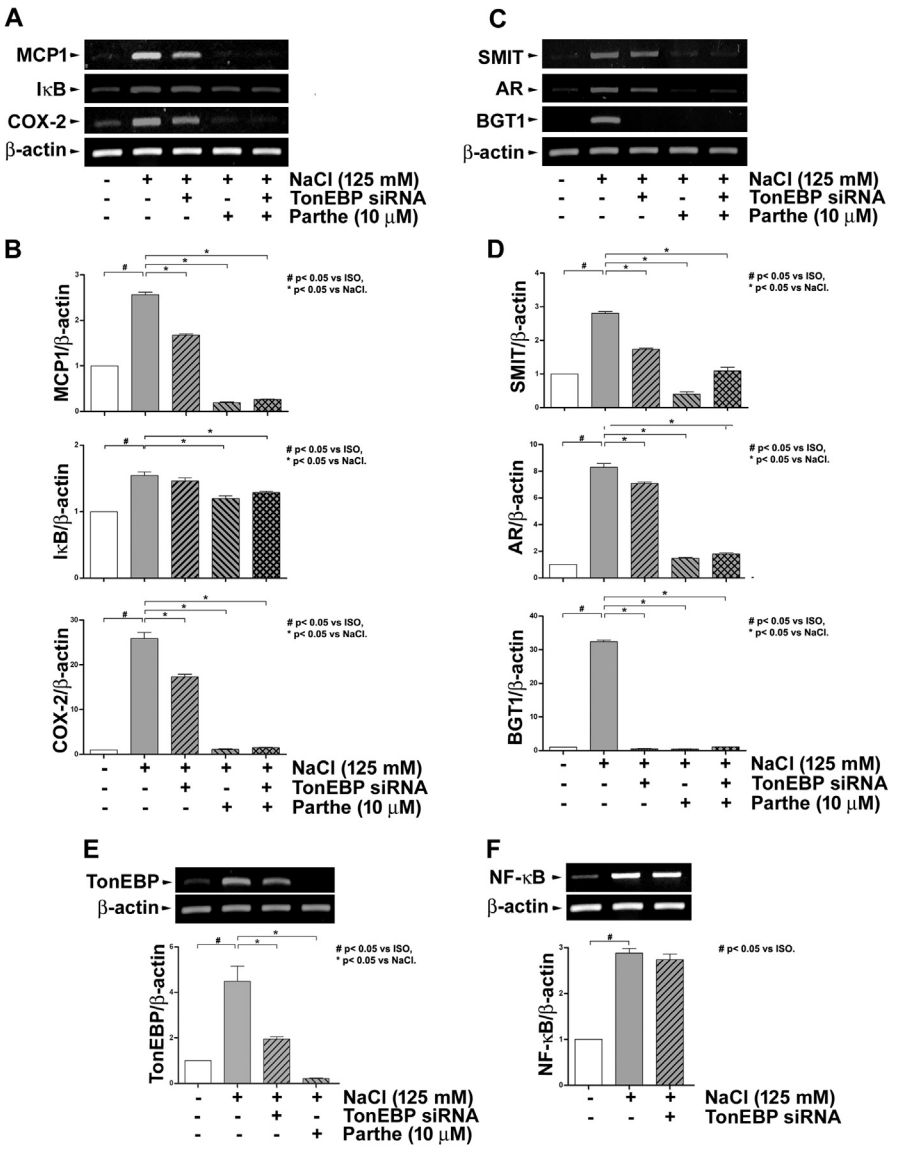

Fig. 4. NF-κB and TonEBP activities under hypertonic conditions. MDCK cells were grown, treated with TonEBP siRNA or 10 μM Parthenolide (Parthe), and subjected to 125 mM NaCl (~512 mosm/kg H_2O) as described in Methods. After 16 h treatment, cells were collected, and lysates were subjected to total RNA isolation followed by RT-PCR for TonEBP, NF-κB (p65/RelA), MCP1, IκB, COX-2, AR, BGT1, SMIT and β-actin. Panels A and B show the mRNA analysis of NF-κB target genes MCP1, IκB and COX-2. Panels C and D shows the mRNA analysis of TonEBP target genes SMIT, AR and BGT1. Panels E shows the mRNA analysis of TonEBP and panel F, the mRNA analysis of NF-κB (p65/RelA). Each image is representative of three independent experiments eliciting similar pattern. The results are expressed as the mean ± SEM of three independent experiments.

target gene MCP1 (Fig. 4A and B). TonEBP target genes were also down-regulated when NF-κB was inhibited by parthenolide (Fig. 4C and D).

These results indicate that TonEBP expression is necessary for NF-κB activation and/or expression since MCP1 fell down after TonEBP silencing. In addition,

https://doi.org/10.1016/j.heliyon.2018.e01072

they show that NF-κB activity regulates TonEBP activity and/or expression because its inhibition blocked SMIT, AR and BGT1 mRNA expression. Fig. 4E demonstrates that NF-κB regulates the expression of TonEBP and, in this way, NF-κB abolished TonEBP activity. This finding goes along with the prediction of potential transcription factor binding sites (κB-response element) performed by TRANSFACT® using the software P-Match 1.0 (Fig. 5). In contrast, the silencing of TonEBP did not affect the expression of NF-κB transcription factor (Fig. 4F).

The fact that TonEBP silencing impaired NF-κB activity but not its expression led us to think that TonEBP protein, but not its activity, is necessary for a complete NF-κB activation. To evaluate the possible interaction between both transcription factors, co-immunoprecipitation assays followed by western blot analysis were performed as described in Methods (Fig. 6). As is seen in Fig. 6A, antibodies against TonEBP were found to coimmunoprecipitate NF-κB (p65/RelA) and antibodies against p65/RelA coimmunoprecipitate TonEBP, under hypertonic conditions. The faint intensity observed for the immunoprecipitated band associated to p65/RelA antibody might be related to reduced amount of p65/RelA detected in nuclear compartment (Fig. 1C and 6B). This experiment suggests proximity of both transcription factors. TonEBP and p65/RelA collocation was evidenced by confocal microscopy (Fig. 6B). Hence, it is possible that the interaction between TonEBP and NF-κB (p65/RelA) could be involved in their regulatory action.

4. Discussion

The aim of the present work was to determine whether hypertonicity-induced gene transcription mediated by NF-κB and TonEBP occurs by an independent action of each transcription factor or by acting together. The present results show that a

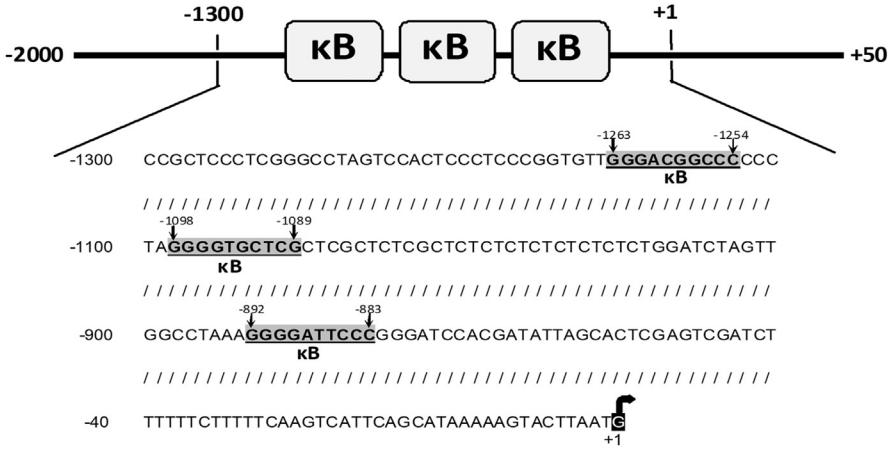

Fig. 5. Putative κB-sites in the TonEBP promoter. The scheme shows the location of the three putative κB-sites in the promoter of TonEBP predicted by TRANSFACT®, with regards to the distance to the transcription start site (+1).

https://doi.org/10.1016/j.heliyon.2018.e01072

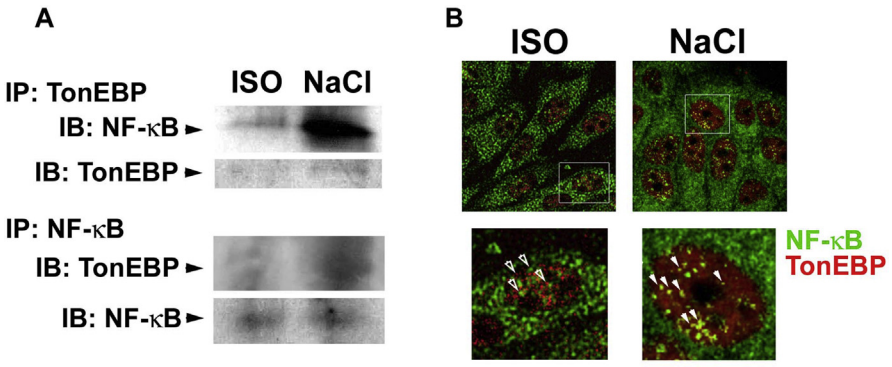

Fig. 6. NF-κB (p65/RelA) interacts with TonEBP under hypertonic conditions. Panel A shows western blot analysis of NF-κB (p65/RelA) protein immunoprecipitated by TonEBP antibody (upper image) and the western blot analysis of TonEBP protein immunoprecipitated by NF-κB (p65/RelA) antibody (lower image), from cells treated or not with hypertonic medium (125 mM NaCl, ~512 mosm/kg H_2O) for 16 h. Panel B shows the confocal microscopy images of MDCK cells stained with rabbit polyclonal TonEBP (1:75) and mouse monoclonal NF-κB (p65/RelA) (1:100) antibodies and revealed by using a FITC- and Alexa 546- conjugated secondary antibody (1:200) as described in Methods. Samples were mounted with Vectashield Mounting Medium. Fluorescence images were obtained with Olympus FV300 Confocal Microscope (Model BX61), with acquisition software FluoView version 3.3 provided by the manufacturer and processed as described in Methods. The figure shows a representative image of three independent experiments.

sequential and synchronized action NF-κB (p65/RelA) → TonEBP would be necessary for the expression of the hypertonicity-induced protective genes SMIT, BGT1, AR and COX-2. Thus, NF-κB (p65/RelA) modulates TonEBP expression that in turn activates the expression of its target genes. Moreover, nuclear p65/RelA-TonEBP colocalization suggests that latter interaction is required for NF-κB transcriptional activity.

It has previously shown that hypertonic environment activates NF-κB in primary cultures of renal interstitial cells [22] and in lines of cortical collecting duct cells [24]. MDCK cells subjected to high sodium hyperosmotic medium behave as inner medullary collecting duct cells, for this reason they are usually used as a model system for studying medullary cell physiology and adaptation to osmotic stress [28, 29, 30, 31]. Coinciding with that reported for other renal-experimental systems, our results show that high NaCl activates NF-κB (p65/RelA) expression and transcriptional activity in MDCK cell line (Fig. 1). Such activation leads the transcription of NF-κB target gene COX-2, which is considered a renal osmoprotective gene. It is well established that hypertonicity up-regulates COX-2 expression in renal cells and that NF-κB mediates COX-2 transcription in different cell types. However, the participation of NF-κB in the induction of COX-2 by hypertonicity is still controversial. Numerous reports describe the participation of NF-κB in COX-2 expression after IL1-β [44, 45], PMA [46], LPS [47, 48] and resistin [49] stimulation. Hypertonic induction of COX-2 in mouse cortical collecting duct cells and in human

https://doi.org/10.1016/j.heliyon.2018.e01072

pulmonary epithelial cell line A549 was independent of NF-κB activity [36, 50]. In contrast, it was shown that hypertonic stress activates an NF-κB-COX-2-linked survival mechanism in renal medullary interstitial cells [22, 51] agreeing the present study. The present results not only show that NF-κB (p65/RelA) is mediating COX-2 expression, but also that in this action NF-κB requires TonEBP coordinated action.

In the kidney, COX-2 is considered an osmoprotective gene against changes in interstitial osmolarity [52]. Various reports demonstrate that COX-2 expression is regulated by environmental osmolarity in renal cells. In animal models it was demonstrated that medullary COX-2 expression decreases significantly with salt depletion and increases with a high-salt diet [11, 53, 54] and with water deprivation [54]. In *in vitro* experiments we and others demonstrated that high-NaCl media significantly increases COX-2 expression in medullary interstitial cells and in collecting duct cell lines [9, 35, 54]. Moreover, in the kidney, COX-2 is present throughout the different regions being maximal its expression in renal medulla where the interstitial osmolarity is the highest [55]. COX-2 osmoprotective actions may be dependent or non-dependent on prostaglandin synthesis. Neuhofer et al demonstrated that PGE_2, induces COX-2 expression in an autocrine-positive feedback loop being such an increase necessary for cell survival [56, 57]. Küper et al found that PGE_2 promotes cell survival by phosphorylation-mediated Bad inactivation during hypertonic treatment of MDCK cells [58]. In a previous work we demonstrated a PG-nondependent COX-2 action, since its silencing in hypertonic-cultures of MDCK reduced the number of cells that survive to treatment [9]. In addition, other authors demonstrated that COX-2 binds to and stabilizes survivin preventing its proteasomal degradation [59, 60].

TonEBP is a key regulator of osmoprotective responses [14] and, similar to NF-κB, belongs to the Rel-family of transcription factors [17]. Roth and coworkers have shown that the activity of NF-κB after hypertonic challenge is modulated by TonEBP. In contrast to these authors, our results suggest that p65/RelA directs TonEBP expression and in consequence its activity. Such affirmation is based on the fact that parthenolide completely blocked the expression of TonEBP after 12 h of incubation with NaCl-enriched media (Fig. 4).

As it is seen in Fig. 2, TonEBP protein shows a biphasic expression, that is minimal after 6 h of treatment and maximal at 24 h. The sharp protein increase observed at 12 h was preceded by its mRNA rise (6 h) reflecting that the rate of protein translation was due to the mRNA abundance. It is well described that during the first hours of high NaCl, cells suffer several morphologic and biochemical alterations, decreasing most of the metabolic processes. During this period there is an activation of protective and adaptive mechanisms to restore cell homeostasis [2, 3, 12, 61].

https://doi.org/10.1016/j.heliyon.2018.e01072

Hypertonicity profoundly inhibits protein synthesis (transcription and translation) except for those genes involved in the adaptive response to hyperosmolar stress [62]. Among them TonEBP, that is the transcription factor considered the master regulator of osmoprotective genes. The half-life of TonEBP protein is ~10 h. Thus, the TonEBP protein level decrease after 6 h may be a consequence of protein degradation and protein synthesis inhibition. Therefore, early response to high NaCl in MDCK cells may be mediated by pre-existing TonEBP protein (as is seen in Fig. 2B, C and D) that can translocate to the nucleus to induce gene expression. During this period (before 6 h) it is probable that no TonEBP transcription and translation occur. After 6 h, hypertonicity increased mRNA but not its translation. As is seen in Fig. 2A and B, the level of TonEBP protein after 12 h was probably due to the activation of TonEBP mRNA translation. The TonEBP mRNA increase observed at 6 h, followed by the protein expression, coincide with the fact that p65/RelA increased its presence in nuclear compartment at this time (Fig. 1). Moreover, by TRANSFACT® searching, three potential transcription factor binding sites (κB-RE) can be predicted in the canine promoter of TonEBP/NFAT5 (Fig. 5). The predicted κB-sites are slight variations of the consensus DNA sequence 5′-GGGRNYYYCC-3′, in which R is a purine, Y is a pyrimidine, and N is any nucleotide [63]. Therefore, as was mentioned before, early reaction to high NaCl may be mediated by pre-existing TonEBP protein, but latter response (between 12 and 24 h) would require newly synthesized protein whose synthesis is mediated by NF-κB. Thus, the expression of the target genes AR, SMIT and BGT1 at short periods of hypertonic treatment is induced by TonEBP protein that is already expressed in the cell. Then, the activation of NF-κB induces the expression of TonEBP. These newly TonEBP-protein synthetized together with NF-κB (p65/RelA) may be responsible for the latter COX-2 mRNA increase after 24 h (Fig. 2).

The present results also suggest that both transcription factors have a synergy action since TonEBP silencing completely abolished MCP1 expression. So, despite NF-κB is still present after TonEBP knock down, it cannot activate the expression of its target genes if TonEBP is not present. In some experimental models, such as mouse embryo fibroblasts (MEFs), peritoneal macrophages and RAW264.7 cells, TonEBP is recruited to the LPS-induced NF-κB enhanceosome critical for expression of pro-inflammatory genes [64]. In Mouse Cortical Collecting Duct Cell Line (mCCD), it has been described that TonEBP can interact with NF-κB [24, 64]. Moreover, the formation of NF-κB (p65-RelA)-TonEBP complexes is required for the binding to κB elements in NF-κB responsive genes [24, 43]. In agreement with this report, present results show the co-distribution of both transcription factors (Fig. 6). Based on the present results, we can speculate that NF-κB (p65-RelA) -TonEBP complexes are the real protagonists in the hypertonic induction of COX-2 in MDCK cells.

https://doi.org/10.1016/j.heliyon.2018.e01072

Declarations

Author contribution statement

Cecilia Casali: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data, Wrote the paper.

Luciana Erjavec: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Marìa Fernández Tome: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Funding statement

This work was supported by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), PICT 2013-1132.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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