3.4.1 The expression of AQP2 correlates with extracellular sodium concentrations

The experiment shown beforehand (Fig. 12) pointed at the importance of elevated tonicity in increasing the expression of AQP2. No increase in AQP2 expression was yielded by a mere elevation of osmolality by urea. The promoting effect of sodium-derived tonicity on AQP2 expression was more pronounced than the effect of sorbitol. Therefore, the effect of sodium concentrations on AQP2 protein expression was further analyzed. For this purpose, IMCD cells were seeded in 600N medium (control, CTRL) and grown for 6 days in 300N elevated by 10, 20, 35, 50, 100, 150, 200, 250, and 300 mM NaCl. AQP2 protein expression was analyzed by Western blot analysis. A representative set of experiments is shown in Fig. 13 A. The results are summarized in Fig. 13 B.

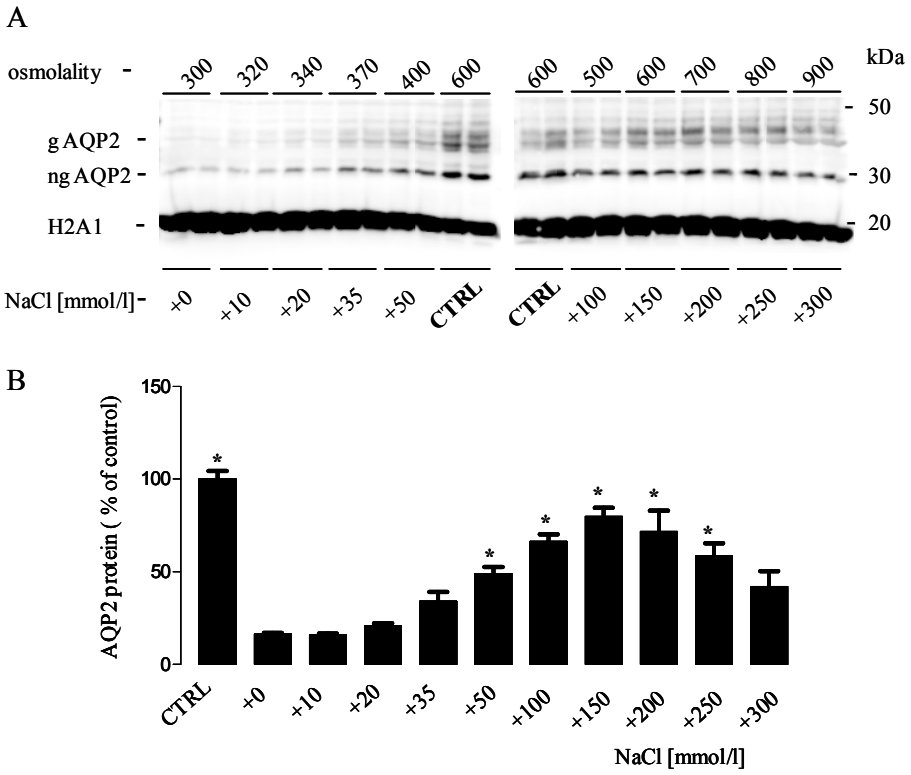

Fig. 13. AQP2 protein expression correlates with extracellular sodium concentrations. IMCD cells were cultured for 6 days in 24-well plates in the presence of 500 μM DBcAMP in 600N (control, CTRL) and 300N elevated to the indicated osmolalities with 0 mM (300N), 10 mM, 20 mM, 35 mM, 50 mM, 100 mM, 150 mM, 200 mM, 250 mM and 300 mM NaCl. A: AQP2 (g, glycosylated; ng, non-glycosylated) and histone H2A1 (as indicated) were detected by immunoblotting with anti-AQP2 antiserum. Per lane of an SDS-PAGE gel, total homogenate (protein) from 1 cm^2 confluent cell monolayer was loaded. B: Densitometrical analysis of AQP2 protein levels (glycosylated and non-glycosylated) detected in total homogenates. Values are means +/- SE (7>n>12) of control (CTRL). *P < 0.05 vs. 300N.

Statistical evaluation shows that baseline AQP2 protein levels (as observed in cells kept in 300N) started to increase significantly when medium tonicity was elevated to 400 mosmol/l by NaCl. The expression of AQP2 increased with sodium concentrations up to 150 mM and appeared to decrease when concentrations higher than 200 mM were applied. The decrease in AQP2 expresssion observed at a sodium-derived tonicity above 700 mosmol/l might be due to a initial reduction in cell volume which interferes with normal cell function. At a sodium-derived tonicity of 800 mosmol/l, AQP2 expression was less decreased when urea (100 mM) was additionally present (see Fig. 12).

3.4.2 The effect of hypertonic challenge on AQP2 expression is enhanced by urea

The results presented in Fig. 12 suggested that urea in combination with elevated sodium concentrations, has a potency to enhance the expression of AQP2 (compare 600 NaCl/U 50/200 to 600 NaCl/S 50/200). Therefore, it was tested whether a combination of urea and sorbitol would increase the expression of AQP2 in IMCD cells compared to cells kept in medium elevated to 600 mosmol/l exclusively by sorbitol.

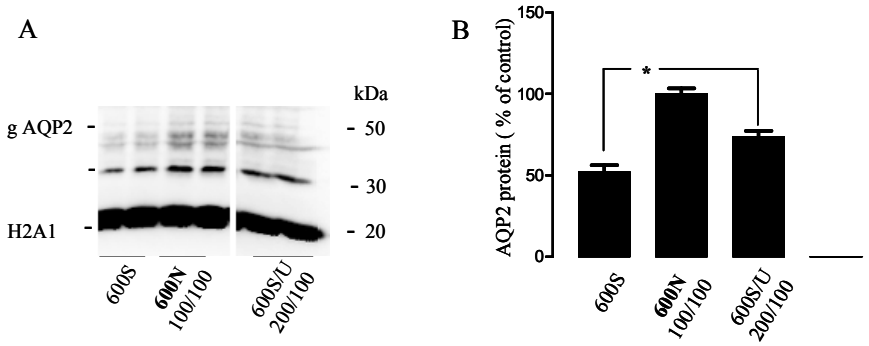

Fig. 14. Urea enhances the promoting effect of hypertonic sorbitol on AQP2 expression.
IMCD cells were cultured for 6 days in 24-well plates in the presence of 500 μM DBcAMP in 600S, 600N 100/100 (control) (*left*) or 600 S/U 200/100 medium (*right*). Numbers in front of and behind slash indicate added concentrations (to 300 N medium) of sodium (NaCl), urea (U) or sorbitol (S), (in mM), respectively. A: AQP2 (g, glycosylated; ng, non-glycosylated) and histone H2A1 (as indicated) were detected by immunoblotting with anti-AQP2 antiserum. All lanes shown are from a single blot. Per lane of an SDS-PAGE gel, total homogenate (protein) from 1 cm^2 confluent cell monolayer was loaded. B: Densitometrical analysis of AQP2 protein levels (glycosylated and non-glycosylated) detected in total homogenates. Values are means +/- SE (8>n>16). *P < 0.05.

The results presented in Fig. 14 show that the expression of AQP2 is significantly increased in IMCD cells when urea is present in the culture medium at the expense of the total sorbitol concentration, although both media compared (600S and 600S/U 200/100) have the same

osmolality (600 mosmol/l). A similar increase in AQP2 expression is effective for the combination of sodium and urea compared to sodium alone (see Fig. 13, compare 600 150/0 to 600N) Together, these findings indicate that an elevation of tonicity by a membrane impermeant compound as sodium or sorbitol increases the expression of AQP2, an effect that is increased when the membrane permeating compound urea accounts for a portion of the total medium osmolality. Intracellular urea accumulation should reduce the extend of cell volume decrease elicited by high extracellular tonicities and might thus facilitate normal cell function under high-tonicity conditions.

3.5 The kinetics of AQP2 regulation in response to hypo- and hypertonic challenge

To show that the effects of hypo- and hypertonic challenge on AQP2 expression were reversible and to assess the time courses of decrease and increase in AQP2 expression, the experiments described below were performed. To analyze the time course of down-regulation in response to hypotonic challenge, IMCD cells were seeded in 600N that was changed to 300N (except for controls) for 144, 72, 48, 24, 6, and 3 h before preparation for Western blot analysis. To investigate AQP2 up-regulation in response to hypertonicity, IMCD cells were seeded in 600N medium and transferred 24 h later to 300N medium (except controls and 0 h time point) that was replaced by 600N medium 72, 48, 24, and 6 h prior to cell lysis. Cells were cultured for a total of 7 days (168 h). AQP2 protein was detected by immunoblotting. Fig. 15 A and 15 B show a representative set of experiment performed to investigate the down-and up-regulation of AQP2, respectively. The results of densitometrical analysis of a series of experiments are summarized in Fig. 15 C and 15 D. The graph was obtained by performing non-linear regression analysis using Graph Pad Prism software.

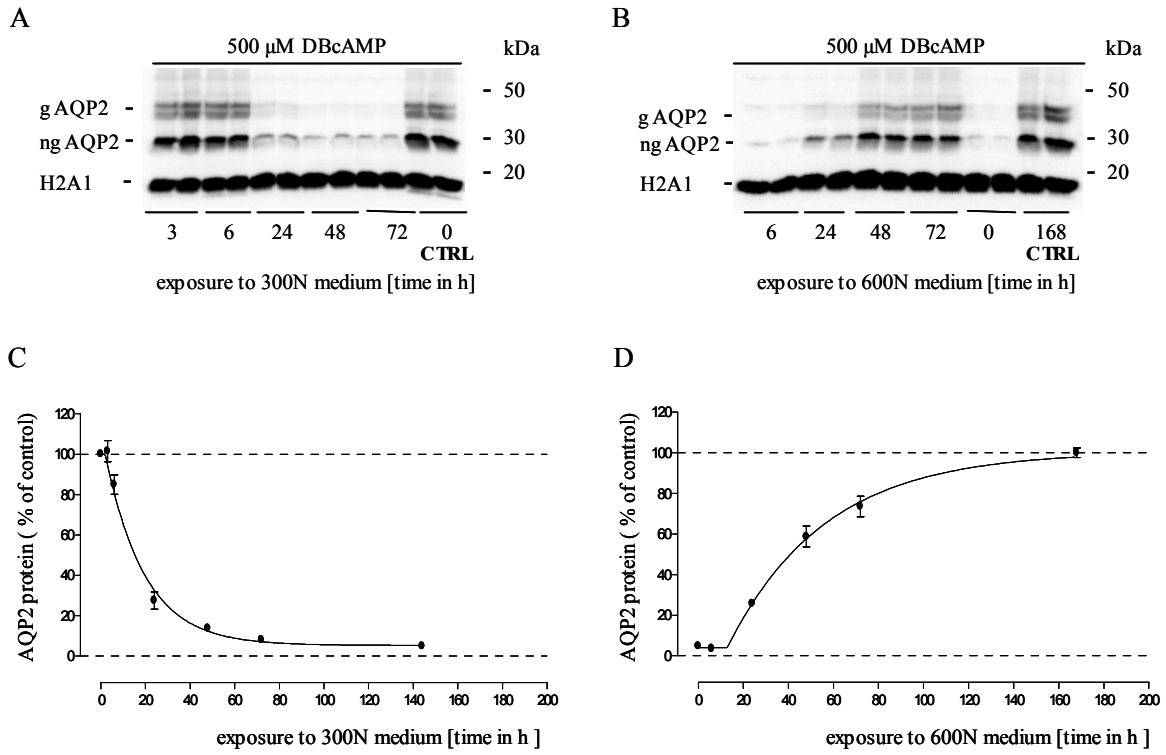

Fig. 15. Kinetics of AQP2 regulation in response to hypo- and hypertonic challenge. IMCD cells were cultured for a total of 168 h (7days) in 24-well plates in the presence of 500 µM DBcAMP. A: Cells were seeded in 600N medium. Down-regulation of AQP2 was induced by changing the culture medium to 300N (except controls; 0-h time point) for 144, 72, 48, 24, 6, or 3 h before cell lysis (168 h after seeding) and Western blot analysis. The 144-h time points were identical to the 0-h time points depicted in B. B: IMCD cells were seeded in 600N medium, which was exchanged by 300N medium 24 h later to induce a down-regulation of AQP2. Medium osmolality was not changed for controls (cells continuously grown in 600N; 168-h time point). Up-regulation of AQP2 was induced by changing the culture medium from 300N to 600N for 72, 48, 24, and 6 h before processing for Western blot analysis (168 h past seeding). AQP2 (g, glycosylated; ng, non-glycosylated) and histone H2A1 (as indicated) were detected by immunoblotting with anti-AQP2 antiserum. Per lane of an SDS-PAGE gel, total homogenate (protein) from 1 cm^2 confluent cell monolayer was loaded. C, down-regulation: Densitometrical analysis of AQP2 protein levels (glycosylated and non-glycosylated) detected in total homogenates. Values are means +/- SE (4>n>8). The graph was obtained by performing non-linear regression analysis [Graph Pad Prism; R^2 (unweighted) = 0.9999]. D, up-regulation: Densitometrical analysis of AQP2 protein levels (glycosylated and non-glycosylated) detected in total homogenates. Values are means +/- SE (7>n>12). The graph was obtained by performing non-linear regression analysis [Graph Pad Prism; R^2 (unweighted) = 0.9990].

The results presented in Fig. 15 C show, that the down-regulation of AQP2 protein was detectable as early as 6 h after the onset of hypotonicity. Within the first 24 h after exposure to hypotonicity AQP2 expression dropped to ~25 % of control levels. Thereafter, AQP2 expression continued to decrease, reaching baseline levels 72 h after exposure to hypotonicity.

No decline in AQP2 protein expression was evident after 3 h of exposure to hypotonicity. As shown in Fig. 15 D, the up-regulation of AQP2 expression in response to hypertonicity is a slower process. Non-linear regression analysis suggests that AQP2 expression increased from baseline levels (cells were kept for 72 h in 300N medium) with a lag time of ~15 h after exposure to hypertonicity. After 24 h, AQP2 expression increased to ~25 % of the level of controls (cells continuously kept in 600N medium) and reached ~75 % within 72 h.

3.6 The kinetics of AQP2 regulation in response to DBcAMP withdrawal and addition

For comparison with the effects of tonicity, the kinetics of the regulation of AQP2 expression by DBcAMP were examined. To analyze the down-regulation of AQP2 expression in response to DBcAMP withdrawal, IMCD cells were seeded in 600N medium with DBcAMP [500µM], which was replaced with 600N medium without DBcAMP 72, 48, 24, 6, and 3 hours before cell lysis and Western blot analysis (Fig. 16 A). For analysis of the DBcAMP elicited up-regulation of AQP2 expression, IMCD cells were seeded in 600N medium without the second messenger analogon (except controls, CTRL). DBcAMP [500µM] was added 72, 48, 24, and 6 h prior to cell lysis and Western blot analysis (Fig. 16 B). The results of densitometrical and non-linear regression analysis are depicted in Fig. 16 C and D for DBcAMP withdrawal and DBcAMP exposure experiments, respectively.

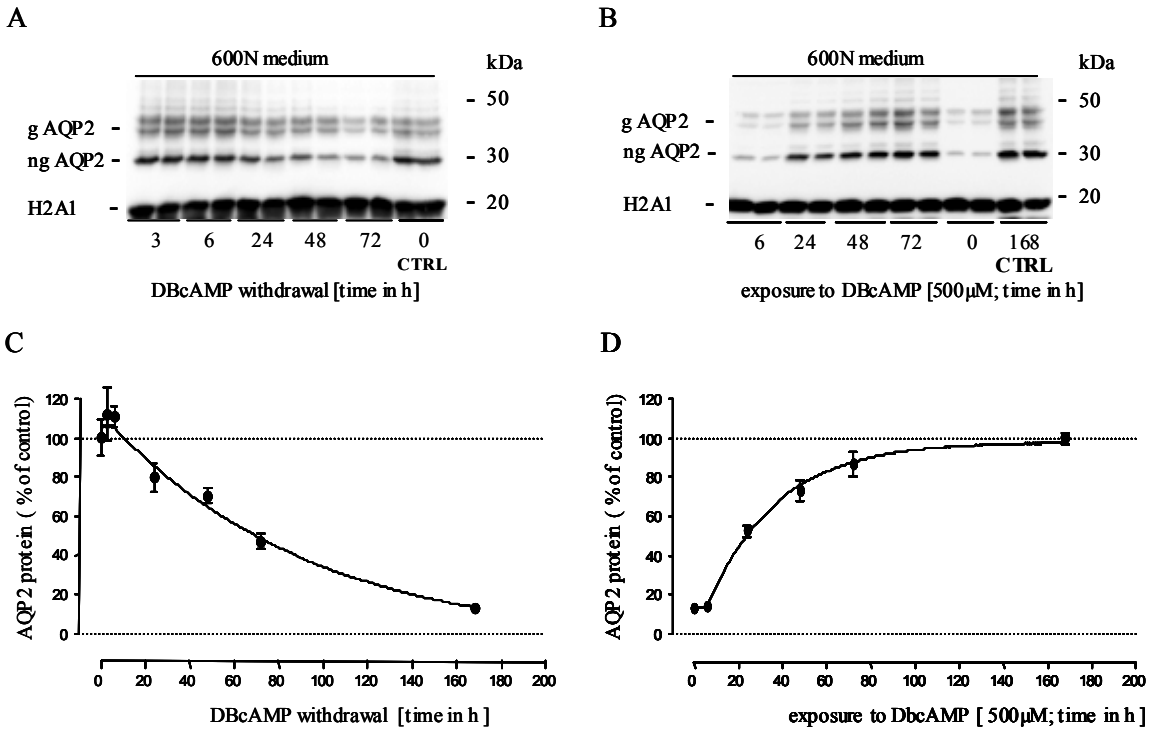

Fig. 16. Kinetics of AQP2 regulation in response to DBcAMP withdrawal or addition. IMCD cells were cultured for a total of 168 h (7days) in 24-well plates in 600N medium. A: Cells were seeded in 600N medium with DBcAMP [500µM] and without DBcAMP (168-h time point). The 168-h time points

were identical to the 0-h time points depicted in B. Down-regulation of AQP2 was induced by changing the culture medium to 600N without DBcAMP (except controls; 0-h time point) for 72, 48, 24, 6, or 3 h before cell lysis (168 h after seeding) and Western blot analysis. B: IMCD cells were seeded in 600N medium without DBcAMP. Up-regulation of AQP2 was induced by changing the culture medium to 600N with DBcAMP [500μM] for 72, 48, 24, and 6 h prior to processing for Western blot analysis. AQP2 (g, glycosylated; ng, non-glycosylated) and histone H2A1 (as indicated) were detected by immunoblotting with anti-AQP2 antiserum. Per lane of an SDS-PAGE gel, total homogenate (protein) from 1 cm^2 confluent cell monolayer was loaded. C, down-regulation: Densitometrical analysis of AQP2 protein levels (glycosylated and non-glycosylated) detected in total homogenates. Values are means +/- SE (n=4). The graph was obtained by performing non-linear regression analysis [Graph Pad Prism; R^2 (unweighted) = 0.9948]. D, up-regulation: Densitometrical analysis of AQP2 protein levels (glycosylated and non-glycosylated) detected in total homogenates. Values are means +/- SE (n=4). The graph was obtained by performing non-linear regression analysis [Graph Pad Prism; R^2 (unweighted) = 0.9993].

In comparison with the kinetics of the decrease in AQP2 expression caused by hypotonicity, AQP2 expression dropped more slowly as DBcAMP was withdrawn. When DBcAMP was removed for 3 and 6 h, AQP2 expression slightly increased and dropped thereafter to ~80 % of controls within 24 h. After 72 h of DBcAMP depletion, AQP2 expression was still as high as ~50 % of controls (168 h with DBcAMP). Non-linear regression analysis suggests that AQP2 protein expression increased with greater velocity in response to DBcAMP than to hypertonic challenge (see Figs. 16 D and 15 D). In the presence of DBcAMP, AQP2 protein expression remained at baseline levels up to 6 h, reached ~50 % of control levels within 24 h, ~70 % within 48 h, and increased to above 80 % within 72 h.

3.7 The human, rat, and mouse AQP2 promoter regions contain a conserved tonicity responsive element (TonE)

The absence of an effect of osmolality on the CMV-promoter driven AQP2 expression in WT-10 cells (Fig. 4) indicated that in IMCD cells, the effects of hyper- and hypotonic challenge on endogeneous AQP2 expression were due to altered transcription. Therefore, the available promoter regions of the mouse, human, and rat AQP2 gene were analyzed for motifs that may confer the genes responsiveness to hyper-or hypotonicity.

Within the promoter of the AQP2 gene the consensus sequence for a regulatory element was located, which could potentially mediate the effects of hypo-and hypertonic challenge on AQP2 expression. Fig. 17 shows that the rat, mouse and human AQP promoter regions (NCBI Accession No. D87128, NCBI accession No. D87129, Rai et al., 1997; NCBI accession No. U30469, Hozawa et al., 1996) contain elements matching the consensus sequence for the

tonicity responsive enhancer (TonE; Ferraris et al., 1996; Miyakawa et al., 1998). The TonE consensus sequence is recognized by the recently identified transcription factor TonE-binding protein (TonEBP; Miyakawa et al., 1999; López-Rodríguez et al., 1999). To assess the frequency of TonE in genomic DNA, a fragment of human chromosome 12 with a size of 1,018,700 bp (NCBI Accession No. 27484131), which harbors the AQP2, AQP5 and AQP6 genes, was searched for the presence of TonE consensus sites. Within this fragment, 71 TonE consensus sites (5´TGGAAAnnC/TnC/T 3´) were found on one strand, and 86 on the complementary strand, yielding a frequency of one TonE site every 14.35 kb and 11.84 kb, respectively.

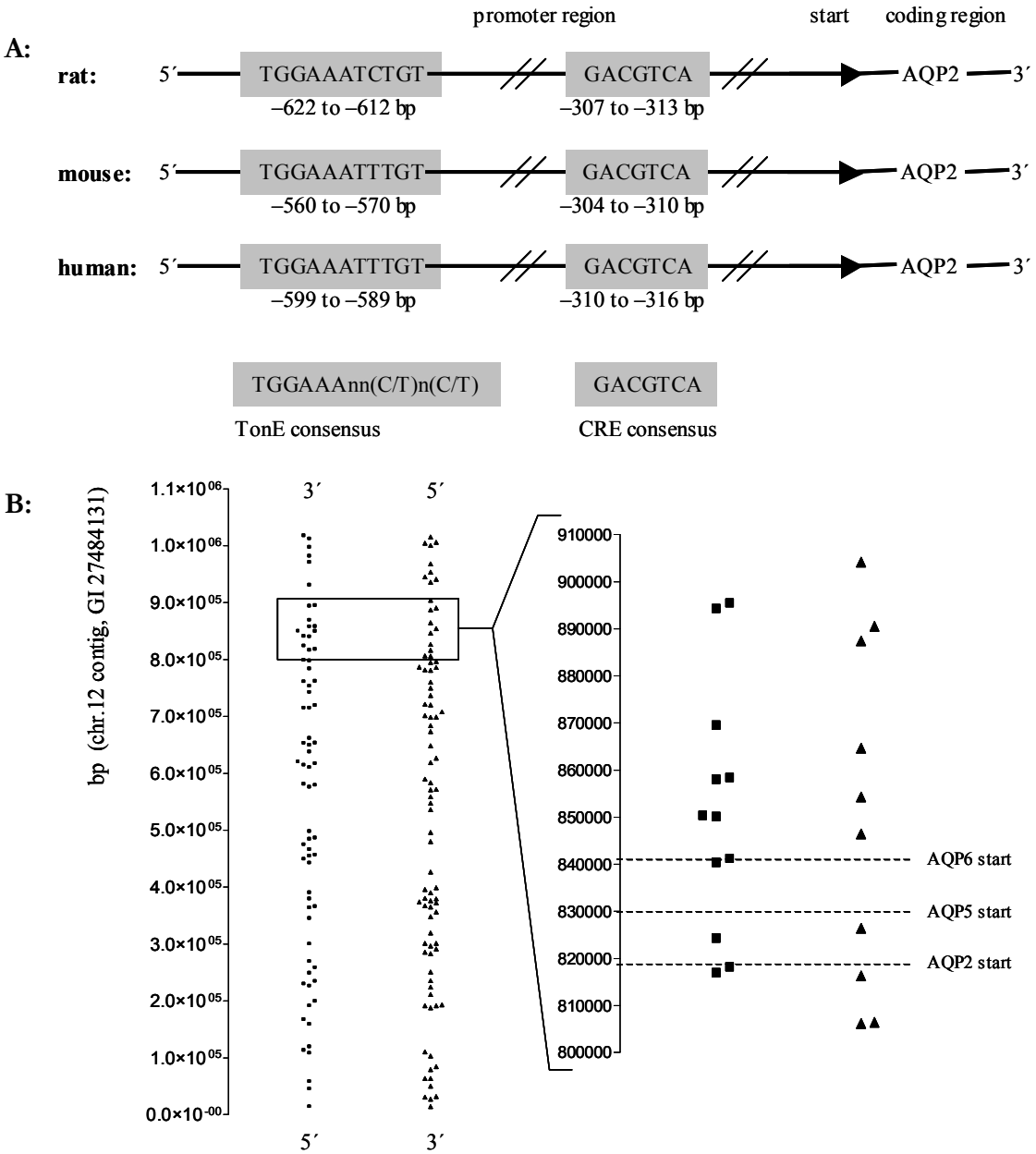

Fig. 17. A Tonicity-responsive element is present in the rat, murine and human AQP2 promoter region. A: Sketch of the rat, mouse and human AQP promoter regions (33, NCBI Accession No. D87128; 33, NCBI accession No. D87129; 13, NCBI accession No. U30469). The TonE consensus sequence (Rim et al. 1998, Miyakawa et al. 1998) as well as the TonE elements and CRE elements found

in the promoters are shaded. Arrowheads, transcription initiation sites. Positions of the transcription factor binding sites are indicated relative to the transcription initiation site. B: Distribution of TonE consensus sites (5´TGGAAAnnC/TnC/T 3´) along a ~1,100 kb fragment of human chromosome 12 (NCBI Accession No. 27484131). Filled squares indicate the positions of TonE sites on one strand, filled triangles represent TonE sites present on the complementary strand. The graph shown on the right represents a magnification of the region outlined on the left. Indicated are the transcription starts for AQP2, AQP5 and AQP6.

3.7.1 The expression of the transcription factor tonicity-responsive element binding protein (TonEBP) is regulated by osmolality and solute composition

The tonicity-responsive element binding protein (TonEBP) is the first mammalian transcription factor known to regulate the expression of genes whose products are involved in osmoregulation in response to osmotic stress. Moreover, hypertonicity, but not mere hyperosmolatity activates TonEBP dependent transcription (Miyakawa et al., 1999). TonEBP, also termed nuclear factor of activated T cells 5 (NFAT5, López-Rodríguez et al., 1999) because of its conserved rel homology region shared with transcription factors of the NFAT family (NFAT1-4) is a large protein (1,455 aa) with a calculated molecular mass of ~160 kDa, which appears at a size of ~200 kDa in SDS-PAGE. TonEBP shows no sequence similarities to transcription factors regulating osmoprotective genes in prokaryotic, yeast or plant cells. The signaling cascade leading to activation of TonEBP in response to hypertonicity is yet unknown. TonEBP RNA was found in virtually all tissues and highest expression levels were found in brain, heart and renal medulla. TonEs have been found in the 5´ regions to the promoters of the sodium-myo-inositol transporter (SMIT), the glycine-betaine transporter (BGT1), aldose-reductase (AR). These transporters mediate the intracellular accumulation of compatible osmolytes in response to hypertonic challenge, enabling mammalian cells to restore and maintain their cell volume and a defined intracellular ion composition required for proper function of cell metabolism.

It was tested, whether TonEBP was abundant in IMCD cells and if the alterations in AQP2 expression elicited by changes in osmolality and solute composition are accompanied by similar changes in the expression of TonEBP. A specific rabbit anti-TonEBP/NFAT5 antiserum was kindly provided by Drs. C. López-Rodríguez and A. Rao of Harvard Medical School. IMCD cells were seeded in 600N medium and were transferred 24 h later to 300N medium, or media based on 300N elevated by the addition of NaCl and/or urea to 400 mosmol/l, 500 mosmol/l, 600 mosmol/l and 800 mosmol/l. All media used contained 500µM DBcAMP throughout the experiment.

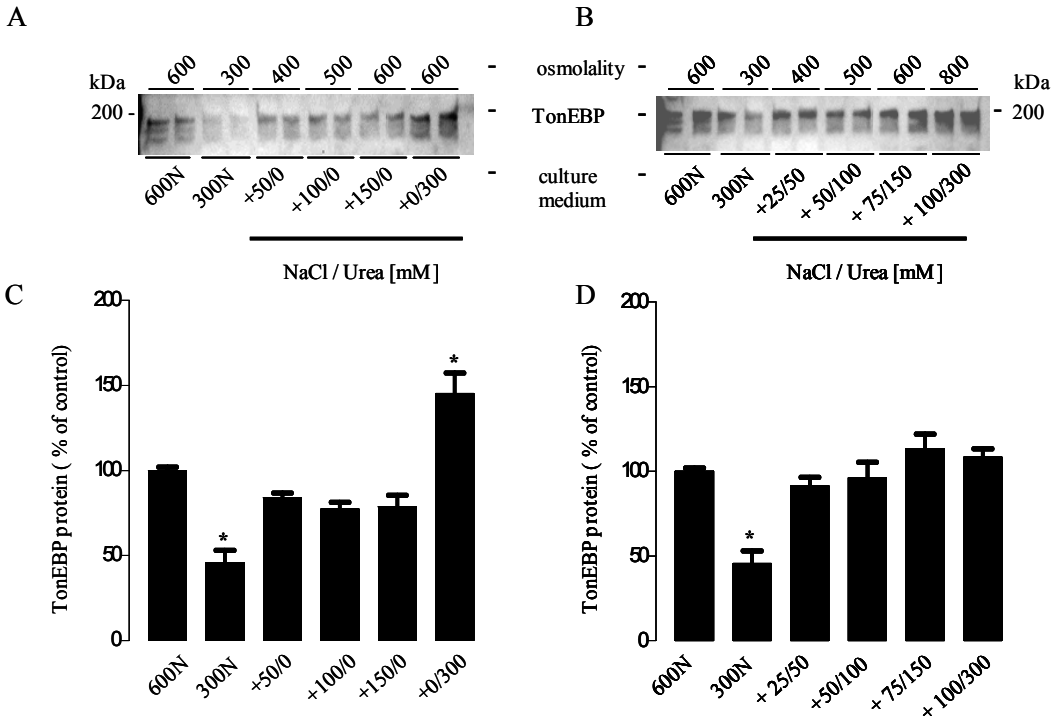

Fig. 18. The expression of TonEBP in IMCD cells is modulated by solute composition and osmolality. IMCD cells were seeded in 24-well plates in 600N. Indicated media were applied 24 h after seeding. The first number indicates the osmolality of the medium, numbers in front of and behind slash (in parenthesis) indicate added concentrations (to 300N medium) of NaCl and urea (in mM), respectively. Cells were cultured for a total of 7 days in the presence of 500 μM DBcAMP in A: 600N (100/100, control) medium, 300N, 400 (50/0), 500 (100/0), 600 (150/0), 600 (0/300) and B: 600N (100/100, control) medium, 300N, 400 (25/50), 500 (50/100), 600 (75/150), 800 (100/300). A and B: TonEBP (running at 200 kDa) was detected by immunoblotting with specific anti-TonEBP antiserum (López-Rodríguez et al., 1999). Per lane of an SDS-PAGE gel, total homogenate (protein) from 1 cm^2 confluent cell monolayer was loaded. C and D: Densitometrical analysis of TonEBP protein levels detected in total homogenates. Values are means +/- SE (4>n>8). *P < 0.05 vs. controls.

Fig. 18 shows that the expression of TonEBP is modulated by the solute composition of the culture medium and its osmolality. Compared to controls (IMCD cells cultured in 600N medium), cells cultured in 300N exhibited a weak TonEBP expression (~ 50%), which was nearly restored by cultivation of cells at 400 mosmol/l, regardless of whether sodium alone (Fig. 18 C) or an equiosmolar combination of sodium and urea (Fig. 18 D) constituted for increased osmolality of the culture medium. A further increase of medium osmolality by sodium alone (up to 600 mosmol/l) or in combination with urea (up to 800 mosmol/l) did not yield significantly higher TonEBP expression levels. No statistically significant difference in TonEBP expression was evident comparing the effect of media with identical osmolalities but different constituents, as determined by ANOVA. A significant increase in TonEBP expression (compared to controls) was yielded only when medium osmolality was elevated to 600 mosmol/l with urea.

3.7.2 TonEBP activity (nuclear localization) corresponds with AQP2 expression in response to hyper- or hypotonic challenge

Immunofluorescence studies were performed to investigate whether the localization of the transcription factor TonEBP is dependent on medium tonicity in IMCD cells. Therefore, IMCD cells were seeded on collagen type IV-coated cover slips and cultured for 6 days in the continuous presence of 500 μM DBcAMP. IMCD cells were seeded in 600N medium and challenged (except controls [0-h time point]) with hypotonicity (300N medium) for 16 h (Fig. 20), 40 h and 122 h before fixation (Fig. 19). Vise versa, cells seeded in 600N medium, were transferred after 24 h to 300N medium for consecutive 72 h and then challenged with hyperosmolality (600U medium or 600N medium) for 19 h or 40 h (600N only) before fixation (Fig. 21).

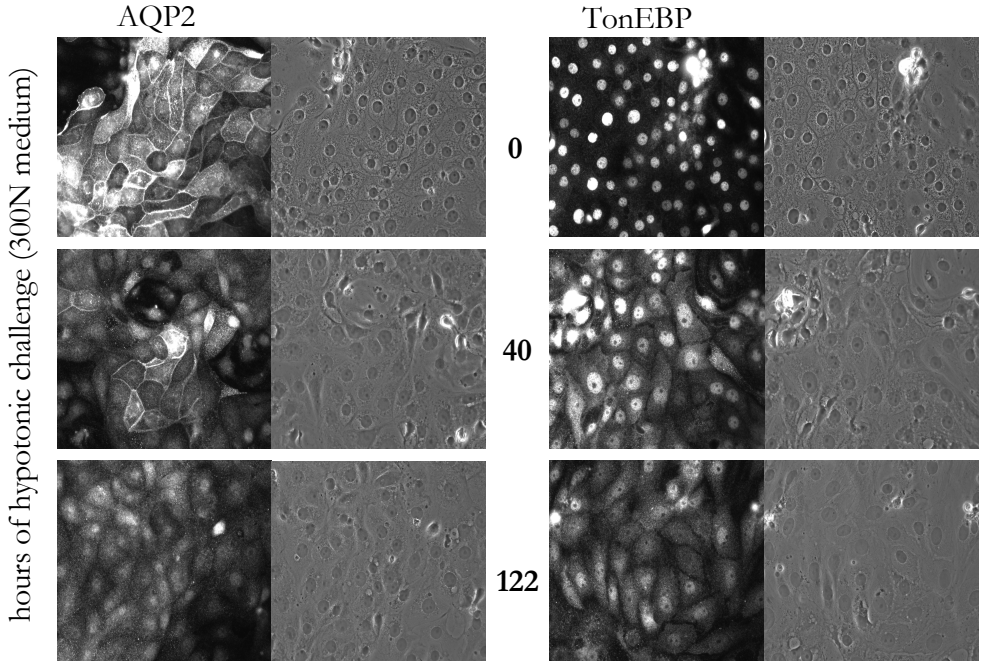

Fig. 19. Hypotonic challenge leads to diminished nuclear, and increased cytosolic TonEBP abundance, concommitant with decreased AQP2 expression. IMCD cells were cultured for 6 days in 600N medium and challenged with hypotonicity (300N medium) 0, 40 and 122 h before fixation. *Left panels*. Visualization of AQP2 (*left*) in IMCD cells challenged with hypotonicity for the time indicated [in h]; the corresponding light micrographs are displayed on the right. *Right panels:* Visualization of TonEBP (*left*) in IMCD cells challenged with hypotonicity for the time indicated [in h]; the corresponding light micrographs are displayed on the right. AQP2 was detected with specific rabbit anti-AQP2 antiserum. TonEBP was detected with a specific rabbit anti-TonEBP antiserum (kindly provided by Drs. López-Rodríguez and Rao). Cy3-conjugated goat anti-rabbit antibodies were used as secondary antibodies.

Fig. 19 *(right panels)* shows that the transcription factor TonEBP is mainly localized within the nuclei of cells kept in medium with 600 mosmol/l (600N). Upon 40 h of hypotonic challenge (300 mosmol/l, 300N), the signal intensities for TonEBP were diminished in the nuclei and increased clearly in the cytoplasm. After 122 h of hypotonic challenge appeared even more decreased. AQP2 signals were diminished with increased time of exposure to hypotonicity *(left panels)* as could be expected from the Western blot data (Fig. 15). The reduction of nuclear TonEBP signals, concommitant with the increase in the cytoplasm indicates that upon hypotonic challenge, TonEBP translocates from the nucleus to the cytoplasm. Thus, in IMCD cells, the hypoosmolality-induced translocation of TonEBP from the nuclei to the cytoplasm was paralleled by a decrease in AQP2 expression.

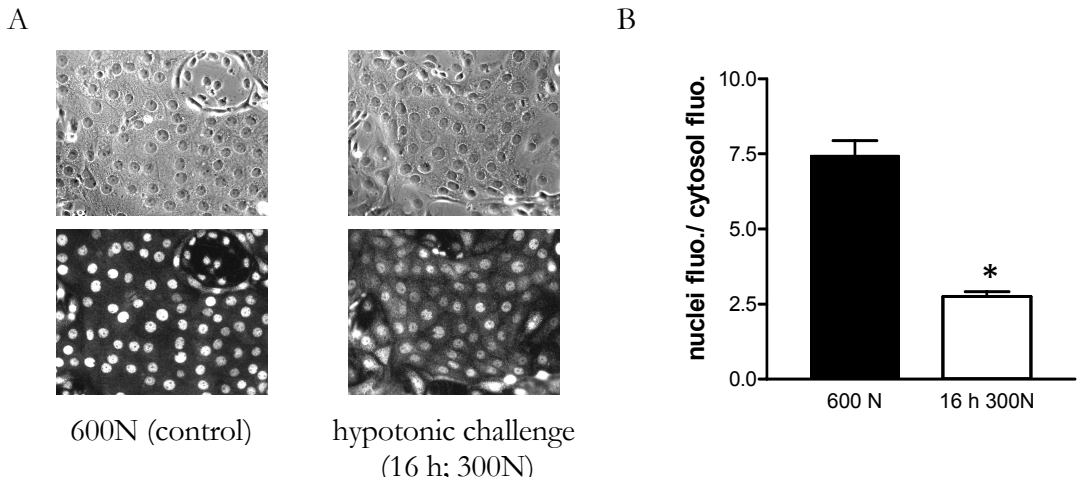

Fig. 20. Quantification of TonEBP redistribution in response to hypotonic challenge in IMCD cells. Cells were cultured for 6 days in 600N medium and challenged with hypotonicity (300N medium) 16h before fixation. A: Visualization of TonEBP (*lower panels*) in IMCD cells left untreated *(left)*, or challenged with hypotonicity for 16 h *(right)*; the corresponding light micrographs are displayed above. B: Quantification of TonEBP fluorescence represented as the ratio between signal intensities detected in the nucleus and in the cytoplasm of individual cells reveal statistically significant differences. Values are means +/- SE (n=20). *P < 0.001. TonEBP was detected with a specific rabbit anti-TonEBP antiserum (kindly provided by Drs. López-Rodríguez and Rao). Cy3-conjugated goat anti-rabbit antibodies were used as secondary antibodies.

Fig. 20 A shows that IMCD cells display a marked reduction in TonEBP fluorescence signal in the nuclei and a clear increase in the cytosolic compartment compared to control cells within 16 h of hypotonic challenge. The diagram in Fig. 20 B, displaying the ratio of TonEBP signals whithin nuclei to cytoplasm illustrates that hypotonicity induced a significant shift of TonEBP from its site of action, the nucleoplasmic compartment to the cytoplasmic compartment.