ISSN: 2279 - 0594

Journal of Biomedical and Pharmaceutical Research

Available Online at www.jbpr.in

CODEN: - JBPRAU (Source: - American Chemical Society)

Volume 4, Issue 5: September-October: 2015, 10-19

Review Article

Effect of TonEBP on cardiovascular manifestations; influence of signaling mediators

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Received 10 September 2015; Accepted 14 October 2015

ABSTRACT

Tonicity-responsive enhancer binding-protein (TonEBP or NFAT5), which belongs to the Rel/NFAT family of transcription factors, plays a critical role in the kidney by controlling the expression of osmoprotective genes including heat-shock protein 70 and genes that mediate the intracellular accumulation of small organic osmolytes that reduce intracellular ionic strength without affecting protein function, such as aldose reductase (AR), sodium-chloride-betaine cotransporter (BGT1), and sodium-myo-inositol cotransporter (SMIT). Numerous cell types throughout the body are exposed to aniosmotic environments. Environmental osmolality is greatest in the kidney where levels as high as 1200 mOsmol/kg can be reached at the tip of the inner medulla in humans. This review examines the importance of TonEBP on cardiovascular manifestations and influence of signaling molecules.

Key words: TonEBP, Heart, SUMO, vascular, kidney, dopa decarboxylase

INTRODUCTION

The fundamental interspecies event of the signaling mechanisms and nature of accumulated osmolytes vary between different species. In mammalian cells, the importance of TonEBP in osmolyte accumulation is well known. The effect of hypertonicity on NF-κB activity, in contrast, is less obvious [1,2]. Altered NF-κB activity in response to hypertonicity has been documented in various cell types, including skin fibroblasts, monocytic cells, gastric epithelial cells, and vascular smooth muscle cells [3-5] . In the kidney, similar to several observations made in renal principal cells[6], hypertonicity was shown to increase NF-κB activity in renal medullary interstitial cells[7]. Increased NF-κB activity may represent a protective measure against hypertoncity-induced apoptosis. TonEBP is an important transcription factor in cellular adaptation to hypertonic stress, and also in macrophage activation. Tonicity-responsive enhancer binding protein (TonEBP), also known as nuclear factor of activated T cells 5 (NFAT5), belongs to the Rel family of transcription factors along with nuclear factor κB (NFκB) and NFAT1 to NFAT4. Members of this family share the Rel-homology domain of ~250 amino acids involved in DNA binding. TonEBP was originally identified as the central regulator of transcriptional response to hypertonic stress. Recent studies have revealed that TonEBP is also involved in the pro-inflammatory activation of macrophages by promoting expression a host of pro-inflammatory genes in response to Toll-like receptor 4 activation[8], as well as by stimulating the activity of NFκB. This review examines the importance of TonEBP on cardiovascular manifestations and the influence of signalling molecules.

TonEBP and cardiovascular system:

Heart

Cardiovascular diseases are the leading cause of premature deaths worldwide, being responsible for about 17 million deaths in 2008 alone[9]. Hypertension is a major risk factor for coronary heart disease[10] and eventual mortality. Osmo receptor activation in the central nervous system can elevate blood pressure through a mechanism involving increased sympathetic nerve activity, a process thought to contribute to salt-induced hypertension [11]. In rats, a high salt diet activates NFAT5 in macrophages and results in VEGFC secretion [12]. VEGFC then diminishes interstitial hypertonic volume retention by inducing hyperplasia of the lymph capillaries and expression

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of endothelial nitric oxide synthase. In isolated vascular smooth muscle cells, NFAT5 was shown to be activated by hypertonicity, the vasoconstrictor angiotensin II, and the mitogen platelet-derived growth factor-BB. These effects were selective in that NFAT5 was not affected by other vasoconstrictors, mitogens, or a variety of cytokines, including IL1β, IL8, IL10, TNF, or IFN [13]. NFAT5 is also significantly up-regulated in models of vascular injury, such as atherosclerotic lesions and neointimal hyperplasia. It was proposed that NFAT5 was involved in the regulation of the vascular smooth muscle cell phenotype [14]. NFAT5 also appears to have a role to play in the heart. In cardiac myocytes, NFAT5 degradation was found to be a key event mediating doxorubicin cytotoxicity [15,16]. An Exposure of myocytes to hypertonic media can cause NF-κB and caspase activation through a mechanism involving ROS [17]. This would suggest that the hyperosmotic stress triggers oxidative stress and ROS production within the cells. Hyperosmotic stress also triggers apoptosis in cardiac myocytes through a p53-dependent manner. In this respect, it appears to be a more potent stimulus than other factors known to induce cardiac myocyte death, including doxorubicin or angiotensin II [18]. NFAT5 mRNA and protein is up-regulated in cardiac myocytes exposed to hyperosmotic media *in vitro* [19]. After hyperosmotic challenge with sorbitol, cardiomyocytes also exhibited an increase in AR expression that was accompanied by AR-mediated activation of apoptotic signaling pathways [20].

The osmolyte taurine a non essental amino acid and is the most abundant free amino acid in cardiac tissues and in skeletal muscle. In response to hyperosmotic stress, cells take up taurine to counter the elevations in extracellular osmolarity. One mechanism responsible for the intracellular taurine accumulation is the NFAT5-mediated up-regulation of the taurine transporter SLC6A6. Consistent with this observation is the finding that SLC6A6 has a NFAT5 binding site within its promoter [18]. Several studies have shown that taurine deficiency leads to cardiomyopathy, suggesting an important role for this osmolyte in maintaining heart health [16,21]. Because of the multifaceted functions of taurine (including antioxidant and osmoprotectant properties) the precise mechanisms mediating protection of cardiac tissues remains to be determined [22]. Using SLC6A6 knockout mice, recent studies show evidence that the cytoprotective actions of taurine in cardiac and skeletal muscle are dependent on its ability to act as an osmolyte. These mice exhibited decreased cell volume, a condition often associated with the cell's inability to effectively counter extracellular osmolarity [23].

Vascular smooth muscle

The hypertension-induced arterial remodeling, a chronic increase in wall stress or biomechanical stretch and is likely to act as the most important determinant promoting activation and migration of VSMCs within the media of the artery [24]. Directed migration requires a polarized reorganization of the actin cytoskeleton to define the cell's front with a loose actin scaffold and the cell's rear with stabilized fibers. Depending on the local balance of stabilizing and destabilizing mechanisms, globular actin (G-actin) rapidly polymerizes to form filamentous actin (F-actin) or depolymerizes to liberate G-actin monomers. From amonst the six mammalian actin genes, the regulation of cytoskeletal actins ACTB (β-actin) and ACTG2 (γ-actin) has been studied in some detail whereby the later encodes for the largest actin isoform and its expression is regulated by CArG promotor elements as a target of the transcriptional coactivator myocardin [25]. Moreover, the expression of the VSMC-specific α-smooth muscle actin (αSMA) appears to be controlled by both myocardin [26] and the hypertonicity-responsive transcription factor nuclear factor of activated T-cells 5 (NFAT5) (Nuclear factor of activated T cells 5 regulates the vascular smooth muscle cell phenotypic modulation [13].

TonEBP/NFAT5 has also been reported to control gene expression in angiotensin II- or PDGF-BB-stimulated VSMCs [13]. Henceforth, it contributes to a phenotypic switch of these cells which includes the transition from a quiescent and resting to an activated and motile phenotype that is usually associated with vascular remodeling processes. An important determinant mediating this phenotype change and realignment of VSMCs in the arterial media is a chronic increase in wall stress or bio-mechanical stretch. Stretch-stimulated VSMCs respond by enhancing protein abundance and

nuclear translocation of NFAT5[27]. Several kinases have been reported to control NFAT5 activity[28], p38 MAP kinase and ERK1/2-dependent signaling appear to affect neither expression nor translocation of NFAT5 under these conditions. However, its palmitoylation appears to be crucial for the stretch-induced nuclear translocation—a covalent attachment of fatty acids to cysteine, sometimes serine and threonine residues of the protein core. Such a modification has been reported to regulate the entry of NFAT5 into the nucleus in response to osmotic stress [29]. Additionally, the activity of type 1 carnitine palmitoyltransferases (CPT1) is rate-limiting for the translocation process occurring in VSMCs upon exposure to biomechanical stretch [30].

Kidney

The Renal medullary cells are normally exposed to high NaCl concentrations in their interstitial fluid, which powers urinary concentration. Such high salt concentrations can damage and even kill cells. The survival and function of renal medullary cells depend on the transcription factor nuclear factor of activated T cells 5 (NFAT5; also called tonicity-responsive enhancer-binding protein or osmotic response element-binding protein), which activates the expression of osmoprotective genes that code for proteins, such as aldose reductase (AR) and betaine/glycine transporter 1 (BGT1). AR and BGT1 cause the cellular accumulation of protective organic osmolytes sorbitol and glycine betaine, respectively [16,31] (Fig.2). NFAT5 also plays an important role in salt-induced or associated inflammation [32], hypertension [33], and experimental autoimmune encephalomyelitis [34]. Although hypertonicity in the renal medullary interstitial fluid provides anosmolar gradient driving water absorption, it is extremely stressful to the medullary cells and can cause cell death. The Survival and function of the kidney medulla are dependent on NFAT5. In the renal medulla, NFAT5 activates expression of two groups of genes that are essential for urinary concentration. The first group of the genes is called osmoprotective genes, including betaine/glycine transporter 1 (BGT1), sodium-dependent myo-inositol transporter (SMIT) and aldose reductase (AR) [18]. Survived NFAT5 homozygous knockouts have profound renal medullary hypotrophy with reduced expression of the osmoprotective gene [35]. The second group of genes is directly involved in urinary concentration such as aquaporin-2 (AQP-2) and possible aquaporin-1 (AQP-1), which are necessary for maintaining adequate water permeability in the kidney medulla, and urea transporter 1 (UTA1) for building hyperosmolarity in the renal medullary interstitium **(Fig.1)**. Expression of a dominant negative mutant of NFAT5 in the kidney epithelial cells reduces expression of AQP-2 and UTA1, and impairs urinary concentration [36]. Thus, NFAT5 is tightly regulated in the kidney medulla to ensure normal process of urinary concentration. Hypokalemia, cyclosporine A and sepsis-induced urinary concentration defect is associated with reduced NFAT5 activity in the region [37]. Altogether these may affect the total body volume and have influence on cardiovascular homeostasis.

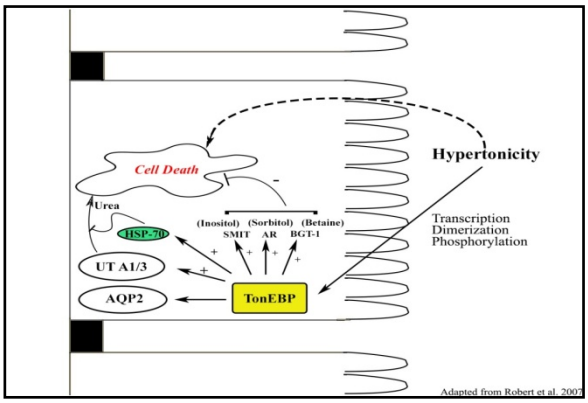

Figure 1: TonEBP and signaling mechanisms

Endoplasmic reticulum (ER) stress is a fundamental stress response that allows cells to adapt to environmental change by restoring cellular homeostasis. It arises when the processing and folding capacities of the ER are overwhelmed, either by an overload of nascent proteins or by exogenous disruption of the protein folding and trafficking system [38]. The accumulation of unfolded protein in the ER lumen triggers the cytoprotective unfolded protein response (UPR) that transiently attenuates protein synthesis, degrades mis-folded proteins and increases ER protein folding capacity. This allows cells to adapt to environmental change by restoring cellular homeostasis. If the stress persists and homeostasis cannot be re-established, for instance when proteotoxic stimuli are excessive, UPR signaling can activate cell-death pathways. In the kidney, extracellular osmolality increases along the cortico-medullary axis and is enhanced by arginine

vasopressin (AVP) in response to an increase of plasma osmolality [39]. Several pieces of experimental data have shown that alterations of inner medullary osmolality are accompanied by altered expression of genes that are typically induced by ER stress [40]. This strongly suggests that hypertonicity induces ER stress. Water restriction or dDAVP infusion both increase medullary osmolality, increased Grp78 and ATF4 expression in the inner medulla [40]. Conversely, GRP78 protein abundance decreased in IMCD of animals subjected to vasopressin escape [41]. Expression levels of ER stress responsive genes CHOP and ATF3 were increased by either NaCl or urea challenge in cultured IMCD cells [42] and several ER stress-responsive genes were up regulated by NaCl challenge in renal fibroblast cell lines [43]. Their study shows that the extent of induction of ER stress-responsive genes by either Tg or Tun was significantly higher than that induced by hyper osmolality. However, it should be appreciated that induction of ER stress by either chemical agent would be expected to be much higher than that induced by a more physiological stimulus since the effects of both chemical agents accumulate over time and eventually induce apoptosis. In this respect, the situation for hyperosmotic stress is very different. Indeed, cells recover from osmotic stress and hyper osmolality may be expected to only transiently increase ER stress-responsive gene expression, as was observed. The observation that ER stress-responsive genes were similarly induced by NaCl and urea indicates that these effects are induced by an increase of intracellular osmolality and not by NaCl *per se*. Increased UPR activity by NaCl is primarily associated with activation of PERK and ATF6 but not IRE1α in cultured CD principal cells. IRE1α activity was previously found to be inhibited when bound to an aberrant splicing iso form of presenilin- 2 caused by hypoxia or oxidative stress [44]. Possibly, a similar mechanism may occur in osmotically challenged cells. Upregulated expression of only relatively few genes by ER stress can be tied definitively to a specific canonical UPR transducer [45]. Their data indicate that unlike chemical chaperones that blunted hyperosmolality-induced EnaC down regulation, siRNA against canonical ER stress transducers only partly reduced this effect. This can likely be accounted for by crosstalk between canonical UPR arms and/or activation of non-canonical UPR pathwaysby hyperosmolality. For instance, nuclear accumulation of factors like bZIPs and C/EBPα,β by PERK [46] might occur in osmotically challenged cells independently of the PERK canonical downstream target ATF4. Although IRE1α activation by hyperosmolality might be too mild or transient to be detected by our assays, this may also help explain how XBP-1 translocates to the nucleus upon hypertonic challenge independently of an increase of IRE1α activity. On the other hand, manipulation of IRE1α affected downregulated ENaCαexpression by chemical ER stress induction, suggesting that other sources of ER stress that induce the IRE1α pathway may affect ENaCα expression.

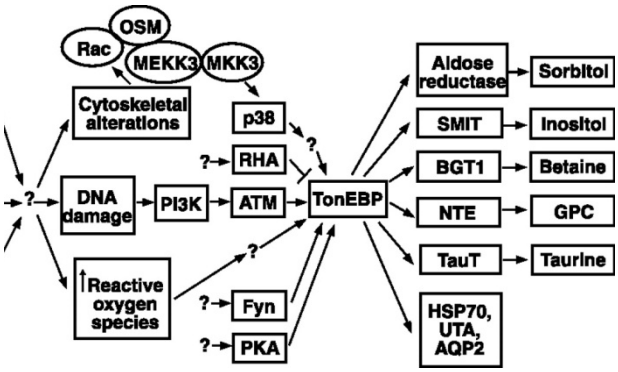

Figure 2: TonEBP and molecular cascade

(Courtesy: Maurice B. Burg, Joan D. Ferraris, Natalia I. Dmitrieva

Physiological Reviews, October 2007 Vol. 87 no. 4, 1441-1474)

TonEBP and signaling molecules

TonEBP and Sumoylation: SUMO (small ubiquitin-like modifier) is structurally related to ubiquitin. SUMO modification or sumoylation is an essential post-translational modification that regulates protein functions including transcription, signal transduction, and DNA repair. In mammals, SUMO2 and SUMO3 are 97% identical to each other and about 50% identical to SUMO1.Because of the transient nature of sumoylation in general, author used over expression of SUMO E2 ligase Ubc9 in combination with SUMO isoforms to demonstrate that TonEBP is di-sumolyated on lysine 556 and lysine 603. The sumoylation appears to take place in the nucleus because DNA binding is required. This is supported by the time course of sumoylation in response to hypertonicity which mirrors the previously reported time course

of binding to the TonE sites [47]. Studies using site-directed TonEBP mutants incapable of sumoylation and SUMO-conjugated TonEBP constructs reveal that TonEBP is inhibited by sumoylation in a dose-dependent manner regardless of the position of SUMO conjugation on the TonEBP molecule. Sumoylation inhibits TonEBP by reducing its transactivation without affecting nuclear translocation or DNA binding. From these data we can envision the following scheme of events after a cell is exposed to hypertonicity. Initially the nuclear TonEBP abundance rises due to enhanced expression in combination with nuclear translocation leading to DNA binding and expression of TonEBP target genes. Later, the TonEBP molecules bound to DNA are sumoylated and their transactivation decreases. Such tempering of TonEBP activity might be important for homeostasis in the renal medulla where TonEBP expression is very high most of the time. It was reported previously that the high concentration of nitric oxide in the renal medulla also inhibits TonEBP by direct *S*-nitrosylation [48]. Thus, the high level of TonEBP expression in the renal medulla is counterbalanced by inhibitory post-translational modifications including sumoylation and *S*-nitrosylation. SUMO (small ubiquitin-like modifier) is structurally related to ubiquitin. SUMO modification or sumoylation is an essential post-translational modification that regulates protein functions including transcription, signal transduction, and DNA repair [49]. In mammals, SUMO2 and SUMO3 are 97% identical to each other and about 50% identical to SUMO [50].The sumoylation appears to take place in the nucleus because DNA binding is required. Studies using site-directed TonEBP mutants incapable of sumoylation and SUMO-conjugated TonEBP constructs reveal that TonEBP is inhibited by sumoylation in a dose-dependent manner regardless of the position of SUMO conjugation on the TonEBP molecule. Sumoylation inhibits TonEBP by reducing its transactivation without affecting nuclear translocation or DNA binding. From these data we can envision the following scheme of events after a cell is exposed to hypertonicity. Initially the nuclear TonEBP abundance rises due to enhanced expression in combination withnuclear translocation leading to DNA binding and expression of TonEBP target genes. Later, the TonEBP molecules bound to DNA are sumoylated and their transactivation decreases. Such tempering of TonEBP activity might be important for homeostasis in the renal medulla where TonEBP expression is very high most of the time [50].

MAPK and TonEBP:

Mitogen activated protein kinases (MAPKs) have three major subcategories: p38, extracellular signal-regulated kinases (ERK) and c-Jun NH_2-terminal protein kinases (JNK). Each family of MAPKs has multiple isoforms. MAPKs regulate a wide variety of cellular activities, including proliferation, apoptosis and differentiation. Some of these distinct functions are carried out by specific isoforms, whereas some of the specific even opposing functions are performed by the same isoform. Hypertonicity activates some isoforms in all three subcategories in cultured cells [51]. Similarly, water restriction increases the stimulatory phosphorylation of p38, ERK1/2 and JNK1/2 in the rat kidney [36]. MAPKs involve in regulation of hypertonicity-induced expression of an array of genes ranging from osmoprotective to inflammatory ones [52], some of which through NFAT5.

PKC enzyme and high salt:

PKC- is a member of the PKC family. This family of serine/threonine kinases can be divided into three categories based on their structure and biochemical properties: classical or conventional PKCs, including PKC-α, PKC-βI, PKC-βII, and PKC-γ; novel PKCs, including PKC-δ, PKC-ε, PKC-η, and PKC-θ; and atypical PKCs, including PKC-ζ and PKC-λ. PKCs play critical roles in a wide variety of physiological and pathophysiological processes, such as cell growth, inflammation, cancer metastasis, and cardiac hypertrophy [53]. Knockout of PKC-α impairs urinary concentration [54], thereby lowering urinary osmolality. The effect of PKC-α on urinary concentration is mediated in mouse inner medullary collecting ducts by its contribution to high NaCl-induced increases of phosphorylation of urea transporters (UTs) [55] and urea permeability [56].

In HEK-293 cells, high NaCl increases specific PKC-α activity and siRNA-mediated knockdown of PKC-α reduces NFAT5 transcriptional activity and transactivating activity without significantly affecting NFAT5 nuclear localization or protein

abundance. It is not surprising that NFAT5 transactivating activity is affected independent of NFAT5 nuclear localization and protein abundance, because the measurement of transactivation uses yeast binary GAL4 assay system in which the amino terminus of NFAT5, which has the nuclear localization signal and DNA-binding domain [57], is replaced by GAL4 DNA binding and nuclear localization domains [58]. The NFAT5 transactivating activity assay is also independent of endogenous NFAT5 protein. We cannot determine whether knockout of PKC-α reduces NFAT5

transactivating activity in the kidney inner medulla, since there is no method available to make the measurement *in vivo.* More than a dozen kinases have been demonstrated to contribute to high NaCl-dependent activation of NFAT5 in cell culture [59], but PKC-α is the first kinase directly shown to be involved in the regulation of NFAT5 in the kidney medulla *in vivo.*

Also from the existing studies it is evident that PKCμ activates NF-κB to protect the cells from oxidative stress-related cell death. Src-Abl activated by oxidative stress induces phosphorylation of PKCμ and leads activation of downstream IKKNF-κB signaling [60]. Additionally, mitochondrial ROS also activate PKCμ and induce the expression of manganese-dependent superoxide dismutase (MnSOD) through activating NF-κB [61]. Reduced ROS production by antioxidant suppresses NaCl-induced TonEBP activation and BGT1 expression [62]. PKCμ could be a mediator between ROS and TonEBP activation in hypertonic condition. Since, when PKCμ was inhibited using specific inhibitor or siRNA knock-down method, TonEBP phosphorylation and HSP70 induction were evidently decreased, it could be concluded that increased ROS by hypertonicity might be the main cause of PKCμ activation and TonEBP-mediated HSP70 gene expression [63].

Dopa Decarboxylase activity:

In renal proximal tubule cells, dopamine is produced to regulate sodium excretion. Dopamine synthesis relies on aromatic l-amino acid decarboxylase (AAD), to convert the precursor, L-3, 4-dihydroxyphenylalanine (L-DOPA) into dopamine. The secreted dopamine in the proximal tubule bind to dopamine D1-like receptors (D1Rs) to inhibit Na+/K+-ATPase (NKA) activity through (1) protein kinase C (PKC) phosphorylates the catalytic α-subunit of NKA [64] or (2) endocytosis of NKA from the cell membrane, which leads to a decrease in sodium absorption [65]. Previous studies have demonstrated that hypertonicity enhanced AAD expression to increase synthesis of dopamine [66].

Dopamine has been identified to be an important modulator of central and peripheral physiological functions in humans. Within the kidney, the highest abundance of dopamine receptors is in proximal tubule epithelial cells [67]. In addition, dopamine secretion significantly increases in hypertonic stress, as reported in previous studies [68]. AAD is the key enzyme for converting L-DOPA to dopamine, and its expression is regulated by TonEBP. Thus, it made sense that dopamine secretion is dependent on TonEBP activity. This study connects the regulatory function of TonEBP not only to AAD expression but also to dopamine secretion in proximal epithelial cells upon hypertonic challenge. Furthermore, to reduce reabsorption of salt in the hypertonic condition, NKA activity is inhibited by dopamine [69]. Previous studies illustrate that phosphoinositide 3-kinase (PI3K) binds to NKA α1 and induces the recruitment and activation of endocytosis [70]. The NKA internalization results in reduced Na+ transport in proximal tubule epithelial cells [71]. In previous study, NKA protein expression of HK-2 cells was up-regulated when exposed to the hypertonic condition, and it was not affected by addition of L-DOPA. However, NKA activity dramatically decreased when treated with L-DOPA to elevate dopamine production. When inhibited by TonEBP shRNA, decreased NKA activity was recovered at 8h after exposure to the hypertonic condition with L-DOPA. This finding may be because dopamine production was triggered by hypertonic challenge with TonEBP activation. The pattern of NKA activity did not parallel its protein abundance. Subsequently, to separate cytoplasmic and membrane enriched fractions, author demonstrated that NKA protein abundance was decreased in membrane enriched fractions with L-DOPA treatment. It was presumed that NKA activity was decreased through the endocytotic mechanism [72].

Conclusion:

TonEBP protein is expressed ubiquitously throughout the human body, and only a small number of tissues are slightly hypertonic or become hyperosmotic during extreme stress. Therefore, it is reasonable to hypothesize that tonicity-independent mechanisms exists to regulate TonEBP in isotonic tissues. Many novel TonEBP-stimulating factors have been identified, such as the ionomycin in T-cells, α6β4 integrin clustering, and ionomycin in carcinomas, ionomycin, BMP-2, and TGF-β in nucleus pulposus cells, 1L-1β, and TNF-α in fibroblast-like synoviocytes, angiotensin II and PDGF-BB in vascular SMCs, and superoxide and NO in kidney cells. These stimuli serve to modulate TonEBP protein expression, transactivation, and/or nuclear localization, leading to TonEBP target gene transcription in isotonic (and some hypertonic) environments. Thus TonEBP may have many an unravelled important roles in pathophysiology and especially in the cardiovascular disorders.

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