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Hypertonicity induced modulation of gene transcription and translation of water regulatory molecules

Aniket Kumar and Manoj G Tyagi

Department of Pharmacology, Christian Medical College, Vellore, Tamil Nadu, India

Abstract

The cells in the renal medulla are exposed to very high osmolality which may exceed 3000 mosmol/kg water depending on the hydration status of the animal. This osmotic stress causes numerous perturbations because of the hypertonic effects of high NaCl and the direct denaturation of cellular molecules by high urea. High NaCl and urea result in increase of reactive oxygen species, inhibit DNA replication and transcription. Adaptive responses and subsequent modulation result in changes of gene transcription and translation of water regulatory biomolecules like the vasopressin, aquaporin, atrial natriuretic peptide and urocortin. For e.g urocortin mRNA in the supraoptic nucleus has been shown to be increased in both vasopressin and oxytocin magnocellular cells after chronic salt loading. On the other hand the increase of AQP_2 expression in response to extracellular hypertonicity is independent of the cAMP-PKA pathway. The tonicity responsive enhancer binding protein, TonEBP is activated and leads to the adaptive responses and causes transcriptional and translational modifications of several water regulatory molecules mentioned above.

Key words: Renal medulla, osmolality, tonicity, transcription, translocation

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Introduction

Hypertonicity increases tyrosine phosphorylation of Jak1, Jak2, and Tyk2, as well as phosphorylation of STAT1 and STAT3, and causes nuclear translocation of STAT3 **[1].** Tonicity dependent activation of STAT1 appears to be mediated by p38 and its upstream activator MKK6 **[2].** Hypertonicity-induced phosphorylation of Jak2/STAT3 may lead to activation of the type 1 sodium hydrogen exchanger (NHE-1) **[3].** NF-kB is a Rel family transcription factor that activates an inflammatory cascade, leading to interleukin (IL)-8 productions. Hypertonicity elevates both NF-kB DNA binding and transcriptional activity in intestinal cells, increasing IL-8 production [4].

Numerous transcription factors are regulated by tonicity, as follows. c-fos and c-jun are immediate-early genes (IEGs) and zinc finger transcription factors [5]. They are members of the activator protein-1 family that regulates diverse processes, including cell proliferation, embryonic development, vascular smooth muscle cell growth, and apoptosis. Hypertonicity increases transcription of c-fos and c-jun in cultured cells [6,7,8] phosphorylation of c-jun, and nuclear abundance of the phosphorylated, active form of c-jun [9]. Hypertonicity in the form of high NaCl also increases the DNA binding activity of AP-1 proteins, c-Fos/Fra, and c-jun *in vivo*[10,11,12]. Egr-1, Egr-3, and SNAI1 are other IEGs and zinc finger transcription factors. Hypertonicity increases their mRNA abundance [13,14].

The tonicity gradient in the renal interstitium, which is mainly created by NaCl and urea, is also crucial for urine concentration in the kidney [15]. Hypertonicity is known to activate AQP_2 promoter activity via the TonEBP pathway, and increases the mRNA and protein expression of AQP_2 *in vitro* [16]. The tonicity of the interstitium in the kidney is augmented in the dehydrated state. Thus, it is clear that the expression of AQP_2 is increased in the dehydrated state. It has been previously reported that hyper-

tonicity increased the expression of V_2R in the inner medullary collecting ducts [17]. AQP_1 is induced by hypertonic stress, accompanied by activation of extracellular signal-related kinase (ERK), p38 kinase and c-Jun NH_2-terminal kinase (JNK) [18]. Transcriptional activation of the AQP_1 promoter is attenuated by inhibitors of these pathways, suggesting a role for the hypertonicity response element in the AQP_1 promoter in hypertonicity induced AQP_1 expression [19]. AQP_2, AQP_3, AQP_4, and AQP_9 have also been shown to be upregulated by hyperosmolarity [20,21]. The promoters of both AQP_4 and AQP_9 are regulated by hypertonicity, although the precise hypertonicity response element in these promoters has not been identified and appears to differ from that in the AQP_1 promoter [22]. Upregulation of AQP_5 by osmotic stress has been reported in a mouse lung epithelial (MLE-15) cell line and in hyperosmolar rats, suggesting possible roles for AQP_5 in regulating alveolar surface liquid tonicity and/or maintaining cell volume [23]. Tonicity-responsive enhancer binding protein (TonEBP or NFAT5) plays a key role in this process by stimulating transcription of aldose reductase (AR) [24], sodium-chloride-betaine cotransporter (BGT1), sodium-myo-inositol cotransporter (SMIT), and taurine transporter that mediate intracellular accumulation of sorbitol, betaine, myo-inositol, and taurine, respectively as shown in Figure 1 [25]. TonEBP additionally stimulates transcription of HSP70 [26]. This review article describes methods used to study how tonicity affects gene expression. We start with a brief overview of the osmoadaptive genes that TonEBP/OREBP transactivates, and the effect of hypertonicity on gene expression of water regulatory molecules

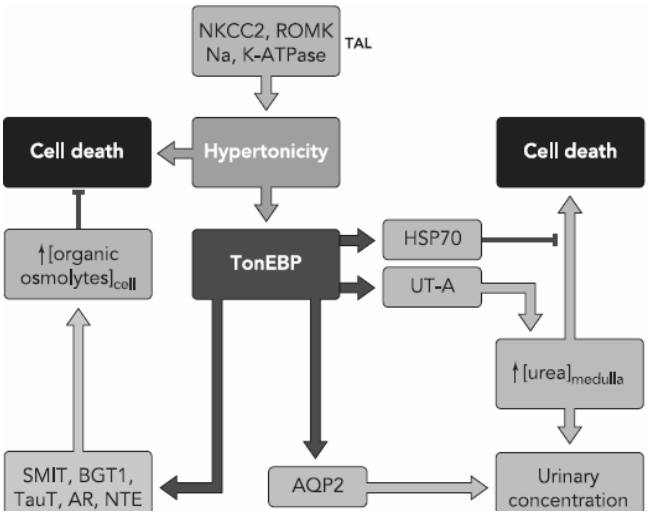

Figure 1. *Physiology of TonEBP in the renal medulla*

i.e vasopressin, aquaporin, natriuretic peptides , and urocortin followed by a description of the mechanisms involved in its tonicity-dependent activation. We concentrate on three aspects of water regulatory molecules activity that are common to all transcription factors, namely nuclear localization, DNA binding, and signal transduction. We emphasize each of these aspects in the overview and in the protocols that follow.

Hypertonicity and natriuretic peptide gene expression

Competitive PCR revealed that ANPR-C mRNA expression was most abundant (ANPR-C>A>B) in glomeruli from control rats. Two days dehydration caused reversible decreases of ANPR-A, B and C mRNAs by 50-80 %. Hyperosmolality induced by NaCl, mannitol or raffinose caused significant increases of ANPR-A, B and C mRNA expression. Studies have been undertaken to explore the acute effect of hyperosmolality on the response of cultured rat inner medullary collecting duct (IMCD) cells to atrial natriuretic peptide (ANP) [27]. In contrast to the stimulatory effect of chronic incubation (12 hr) in hypertonic medium, it was found that short-term incubation (< 2 hr) reversibly suppressed the ANP-dependent cyclic guanosine monophosphate (cGMP) production. Urea, NaC1 and mannitol were equipotent as the osmolyte in suppressing the ANP-dependent cGMP production. Receptor binding assay revealed that hyperosmolality induced a rapid and marked reduction of the maximum binding (Bmax) of ANP without a significant change of the dissociation constant (Kd). with protein kinase C inhibitors (calphostin-C, staurosporine) or with cytoskeleton modulators (cytochalasin-B, colchicine) did not affect the inhibitory effect of hyperosmolality. In conclusion, acute hypertonicity inhibited the ANP-induced cGMP production in contrast to chronic hypertonicity, and reduction of the number of ANP binding sites was considered to be a mechanism responsible for the inhibitory effect of Hypertonicity [28].

Hypertonicity and urocortin gene expression

Urocortin, Urocortin II and Urocortin III are members of corticotrophin releasing factor (CRF) family. Hypertonic treatment increased urocortin mRNA expression in the PVN and SON. In the SON, the urocortin was located to vasopressin and oxytocin neurons. Thus urocortin may exert modulatory effects locally with in magnocellular neurons as well as at the pituitary gland in response to osmotic stimulation. Renal uroguanylin expression was modulated by high salt loading. Because renal uroguany-

lin mRNA expression was increased by saline water but not dehydration, changes in uroguanylin mRNA expression cannot be explained simply in terms of reduced nutrient, but to excessive salt load. The results showed that M-1 cells, originating from cortical collecting ducts and retaining many characteristics of the original cell types constitutively express uroguanylin mRNA. Consistent with the *in vivo* observations M-1 cells responded to hypertonic NaCl solutions with increased expression of uroguanylin mRNA. These data suggest that renal uroguanylin expression is directly influenced by renal hypertonicity and by its natriuretic/kaliuretic properties might contribute to stimulate renal saluresis in response to increased salt load**.** Urocortin and urocortin mRNA exhibit in the SON. Urocortin mRNA in the SON was increased in both the AVP and OXT magnocellular cells after chronic salt loading [29].

Hypertonicity and effect of vasopressin on corticomedullary osmolality

Of all regions of the body, environmental osmolality is by far highest in the renal medulla. The corticomedullary osmolality gradient that drives water reabsorption in the kidney increases along the tubule with a maximum osmolality (1,200 mosmol/kg H_2O in humans) at the tip of the inner medulla. The gradient arises from active NaCl reabsorption in the thick ascending limb of Henle (TAL) via Na-K-Cl cotransporter (NKCC2) and by passive reabsorption of NaCl and urea by the thin ascending limb of Henle (tAL) and the inner medullary collecting duct (IMCD), respectively. It additionally depends on the water impermeability of both TAL and tAL together with low blood flow through the vasa recta. Vasopressin plays a fundamental role in establishing and maintaining the hyperosmotic environment of the kidney medulla by controlling the expression levels of key proteins present in distinct regions of the nephron that together mediate the countercurrent concentration mechanism. Vasopressin controls interstitial NaCl accumulation by stimulating NKCC2 abundance and cell surface expression in the TAL and by promoting the coordinated expression of the epithelial sodium channel (ENaC) and Na^+-K^+-ATPase at the cell surface of collecting duct (CD) principal cells. VP also increases urea permeability by acutely increasing UT-A1 cell surface expression and UT-A3 mRNA abundance in terminal IMCD cells and by increasing UT-A2 expression in thin descending limbs of Henle. Finally, VP tightly regulates osmotically driven water reabsorption by mediating transcriptional activity as well as apical cell surface expression of AQP_2 in CD principal cells [30]. In addition to VP, other hormones act on Na^+ and Cl^- reabsorption in the TAL and CD. TAL Na^+ and Cl^- reabsorption is increased by adrenaline, calcitonin, parathyroid hormone, and glucagon and decreased by prostaglandin E_2 [31] . In the CD, Na^+ and Cl^- reabsorption is increased by aldosterone and decreased by atrial natriuretic peptide and prostaglandins [32,33]. Acute increases in plasma osmotic pressure produced by intraperitoneal injection of hypertonic NaCl are sensed by osmoreceptors in the brain, which excite the magnocellular neurons (MCNs) in the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) in the hypothalamus inducing the secretion of vasopressin (VP) into the general circulation. Such systemic osmotic stimulation also causes rapid and transient increases in the gene expression of c-*fos* and VP in the MCNs [34]. Studies which have evaluated potential signals that might be responsible for initiating these gene expression changes during acute hyperosmotic stimulation. Some studies suggest that *in vivo* paradigm in which stereotaxically delivered putative agonists and antagonists over the SON unilaterally, and use the contralateral SON in the same rat, exposed only to vehicle solutions, as the control SON. Quantitative real time-PCR was used to compare the levels of c-*fos* mRNA, and VP mRNA and VP heteronuclear (hn) RNA in the SON. We found that the ionotropic glutamate agonists (NMDA plus AMPA) caused an approximately 6-fold increase of c-*fos* gene expression in the SON, and some, but not all, G-coupled protein receptor agonists (e.g., phenylephrine, senktide, a NK-3-receptor agonist, and α-MSH) increased the c-*fos* gene expression in the SON from between 1.5 to 2-fold of the control SONs. However, none of these agonists were effective in increasing VP hnRNA as is seen with acute salt-loading. This indicates that the stimulus-transcription coupling mechanisms that underlie the c-*fos* and VP transcription increases during acute osmotic stimulation differ significantly from one another [35].

Effects of Hypertonicity on AQP_2 Abundance

The year AQP_2 was cloned by Fushimi *et al* [35] was also the year that water restriction was first found to affect AQP_2 whole cell abundance independently of VP [36]. This provided one of the first compelling pieces of evidence that factors other than VP control AQP_2 expression, and therefore CD water permeability. The remaining part of this review will focus on the role that osmolality plays in regulating AQP_2 expression. The influence of other non-VP stimuli on AQP_2 expression has been reviewed elsewhere [37].Together with the demonstration that enhanced CD water permeability by VP was due to AQP_2

accumulation at the cell surface, VP was shown to increase AQP_2 protein abundance [38,39]. It was demonstrated soon after that VP increases AQP_2 mRNA content [40] and that a cAMP responsive element (CRE) and an activator protein 1 (AP-1) element located in the AQP_2 promoter, which respectively bind cAMP-responsive element protein (CREP) and c-Fos/ c-Jun, mediate VP-induced AQP_2 transcription [41,42]. Numerous observations from animal studies led to the suspicion that environmental osmolality might also participate in regulating AQP_2 abundance by acting independently of VP. One of the first clues was provided by a study that showed that reduced AQP_2 abundance following lithium treatment could be partly restored in water-restricted animals, and to a greater extent than that achieved by VP treatment [43]. Water restriction was later shown to return AQP_2 abundance to normal levels in animals treated with the V_2 receptor antagonist OPC-31260 [44]. By performing the exact opposite experiment, Ecelbarger *et al* showed that water loading decreased whole cell AQP_2 content despite ongoing V_2 receptor stimulation [45]. The possibility that environmental osmolality regulates AQP_2 abundance independently of cAMP/PKA was further evidenced by the finding that AQP_2 expression decreases with medullary osmolality in senescent animals despite unchanged papillary cAMP levels [46]. AQP_2 expression could be restored in these animals by VP administration, but it was concluded that this event is most likely mediated by an increase of papillary osmolality rather than a direct effect of VP itself [47] . On the other hand, medullary osmolyte washout following long-term (4–5 days) furosemide treatment had no effect on AQP_2 abundance shedding some doubt on the enhancing effect of increased environmental osmolality on AQP_2 abundance [48]. Using cultured outer medullary collecting ducts (OMCD) cells, Furuno *et al* showed, for the first time, that hypertonic challenge alone (24 h) increased AQP_2 mRNA abundance. The effect of extracellular osmolality on AQP_2 abundance was further tested by Storm *et al* who measured AQP_2 mRNA and protein abundance in primary cultured IMCD cells exposed to a hypertonic medium [49]. Some studies suggest that both AQP_2 mRNA and protein expression levels increased in a dose-dependent manner after long-term (days) hypertonic stimulation. The finding that osmolality did not influence cytomegalovirus (CMV) promoter-driven AQP_2 expression provided evidence that osmolality influences AQP_2 abundance by increasing its transcription. Using a luciferase reporter plasmid driven by various fragments of the murine AQP_2 promoter, Kasano *et al.* confirmed the VP-independent enhancing effects of hypertonicity on AQP_2 transcription in Madin-Darby canine kidney cells [50]. Enhanced AQP_2 mRNA and protein expression following 24 h of hypertonic challenge was also observed in cultured CCD $mpkCCD_{cl4}$ cells, a cell line that exhibits many major characteristics of CD principal cells including VP-inducible expression of endogenous AQP_2 [51] . Interestingly, AQP_2 expression levels decreased shortly (≤3 h) following hypertonic challenge implying a time-dependent biphasic effect of hypertonicity on AQP_2 expression [52] as shown in figure 2. What are the mechanisms underlining the effects of hypertonicity on AQP_2 transcription? Hypertonicity does not increase cAMP concentration or cAMP response element-binding protein (CREB) phosphorylation indicating that its effects are not mediated by cAMP [52]. This is further supported by the observation made in $mpkCCD_{cl4}$ cells that the extent of both time-dependent effects of hypertonicity on AQP_2 expression was similar in the absence or presence of VP. Moreover, the extent of stimulatory effect mediated by hypertonicity was not altered by PKA inhibition [52]. Together, these observations indicate that the effects of hypertonicity do not rely on the "classical" cAMP/PKA pathway.

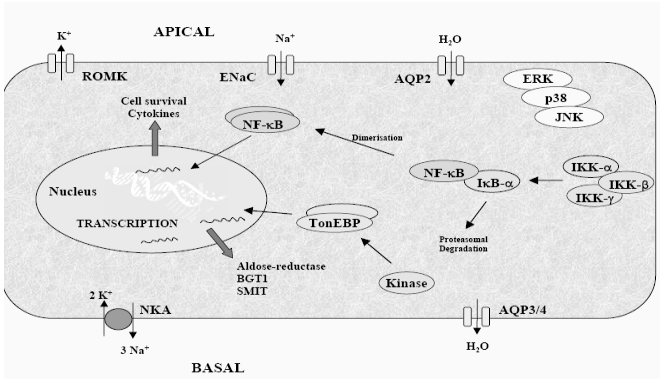

Figure 2. *Signaling in response to hypertonicity in collecting duct principal cells.*

Effect of hypertonicity **on intracellular signal transduction:**

Kidney cells, and particularly medullary cells, are routinely exposed to conditions challenging cell volume as a consequence of urine concentrating disparity that in turn maintains whole body water homeostasis within a restricted range. An accumulation of poorly permeable electrolytes outside the cell gives rise to a hypertonic (>300 mosmol/kgH_2O) environment. Cells exposed to such an environment are subjected to immediate water efflux causing cells to shrink. Conversely, osmotic water influx causes cells to swell when exposed to a hypotonic (<290 mosmol/kgH_2O) environment. A fundamental requirement of all mammalian cells is to maintain a constant volume. Cells react to shrinkage by rapidly accumulating extracellular Na^+ and Cl^- as part of the regulatory volume increase (RVI) mechanism that restores cell volume within minutes by passive water uptake [53]. In hypotonicity-challenged cells, cell swelling is reduced by osmotic water efflux as a consequence of intracellular ion efflux, mainly K^+ and Cl^- and various organic molecules including amino acids and amines, as part of the regulatory volume decrease (RVD) mechanism [54]. RVI occurs at the expense of perturbed protein function that results from

elevated intracellular ionic strength. Depending on the extent of hyperosmotic challenge, cells either adapt to their new conditions of elevated osmolality or, when osmolality is too high, undergo apoptotic-mediated cell death. In the latter case, RVI does not occur, cells continue to shrink and undergo apoptosis within hours of exposure. In cultured renal cells grown in 290 mosmol/kg H_2O medium, such a response is typically induced following an acute increase of environmental osmolality of >600 mosmol/kg H_2O.

Hypertonicity induces cell cycle arrest providing the cell with time (hours) needed for its adaptation to conditions of higher osmolality. Hypertonicity additionally induces DNA breaks, an event that is well known to be associated with cell cycle arrest [55]. Notwithstanding the many DNA breaks observed in renal inner medulla cells exposed to hypertonic medium, adapted cells grow normally but do not display the usual DNA damage response [55]. Only a subset of proteins involved in the classical DNA damage response, including p53, ATM, Gadd 45a, and Gadd 153, is activated by Hypertonicity [55]. Hypertonic adaptation involves increased signaling of osmoprotective responses mediated by reactive oxygen species [56] and COX-2 induction [57]. Increased p53 activity helps reduce hypertonicity-induced apoptosis by restricting DNA replication [58]. Increased expression of Gadd 45 contributes to protect the genomic integrity of renal inner medulla cells [59]. Hypertonicity increases the expression levels of several heat shock proteins (HSPs) including HSP25, HSP70, HSP110, Osp90, and αB-crystalin [60], which collectively enhance cell survival and prevent apoptosis. In addition to increased HSP expression, an important hallmark of hypertonic adaptation is the intracellular accumulation of small organic osmolytes that reduce intracellular ionic strength without affecting protein function. Hypertonicity induces the activation of a network of kinases in a variety of cell types [61]. In yeast, adaptation to osmotic stress is dependent on the p38 kinase homolog high-osmolality glycerol (HOG1) kinase [62]. Numerous studies have since demonstrated the pivotal role that p38 kinase plays in mammalian cells in numerous processes including transcriptional regulation, apoptosis, cytokine production, and cytoskeletal reorganization that characterize the adaptive response to osmotic stress [61]. Transcriptional regulation by ERK kinases, via activation of targets such as *c-myc*, *c-jun,* and *c-fos* [63], may counter hypertonicity-induced apoptosis by enhancing cell survival and proliferation. Enhanced transcription of HSP70, COX-2, and the γ-subunit of Na^+-K^+-ATPase by JNK_2 kinase may additionally contribute to kidney cell survival under hypertonic conditions [64.65]. It should be noted that mitogen-activated protein (MAP) kinase activation appears to depend on the cell type. For instance, hypertonicity activates ERK1/2 in inner medullary collecting duct cells and in cells of the medullary TAL [66] but decreases ERK1/2 activity in NIH 3T3 fibroblasts [67]. In addition to transcriptional activity, hypertonicity-induced MAP kinase signaling has been shown to modulate intracellular trafficking of a number of receptors and transporters. Cumulating evidence, gathered in both renal and nonrenal cells, supports a role for MAP kinases in synaptic receptor and transporter trafficking [68], as illustrated by the role that p38, ERK1/2, and JNK kinases may play in the cell surface expression of serotonin and dopamine transporters and the AMPA-sensitive glutamate receptor [69]. In neurons, Kv 2.1 potassium channel cell membrane insertion relies on p38 MAP kinase activity [70], whereas ERK-dependent inhibition of ENaC activity in renal cortical collecting duct (CCD) cells may result from reduced expression of ENaC at the cell surface [71]. In addition to MAP kinases, hypertonicity alters the activities of numerous other signaling pathways, including phosphatidylinositol phosphate 5-kinase (PIP5K)β kinase, which contributes to hypertonicity-induced phosphatidylinositol 4,5-bisphosphate-dependent cytoskeletal rearrangement [72], PI_3K-1A, which activates TonEBP via PIP_3-dependent ATM activation [73] and Src tyrosine kinases that mediate various cellular responses to hypertonicity including RVI, cytoskeletal reorganization [74], and TonEBP activation. Activation of the catalytic subunit of PKA (PKAc) by hypertonicity has been demonstrated in a liver cell line (HepG2) in the absence of an increase of intracellular cAMP. A similar increase of PKA activity was observed in cultured renal CD cells in the absence of increased intracellular cAMP concentration [75]. Of particular pertinence to the present review, PKAc has been shown to be part of a NF-κB-IκB-PKAc complex that dissociates following stimulation of the nuclear factor (NF)-κB pathway, leading to the release of active PKAc [76]. Hypertonicity is one such factor that stimulates NF-κB activity in renal CD principal cells.

Signaling pathways and downstream effectors induced by hypertonicity are being increasingly identified and understood. However, the specific osmosensor involved in initiating the hypertonicity response has yet to be identified in mammalian cells. In yeast, an increase of environmental osmolality activates putative osmotic sensor proteins (Sln1p, Sho1p, and Msb2p) that activate the MAP

kinase Hog1p that in turn activates transcriptional responses involved in glycerol synthesis, leading to an increase of intracellular compatible osmolyte concentration [77]. Several mechanisms have been proposed to play the role of an osmosensor in mammalian cells [4]. These include cell shrinkage *per se*, increased intracellular ionic strength, molecular crowding, DNA breaks, cytoskeletal perturbations, cytoskeletal stress, and altered intracellular signaling. Transient receptor potential vanilloid (TRPV) ion channels have additionally been proposed to play an osmosensing role [78]. On the other hand, osmotic sensing could by achieved by several elements acting in tandem with each other. Nuclear factor of activated T cells $_5$ ($NFAT_5$) is a member of the Rel family of transcription factors and is an essential inducer of osmoprotective gene products in mammalian cells. Its activation by hypertonicity requires p38 mitogen-activated protein kinase (MAPK) signaling and other pathways. A study now elucidates a signaling cascade regulated by the guanine nucleotide exchange factor Brx that leads to the activation of p38α MAPK and the induction of *nfat5* messenger RNA in response to osmotic stress in lymphocytes and renal medullary cells. *Brx*-deficient lymphocytes showed impaired responses to hypertonicity, and $brx^{+/-}$ mice exhibited immune defects similar to those of *nfat5*-deficient mice. These findings support a major role for Brx in regulating the osmoprotective function of $NFAT_5$ in different cell types [79].

Conclusion

This review article illustrates the importance of hypertonicity on the gene expression of water regulatory molecules and intracellular signal transduction. The results of this study indicate that increasing extracellular tonicity specifically first inhibits and then enhances AQP_2 expression, most likely through transcriptional control of the AQP_2 gene. Hypertonicity is also able to significantly alter the gene expression of water regulatory molecules, TonEBP, vasopressin, natriuretic peptides and urocortin.

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Correspondence:

Manoj G Tyagi
Department of Pharmacology
Christian Medical College
Vellore 632002, Tamil Nadu
India