TonEBP/OREBP IS A REGULATOR OF NUCLEUS PULPOUS CELL FUNCTION AND SURVIVAL IN THE INTERVERTEBRAL DISC
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Running Title: Role of TonEBP in the Intervertebral Disc

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The nucleus pulposus is an aggrecan-rich hydrated tissue that permits the intervertebral disc to resist compressive loads. Adaptation to loading is achieved through an elevation in disc osmolarity mediated by the numerous charged glycosaminoglycan side chains of the aggrecan molecule. The goal of this investigation was to determine the functional role of the osmoregulatory protein, TonEBP, in cells of the nucleus pulposus. We found that TonEBP and its downstream target genes were robustly expressed in the tissues of the disc. Above 330mOsm/kg, cultured nucleus pulposus cells upregulated target genes TauT, BGT-1 and SMIT; above 450mOsm/kg, there was raised expression of HSP-70. In hypertonic media there was activation of TauT and HSP-70 reporter activity and increased binding of TonEBP to the TonE motif. When cells were transfected with DN-TonEBP there was suppression of TauT and HSP-70 reporter gene expression; pTonEBP enhanced reporter gene expression. Moreover, in hypertonic media, forced expression of DN-TonEBP induced apoptosis. We suppressed TonEBP using siRNA technique and noted a decrease in TauT reporter activity in isotonic as well as hyperosmolar media. Finally, we report that the aggrecan promoter contains two conserved TonE motifs. To evaluate the importance of these motifs, we over-expressed DN-TonEBP and partially silenced TonEBP using siRNA. Both approaches resulted in suppression of aggrecan promoter activity. It is concluded that TonEBP permits the disc cells to adapt to the hyperosmotic milieu while autoregulating the expression of molecules that generate the unique extracellular environment.

The intervertebral disc is a specialized biomechanical structure that permits movement between vertebrae and protects the vertebral bone from mechanical forces. It consists of an outer ligament, the annulus fibrosus that encloses a gel-like tissue, the nucleus pulposus. While sparse, cells in the nucleus pulposus secrete a complex extracellular matrix that contains fibrillar collagens and the proteoglycan, aggrecan. The numerous glycosaminoglycan side chains of the aggrecan molecule bind cations thereby raising the osmolarity of the disc tissues (1-4). While the hydration properties of the nucleus pulposus permits dynamic loading and unloading, the mechanism by which these cells adapt to elevated osmotic forces is poorly understood.

In a number of tissues, cellular adaptation to hyperosmotic stress is mediated by the Tonicity Enhancer Binding Protein (TonEBP) also called OREBP (5), or NFAT5, (5, 6). Upon activation, TonEBP binds to the tonicity responsive enhancer element (TonE) of genes required for osmotolerance and cell survival. These genes include the betaine/γ-aminobutyric acid transporter, sodium-myoinositol co-transporter (7-9), taurine transporter (10, 11) and aldose reductase (6). By regulating levels of betaine, myo-inositol, taurine and sorbitol, these genes control the osmotic properties of the cytosol. HSP70, a molecular chaperone that maintains
cellular function under hypertonic stress is also induced by TonEBP (12, 13).

Most homozygous TonEBP knockout mice evidence mid-gestational lethality. Of the few that survive, all exhibit severe growth retardation and kidney dysfunction (14). A transgenic mice expressing a dominant-negative form of TonEBP (DN-TonEBP) in collecting duct epithelial cells demonstrates an absolute requirement of TonEBP for expression of the urea transporter gene and aquaporin-2 (15). Aside from osmoregulation, TonEBP is required for T cell proliferation and function (16, 17), and it is implicated in cancer cell migration and metastasis (18). A recent study by Wang et al. showed that expression of DN-TonEBP in lens fiber cells promotes cataract formation by causing defects in their elongation (19). Since TonEBP is expressed by a number of cell types, it is reasonable to assume that it serves a variety of physiologic functions, especially those that impact on tissue hydration and the osmotic environment (20).

The goal of the present study is to examine the role of TonEBP in cells of the postnatal intervertebral disc. We show that TonEBP, and its downstream target genes, are expressed in the nucleus pulposus and the annulus fibrosus. Importantly, our data indicates that TonEBP binds to a TonE motif in the aggrecan promoter and regulates its transcriptional activity. This finding lends credence to the view that TonEBP regulates the hydration status of the disc thereby enhancing cell function in the hyperosmotic environment.

Experimental Procedures

Isolation of nucleus pulposus cells - Rat nucleus pulposus cells were isolated using method reported earlier by Risbud et al. (21). Briefly, male Wistar rats, (250 g) were euthanized with CO₂ and lumbar intervertebral discs were removed from the spinal column. The gel-like nucleus pulposus was separated from the annulus fibrosus, using a dissecting microscope, and treated with 0.1% collagenase and 10 U/ml hyaluronidase for 4-6 h. The partially digested tissue was maintained as an explant in Dulbeccos Modified Eagles Medium (DMEM) and 10% fetal bovine serum (FBS) supplemented with antibiotics. Nucleus pulposus cells migrated out of the explant after 1 week. When confluent, the cells were lifted using a trypsin (0.25 %) EDTA (1 mM) solution and subcultured in 10 cm dishes.

RT-PCR analysis - RNA was isolated from cells using Trizol reagent (Invitrogen, CA) following the manufacturer’s instructions. Briefly, 2 µg of total RNA was reverse transcribed into cDNA using Superscript II RT enzyme (Invitrogen, CA) and oligo (dT) primers. PCR reactions were performed using cDNA samples (1 µl) with Superscript DNA polymerase (Invitrogen, CA). Primers for rat genes were custom designed (see Table I, supplementary data) and synthesized by Integrated DNA Technologies (Coralville, IA). The PCR product was run on a 1.2 % agarose gel and the amplicon visualized on a Kodak 440 imaging station.

Immunohistological studies - Freshly isolated discs were immediately fixed in 4% paraformaldehyde in PBS and then embedded in paraffin. Transverse and coronal sections, 6-8 µ in thickness, were deparaffinized in xylene, rehydrated through graded ethanol and stained with alcian blue, and with osin and propidium iodide. For localizing TonEBP, sections were incubated with the anti-TonEBP antibody (Abcam, MA) in 2% bovine serum albumin in PBS at a dilution of 1:100 at 4 °C overnight. After thoroughly washing the sections, the bound primary antibody was incubated with biotinylated universal secondary antibody, at a dilution of 1:20 (Vector Laboratories, CA) for 10 min at room temperature. Sections were incubated with a streptavidin/peroxidase complex for 5 min, washed with PBS and color was developed using 3'-3-diaminobenzidine (Vecta Stain Universal Quick Kit, Vector Laboratories, CA).

Immunofluorescence microscopy - Cells were plated in flat bottom 96 well plates and maintained in isotonic or hypertonic medium for 24 h. After incubation, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% triton-X 100 in PBS for 10 min, blocked with PBS containing 5% FBS, and incubated with anti-TonEBP antibody (1:200) (Abcam, MA) at 4°C overnight. As a negative control, cells were reacted with mouse isotype IgG under similar conditions. After washing, the cells were incubated with Alexa fluor-488 conjugated antimouse secondary antibody (Molecular Probes, St Louis, MO), at a dilution of 1:50 and 10 µM.
propidium iodide for 1 h at room temperature. Quantitative image analysis was performed using 9 random fields of cells per experimental group. The 36 bit color images were recorded by confocal microscopy using the green and red channel for TonEBP and propidium iodide (nuclear) respectively. Image Pro-plus software (Media Cybernetics, Silver Spring, MD) was used to calculate the threshold for all cells in the field. The mean density of TonEBP was then plotted as a histogram. Caspase-3 activation was evaluated by visualizing cleavage of the PhiPhiLux-G1D2 substrate (OncoImmune Inc., MD) following the manufacturer’s protocol. Cells were imaged using a laser scanning confocal microscope (Olympus Fluoview, Japan) and images were analyzed as described above.

**Nuclear extracts and Western blotting** - Nuclear extracts were prepared according to the method of Dignam et al. (22) using the Celllytic NuCLEAR extraction kit (Sigma-Aldrich, St. Louis, MO). After culture in isotonic or hypertonic medium, cells were immediately placed on ice. Cells were treated with a hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, and 0.5 mM DTT) and incubated on ice for 15 min. Igepal CA-630 was added to a final concentration of 0.6% and the mixture was vortexed vigorously for 10 sec. Nuclei were recovered by centrifugation at 3,300 x g for 30 sec at 4°C and extracted by gentle shaking in buffer containing 20 mM HEPES pH 7.9, 0.42 M NaCl, 25% glycerol, 1.5 mM MgCl2, 0.2 mM EDTA, and 0.5 mM DTT for 30 min at 4°C. The extract was then centrifuged for 15 min at 25,000 x g, and the supernatant was snap frozen at -70°C. All buffers contained a protease inhibitor cocktail.

Nuclear extracts were resolved on 10% SDS-polyacrylamide gels. Proteins were transferred by electroblotting to nitrocellulose membranes (Bio-Rad, CA). The membranes were blocked with 5% non-fat dry milk in TBST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% tween 20) and incubated overnight at 4°C in 3% non-fat dry milk in TBST with the anti-TonEBP antibody (Abcam, CA) at a dilution of 1:500. Immunolabeling was detected using the enhanced chemiluminescence reagent (ECL, Amersham Biosciences).

**Reporter plasmids** - PCR amplification of a 2.5 kb fragment of the upstream mouse aggregan promoter spanning -2204 to + 290 was performed using LA Taq polymerase (Takara Mirus Bio) using the following primers: Forward 5’ GAGGAGCTCCAGCAATATCGATGCTCC AGCTAGTGTGCAGAAAAT-3’, Sac I site underlined; Reverse 5’-GGTAGATCTGAGGGTCTGAGAAGGAAG GGACACAGAAAAGGTG-3’, Bgl II site underlined. The resulting DNA fragment was subcloned into pCR2.1 (Invitrogen, CA), isolated by restriction digestion with Sac I and Bgl II, and ligated into the luciferase basic expression vector, pGL3 (Promega). As an internal transfection control, vector pRL-TK (Promega) containing Renilla reniformis luciferase genes was used. The amount of transfected plasmid, the pre-transfection period after seeding, and the post-transfection period before harvesting, were optimized for rat nucleus pulposus cells using pSV β-galactosidase plasmid (Promega). Reporter plasmids were kindly provided by Dr. Takashi Ito, Osaka University, Japan [TauT (Wt), TauT (Mt)] (11) and Dr. H.M. Kwon, University of Maryland, USA [HSP-70 (Wt), HSP-70 (Mt)] (12); pTauT (Wt) and pHSP-70 (Wt) contains active TonE, whereas the base mutations in pTauT (Mt) and pHSP-70 (Mt) disrupt TonEBP binding to the TonE site. Dr. Ben C. Ko, University of Hong Kong, P.R China, kindly provided FLAG-DN-TonEBP (dominant negative expression vector), FLAG-TonEBP and FLAG-CMV2 expression plasmids (15).

**Transfections and dual luciferase assay** - Nucleus pulposus cells were transferred to 24-well plates at a density of 7.5 × 10⁴ cells/well one day before transfection. LipofectAMINE 2000 (Invitrogen, CA) was used as a transfection reagent. For each transfection, 330-500 ng of reporter gene plasmid, and 330-500 ng of control plasmid pRL-TK, and where applicable, 330 ng of either DN-TonEBP or FLAG-TonEBP or FLAG-CMV2 (empty vector) expression plasmid were premixed with the transfection reagent. Twenty four hours after transfection, the osmolarity of the medium was increased to 400 or 500 mOsm/Kg by addition of NaCl. The next day, the cells were harvested and a Dual-Luciferase™ reporter assay system (Promega) was used for sequential measurements of firefly and Renilla luciferase activities. Quantification of luciferase activities and calculation of relative ratios were carried out using a luminometer (TD-20/20, Turner Designs).
Sunnyvale, CA, USA). At least three independent transfections were performed, and all analysis were carried out in triplicate.

Electrophoretic mobility shift assay - Electromobility shift assays were performed as previously described with minor modifications (21). Briefly, the binding reaction was carried out in 12.5 mM HEPES, pH 7.9, 50-100 mM NaCl, 5% glycerol, 0.5 mg/ml BSA, 1-2 µg poly-dIdC, 0.1 mM EDTA, 0.1 mM DTT, using 50 fmol of biotin-end-labeled double stranded oligonucleotide (top strand sequence 5’CAAGCTGGTATTTTTCCACCCAGCA3' for TauT and 5’ TGAGAGATTCGGGAGATTTCCACTACACTGCCTGA 3’ for aggrecan) and 10-15 µg of nuclear protein. Following incubation for 45 min at room temperature, extracts were loaded onto 5% acrylamide-0.5X tris-borate-EDTA gels, electrophoresed at 130 V for 1 h and transferred onto a positively charged nylon membrane (Hybond™-N+, Pierce, IL) in 0.5X Tris-borate/EDTA at 100 V for 45 min. Transferred DNAs were cross-linked to the membrane at 120 mJ/cm² and detected using horseradish peroxidase-conjugated streptavidin according to the manufacturer’s instructions (LightShift™ Chemiluminescent EMSA kit, Pierce, IL).

Silencing of TonEBP by SiRNA - The motif chosen corresponded to the second codon initiating site for the rat TonEBP/NFAT5 gene (23). As a negative control, the inverse dDNA sequence of the second motif was utilized (23). The annealed oligonucleotides were ligated into an expression vector (pSuppressorNeo, Imgenex, CA). The plasmid DNA was then linearized with Sca I at a site in the 8 bp loop region and sequenced in both directions as suggested by Ducat et al (24).

Primary rat nucleus pulposus cells were transfected with circular plasmid DNA containing SiRNA sequences using the TransFast transfection reagent (Promega) and clones were selected by culturing in medium containing G418 (400 µg/ml) for 7 days. Following expansion of the clones, total RNA was isolated using RNAeasy columns (Qiagen, CA). RT-PCR was used to determine which clones were partially silenced.

Measurement of cell viability - To measure cell viability, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out as described previously (25). Briefly, after treatment, MTT diluted in PBS was added to the culture medium to a final concentration of 0.5 mg/ml. At the end of the incubation period (2-4 h at 37 °C), the medium was removed, and the precipitated formazan crystals were solubilized in dimethyl sulfoxide. Product formation was measured by reading the absorbance at 560 nm using a microplate reader (Tecan, Spectra Fluor Plus, NC).

Statistical analysis - All measurements were performed in triplicate, data is presented as mean ± standard deviation. Differences between groups were analyzed by the student t test; *p < 0.05.

RESULTS

Figure 1A shows that intervertebral disc tissue of the adult rat robustly express TonEBP mRNA. Cartilage, another aggrecan rich skeletal tissue, also expresses TonEBP mRNA; however, the level of expression is lower than the disc. As might be expected, TonEBP mRNA expression in the kidney is the highest of all the tissues analyzed. The expression of the taurine transporter (TauT), HSP-70, and betain-GABA transporter-1 (BGT-1) is elevated in the disc tissue. However, the level of expression of sodium myoinositol transporter (SMIT) is lower than that of heart, kidney or liver. The level of expression of TauT, HSP-70, SMIT and BGT-1 in the disc is higher than that of cartilage. Immunohistological analysis of neonatal as well as adult rat disc reveals that TonEBP is expressed in neonatal and mature nucleus pulposus cells (Fig. 1B, D). In both cases, much of the staining is nuclear. However, some staining is seen in the cytosol of the nucleus pulposus cells of the mature discs (Fig. 1D). There is also a prominent expression of TonEBP protein in the annulus fibrosus cells of the mature rat disc (Fig. 1F) which are localized in a narrow zone of alcian blue positive matrix (Fig. 1G).

Figure 2A shows that when rat nucleus pulposus cells are cultured in hyperosmotic media (400 mOsm/kg) there is induction of TonEBP mRNA. Cartilage, another aggrecan rich skeletal tissue, also expresses TonEBP mRNA; however, no further increase in mRNA levels are evident when the medium osmolarity is raised to 550 mOsm/kg. A similar pattern of expression is observed for TauT, SMIT and BGT-1. In contrast, an increase in HSP-70 mRNA expression is seen when the osmolarity is raised to 450 mOsm/kg. Figure 2B shows that TonEBP
protein is robustly expressed under hyperosmotic culture conditions. In accord with the immunohistochemical observations (see Fig. 1), most of the protein is localized to the cell nucleus.

Figure 3 A and B shows TauT and HSP-70 reporter activities under hypertonic conditions. When the medium osmolarity is raised, a significant induction in TauT reporter activity is seen. Thus, at 400 mOsm/kg there is 5-6 fold increase in reporter activity. A further increase in medium osmolarity to 500 mOsm/kg causes an additional doubling of the activity. HSP-70 reporter activity is marginally induced at 400 mOsm/Kg; however, when the medium osmolarity is raised to 500 mOsm/kg there is a 5-6 fold increase in activity. To further examine the functional interaction of TonEBP protein with the TauT promoter, a gel mobility shift assay was performed. Figure 3C shows that when the medium is isotonic (330 mOsm/kg) or hypertonic (400-500 mOsm/kg), there is binding of TonEBP to the oligonucleotide probe containing the TonE site of the TauT promoter. The gel shift assay shows that binding of the TonEBP to the TonE probe is increased with raised medium osmolarity. To examine the specificity of this interaction, we used a probe containing mutation in the TonE site; in addition a competition analysis was performed. As expected, there was no/or extremely low binding to the mutant probe. Likewise, excess mutant probe could not compete the binding of TonEBP to wild type TonE.

To confirm that TonEBP is required for the induction of TauT and HSP-70 promoter activity, nucleus pulposus cells were transiently transfected with plasmids encoding DN-TonEBP and full length TonEBP. Figure 4 shows that forced expression of DN-TonEBP significantly suppresses TauT (A) and HSP-70 (C) promoter activity. When the medium is hypertonic, the level of expression in cells transfected with DN-TonEBP is lower than expression under isotonic condition. A significant inhibitory effect of DN-TonEBP expression on TauT reporter activity is seen at a dose of 50 ng, which is further enhanced when the concentration of the DN plasmid is increased to 100 ng. Despite further increases in the DN plasmid dose, the reporter activity is not decreased. On the other hand, overexpression of TonEBP under isotonic conditions, using pFLAG-TonEBP vector, results in a significant increase in both TauT (Fig. 4B) and HSP-70 (Fig. 4D) promoter activities. To ensure that the FLAG-fusion protein is expressed in nucleus pulposus cells, Western blot and immunofluorescence analysis was performed (Fig. 4E). As expected, using both the techniques, anti-FLAG antibody detects FLAG-DN-TonEBP in transfected cells only. Moreover, DN-TonEBP does not alter the level of native TonEBP expression in transfected cells.

To further explore the importance of TonEBP in regulation of the osmotic response, we stably transfected rat nucleus pulposus cells with TonEBP SiRNA. Among different clones screened, one clone displayed a partial suppression of TonEBP mRNA (Fig. 5A). Thus, there was about a 2 fold decrease in TonEBP mRNA expression. We confirmed that TonEBP protein levels are low by immunofluorescence analysis (Fig. 5B, C). Accordingly, the fluorescence yield of the SiRNA treated cells is minimal under isotonic as well as hypertonic conditions (Fig. 5C). The silenced SiRNA cells are smaller in size and exhibit a slow growth rate when compared with the wild type cells (not shown). Similarly, under both isotonic and hypertonic conditions, the silenced cells display a lower TauT reporter activity than control cells (Fig. 5D).

Analysis of mouse and rat aggrecan promoter showed two conserved TonE binding sites at -912 b and -390 b from the transcription start site (Fig. 7A and B). To examine the functional interaction of the TonE site with TonEBP protein, a electromobility gel shift assay was performed using an oligonucleotide probe that contained the wild type and mutant TonE site located at -912 b in the rat aggrecan promoter. As seen in Fig. 7C, there is functional binding of TonEBP protein to the TonE containing probe. Moreover, this binding is enhanced when the
medium osmolarity is raised to 400 and 500 mOsm/kg. To examine the specificity of this binding reaction, a wild type competitor probe was included in the binding reaction. In the presence of excess unlabelled competitor probe, binding of TonEBP protein to TonE site is significantly reduced (Fig. 7C). Likewise, when mutant TonE probe was included in the binding reaction, no binding to labeled probe was evident.

To further explore the role of TonEBP in the regulation of the aggrecan promoter, we analyzed promoter activity in nucleus pulposus cells transiently transfected with DN-TonEBP. Figure 7D shows that forced expression of DN-TonEBP results in approximately a 50% reduction in aggrecan promoter activity, compared to cells that receive the empty backbone plasmid (pFLAG-CMV). Similarly, nucleus pulposus cells expressing TonEBP SiRNA display a marked decrease in aggrecan promoter activity compared to control cells under isotonic conditions.

**DISCUSSION**

The current study is an extension of our earlier work that was directed at identifying key phenotypic markers of nucleus pulposus cells (26). Building on the observation that the cells exist in a unique microenvironment, we evaluated TonEBP and its target gene expression in the discal tissues. At the mRNA level, there was a robust expression of TonEBP. Although costochondral cartilage also expressed TonEBP mRNA, the level of expression was low when compared to disc or kidney. We used immunohistochemistry to probe TonEBP protein expression in the disc. Not surprisingly, there was expression of TonEBP protein in nucleus pulposus cells of both the neonatal and adult rat discs; the protein was also expressed by cells of the annulus fibrosus. This observation was not unexpected as the annulus, like the nucleus, is rich in proteoglycans and hydrodynamically stressed (4). We also showed that TonEBP positively regulates aggrecan gene expression. This latter finding is of some importance as aggrecan, a major constituent of the nucleus pulposus binds water molecules and provides resistance to applied mechanical forces. Accordingly, the expression of TonEBP permits nucleus pulposus cells to autoregulate the osmotic environment, while at the same time accommodating to the hyperosmotic pressure of the intervertebral disc.

To learn if there was transcriptional activation of TonEBP, TauT and HSP-70 reporter activity and protein-DNA binding was studied in nucleus pulposus cells maintained in hyperosmolar media. The results of this study showed that there was an activation of both the reporters and increased binding of TonEBP to the TonE motif indicating that in hypertonic media, TonEBP was transcriptionally active in the nucleus pulposus cells. We examined the hyperosmotic reactivity of the TonEBP by transfecting cells using pDN-TonEBP or pTonEBP. Predictably, pDN-TonEBP suppressed TauT (11) and HSP70 reporter gene activity (12) while pTonEBP enhanced reporter gene expression (11, 12). In a related study, we silenced TonEBP in nucleus pulposus cells and evaluated TauT reporter expression. We noted that the basal level of TauT reporter activity in the stably transfected silenced cells was reduced. Moreover, when the cells were maintained in hyperosmotic media, there was a marked decrease in reporter activity. It should also be noted that, the TonEBP activation is marked by translocation of the protein to the nucleus (5, 27). In nucleus pulposus cells, there was nuclear localization of TonEBP and induction of target gene expression under hypertonic conditions. These observations provide a basis for considering that the intervertebral disc cells possess a functionally active osmolyte system that serves to adapt the nucleus pulposus, and possibly the annulus fibrosus cells, to the high osmotic pressure and possibly the gradient in osmolarity that has been reported to exist within the intervertebral disc (28).

Although a considerable number of water binding molecules contribute to the regulation of the osmotic pressure, we focused on aggrecan as it is the major polyelectrolyte constituent. The charged COO\(^-\) and SO\(_4^{2-}\) groups of N-acetylgalactosamine, glucuronic acid and other substituted sugars bind hydrated Na\(^+\) ions thereby regulating the osmotic properties of the disc. While it is known that aggrecan transcription and osmotic pressure are linked (29, 30), details of the relationship are obscure. Promoter analysis of TonEBP provided a new insight into this relationship. We found two TonE sites at -390bp and -912bp in the aggrecan gene promoter sequence. A similar sequence was noted in the
human aggrecan promoter sequence. The observation that the human sequence was at -890 bp probably reflects differences in species specific organization of the aggrecan promoter sequence. The presence of these conserved motifs provide a direct link between aggrecan expression and tissue osmolarity.

That the aggrecan promoter was responsive to TonEBP was evident from experiments performed using the DN-TonEBP transfected cells. These studies revealed that forced expression of DN-TonEBP in isotonic media resulted in partial loss of aggrecan promoter activity. When TonEBP expression in nucleus pulposus cells was partially silenced by SiRNA, again there was suppression of aggrecan promoter activity. In an ongoing study, we have slowly adapted disc cells to an increase in medium osmolarity. In this case, there is continued induction of aggrecan mRNA expression suggesting that there is a direct relationship between aggrecan expression and the tonicity of the medium. Together, these findings indicate that aside from Sox-9 (31) and other transcriptionally active proteins, TonEBP serves as a regulator of aggrecan expression that constitutes a major functionally active component of the disc matrix.

The nucleus pulposus cells were probably able to survive in a hyperosmotic medium through activation of TonEBP. In addition, the expression of almost all of TonEBP target genes was elevated. BGT-1 and SMIT appeared to be most sensitive to the increased osmolarity, while at 450 mOsm/kg, HSP-70 expression appeared to be further increased. This result suggests that TonEBP can respond to hyperosmolarity by activating the transcription of a series of gene products that adapt the nucleus pulposus cell to changing environmental conditions. When TonEBP activity was inhibited using DN-TonEBP, we noted a marked decrease in cell survival. At 500 mOsm/kg, the number of viable cells was reduced by 50%. Since there was activation of caspase-3, it is probable that the osmotic stress stimulated the intrinsic apoptotic pathway. The observed increase in apoptosis is in agreement with studies of TonEBP null mice and transgenic animals expressing DN-TonEBP in the thymus and in the lens (16, 17, 19). In both these conditions, there was an acceleration of cell death through apoptosis. The relationship between TonEBP and cell death lends strength to the ideas presented here on the critical importance of this transcription factor in the life history of cells of the nucleus pulposus. Aside from its overt role in adapting cells to the microenvironment within the disc, it appears to play a survival role in protecting cells of the nucleus pulposus from the induction of apoptosis as well as regulating the expression of one and possibly more components of the extracellular matrix. Experiments are now in progress to learn if fibrillar proteins are regulated by this transcription factor and whether the osmotic environment promotes induction of water transporting proteins in the disc.

REFERENCES


FOOT NOTES

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FIGURE LEGENDS
**Fig. 1.** A) Expression level of TonEBP and other osmotically active genes in the intervertebral disc and other rat tissues. mRNA was extracted from disc tissue, costochondral cartilage, heart, kidney and liver of adult rats, and subjected to RT-PCR analysis. Note, there is robust expression of TonEBP and its target genes: heat shock protein-70 (HSP-70), betaine/GABA co-transporter-1 (BGT-1) and sodium myoinositol transporter (SMIT) mRNA. Cartilage, another aggregan rich skeletal tissue contains lower levels of TonEBP mRNA than the disc. Kidney maximally expresses TonEBP as well as the target genes. B-G) Saggital and longitudinal sections of disc tissue from neonatal (B) and mature rat (D and F) spines that were treated with anti-TonEBP antibody, or counter stained with alcian blue, eosin and propidium iodide (C, E, G). Note that nucleus pulposus cells in the neonatal (B) as well as skeletally mature disc cells (D) express TonEBP protein; much of the staining is localized to the nucleus (B; arrows). Some staining is also evident in the cytosol of the nucleus pulposus cells of mature discs (D; arrow head). Furthermore, annulus fibrosus cells localized in a narrow zone of alcian blue positive matrix (G; arrow head) express TonEBP protein (F; arrow). Isotype and secondary antibody controls were negative (not shown). Mag. X 20

**Fig. 2.** A) Influence of osmolarity on expression of TonEBP and its target genes by rat nucleus pulposus cells. Following isolation from the disc, nucleus pulposus cells were maintained in culture in hyperosmolar media (400-550 mOsm/kg). The expression of TonEBP and target genes was evaluated by RT-PCR. An increase in medium osmolarity from isotonic to 400 mOsm/Kg results in an elevation in TonEBP, TauT, SMIT, BGT-1, and HSP-70 mRNA levels. Except for HSP-70, further increases in medium osmolarity did not elevate any of the expressed genes. B) Western blot analysis of the expression of TonEBP protein by nucleus pulposus cells at 330-500 mOsm/kg. Most of the TonEBP protein is present in the nuclear fraction of the nucleus pulposus cells. An increase in medium osmolarity from isotonic to 400 and 500 mOsm/kg results in a robust increase in TonEBP protein in the nuclear fraction. Under hypertonic conditions (400 mOsm/kg), there is also a small increase in the cytosolic level of TonEBP, while at 500 mOsm/kg all the protein is localized to the nucleus.

**Fig. 3.** A, B) Evaluation of TonEBP responsive promoter activity in nucleus pulposus cells cultured in hypertonic medium. TauT (A) or HSP-70 (B) reporter plasmids containing a wild type (Wt) or a mutant (Mt) TonE, driving firefly luciferase, were transfected into rat nucleus pulposus cells along with pRL-TK vector. Cells were cultured in isotonic (330 mOsm/kg) or hypertonic (400 or 500 mOsm/kg) media for 24 h and luciferase reporter activity was measured. When the medium osmolarity is raised to 400 mOsm/kg there is a 5 fold increase in TauT reporter activity. At 500 mOsm/kg there is a further doubling of activity. HSP-70 reporter activity does not change until the osmolarity of the medium is increased to 500 mOsm/kg, it then increases 5-6 fold. Cells transfected with (A) mutant TauT or (B) HSP-70 plasmids (Mt) display a very low luciferase activity. Values shown are mean ± SD, of 3 independent experiments; *p<0.05. C) Electromobility shift assay to examine the functional binding of TonEBP protein to TonE. Nuclear protein was isolated from rat nucleus pulposus cells cultured under isotonic or hypertonic conditions. 10 μg of nuclear protein was incubated with 3 fmol biotin-end labeled double stranded oligonucleotide probes containing the TonE motif in the TauT promoter. Binding was resolved on a polyacrylamide gel, transferred to a Nylon membrane and detected using chemiluminescence. Under hypertonic conditions, there is more pronounced binding of TonEBP protein to the DNA probe. When a probe containing mutant TonE site was used in the binding reaction, there is no or very little binding. Furthermore, the mutant competitor probe did not compete with the binding of TonEBP to labeled wild type TonE probe, confirming the specificity of the interaction.

**Fig. 4.** A-D) TonEBP regulation of TauT and HSP-70 reporter activity in nucleus pulposus cells. Rat nucleus pulposus cells were co-transfected with TauT (Wt) or HSP-70 (Wt) reporter plasmids along with either pFLAG-DN-TonEBP or pFLAG-TonEBP or pFLAG-CMV2 (empty vector). The cells were cultured in isotonic or hypertonic (500 mOsm/kg) medium for 24 h and reporter activity was measured. Under hypertonic conditions, cells receiving TauT (A) or HSP-70 (B) reporter plasmid (along with empty
vector) show an approximate 6 and 3 fold induction in luciferase activity respectively. When DN-TonEBP is co-transfected with the reporters, there is a significant suppression of activity. There is significant suppression of TauT promoter activity (A) with 50 ng DN-TonEBP plasmid which is further suppressed at 100 ng. Further increases in DN-TonEBP plasmid concentration did not change TauT reporter activity. When cells transfected with TonEBP expression vector (pFLAG-TonEBP) are compared to cells transfected with empty backbone vector (pFLAG) along with either TauT (C) or HSP-70 (D) a significant induction in promoter activity is seen. Data shown is mean ± SD of 3 independent experiments performed in triplicate (n =3); *p < 0.05. E) Western blotting (Top panel) and immunofluorescence microscopic (bottom panel) analysis of expression of FLAG-fusion protein in nucleus pulposus cells. Cells were transfected with FLAG-DN-TonEBP or did not receive any plasmid (non-transfected control). Whole cell lysates were prepared, resolved on acrylamide gel blotted and probed with antibodies against TonEBP, FLAG and β-actin. Both transfected and non-transfected cells express similar levels of native TonEBP protein. However, only the transfected cells express FLAG fusion protein. When the cells were fixed and reacted with anti-FLAG antibody, there is evidence of cytoplasmic localization of the FLAG signal (E. bottom panel) Original Mag. X 40.

**Fig. 5.** Phenotype and TauT reporter activity of TonEBP silenced rat nucleus pulposus cells. A) Rat nucleus pulposus cells were transfected with plasmid DNA containing SiRNA sequences and G418 resistant cells were selected. RT-PCR analysis of TonEBP mRNA levels in SiRNA transfected nucleus pulposus cells show a 2 fold reduction in TonEBP mRNA abundance; C: Control cells; M: marker. B) SiRNA and control cells were cultured under isotonic and hypertonic conditions for 24 h and TonEBP protein expression was evaluated by immunofluorescence; nuclei were stained with propidium iodide (red signal). Note that SiRNA cells (top panel) exhibit a reduction in TonEBP protein level (green fluorescence) in both isotonic (left) and hypertonic (right) medium when compared to control cells (bottom panel) under isotonic (left) or hypertonic (right) conditions respectively. Note that TonEBP is mostly localized to cell nuclei. C) Quantitative image analysis of TonEBP fluorescence in control (C) and SiRNA cells in isotonic and hypertonic media. Under both isotonic and hypertonic conditions SiRNA cells exhibit significantly lower levels of fluorescence when compared to control cells. *p < 0.05 D) Relative TauT reporter activity of SiRNA cells cultured in isotonic and hypertonic media. SiRNA cells were transfected with either wild type (Wt) or mutant (Mt) TauT reporter plasmid and luciferase activity assayed after 24 h in either isotonic or hypertonic (400 mOsm/kg) media. In isotonic media, basal TauT expression was evaluated by phase contrast (top panels) and fluorescent (bottom panels) microscopy. Note, high number of DN-TonEBP expressing cells exhibit green fluorescence evidencing increased activation of caspase-3, a pro-apoptotic molecule (arrow) Mag. X 20. C) Quantitative image analysis of caspase-3 activity in nucleus pulposus cells. The figure shows that there is significant increase in PhiPhi Lux- G_1D_2 fluorescence yield in cells expressing DN-TonEBP under hypertonic conditions. *p < 0.05.

**Fig. 6.** Osmoprotective role of TonEBP in nucleus pulposus cells. A) Rat nucleus pulposus cells were transfected with DN-TonEBP and cultured under hypertonic media (500 mOsm/kg) for 24 h. Control cells received empty backbone vector. Viability of cells was evaluated by the MTT assay. Note that exposure to the hyperosmolar medium results in decreased viability of the cells expressing DN-TonEBP. B) To assess the mode of cell death, cells transfected with empty vector (FLAG-CMV) and DN-TonEBP expression vector were treated with the caspase-3 substrate PhiPhi Lux- G_1D_2 in hypertonic medium and evaluated by phase contrast (top panel) and fluorescent (bottom panel) microscopy. Note, high number of DN-TonEBP expressing cells exhibit green fluorescence evidencing increased activation of caspase-3, a pro-apoptotic molecule (arrow) Mag. X 20. C) Quantitative image analysis of caspase-3 activity in nucleus pulposus cells. The figure shows that there is significant increase in PhiPhi Lux- G_1D_2 fluorescence yield in cells expressing DN-TonEBP under hypertonic conditions. *p < 0.05.

**Fig. 7.** Regulation of aggrecan gene promoter activity by TonEBP. A) DNA sequence of the promoter region of rat and mouse aggrecan gene. TonE consensus sequence is marked in bold and underlined. B) Promoter organization of the rat aggrecan gene. The transcription start site is marked as +1. TonE sites
are shown as ovals (red) on the either side of a conserved Sox-9 binding site. C) Electromobility shift assay to examine functional binding of TonEBP to TonE motif in the rat aggrecan gene promoter. An oligonucleotide probe containing the TonE motif (-912 b) in the rat aggrecan promoter was incubated with nuclear extracts from rat nucleus pulposus cells cultured under isotonic and hypertonic (400 and 500 mOsm/kg) conditions and binding was detected using chemiluminescence. Specificity was confirmed by inclusion of excess unlabeled wild type probe or a probe containing mutation in the TonE site (Mt probe) in the binding reaction. The binding signal is significantly diminished when either a wild type competitor probe or a mutant probe is used. D) Nucleus pulposus cells were co-transfected with DN-TonEBP and aggrecan reporter plasmids. Twenty four hours after transfection, cells were cultured in isotonic medium for 24 h and luciferase activity measured. Expression of DN-TonEBP results in decreased aggrecan promoter activity compared to control cells that receive empty backbone vector. E) Aggrecan promoter construct was transiently transfected into SiRNA expressing and control cells (C) and reporter activity measured in isotonic media. Compared with control cells, the silenced nucleus pulposus cells elicit a marked reduction in aggrecan reporter activity. Data represents mean ± SD from three independent experiments, performed in triplicate (n = 3); *p < 0.05.
Figure 1
Figure 2

(A) Hypertonicity

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<th>Osm/Kg</th>
<th>TonEBP</th>
<th>TauT</th>
<th>SMIT</th>
<th>BGT1</th>
<th>HSP70</th>
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(B) Hypertonicity

<table>
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<td>Cytoplasm</td>
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<tr>
<td>500</td>
<td>Nucleus</td>
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Figure 3

(A) Fold (Luciferase activity) vs. mOsm/kg for TauT. The graph shows three conditions: 330 mOsm/kg (white), 400 mOsm/kg (gray), and 500 mOsm/kg (black). The fold change is plotted for Wt and Mt conditions.

(B) Fold (Luciferase activity) vs. mOsm/kg for HSP-70. Similar to (A), the graph shows the fold change for Wt and Mt conditions under the three osmotic conditions.

(C) Gel image showing the complex formation. The gel lanes are labeled with different osmotic conditions: 330 mOsm/kg and 500 mOsm/kg. The lanes are marked with Wt probe, Mt probe, and competitor. The figure indicates the formation of a complex under specific conditions.
Figure 4

(A) Fold (Luciferase activity) for TauT under hypertonic conditions with Vector, 50, 100, 200, 300 DN (ng).

(B) Fold (Luciferase activity) for TauT under isotonic conditions with Vector and FLAG-TonEBP.

(C) Fold (Luciferase activity) for HSP-70 under hypertonic conditions with Vector and DN-TonEBP.

(D) Fold (Luciferase activity) for HSP-70 under isotonic conditions with Vector and FLAG-TonEBP.

(E) Western blot images showing anti-TonEBP, anti-FLAG, and β-actin for Transfected (FLAG-DN) and Non-transfected conditions.

Transfected (FLAG-DN)

Non-transfected
Figure 5

(A) 

(B) 

(C) 

(D) 

Mean Fluorescence/Cell

Fold (luciferase activity)
Figure 6

(A) MTT activity (% viability)

Vector DN-TonEBP

* p < 0.05

(B) Empty vector DN-TonEBP

Phase contrast

Fluorescent

(C) PhiPhi Lux intensity/field

Vector DN-TonEBP

hypertonic
Figure 7

(A)  
```
ATTCGGGAGA TTTCCA CTGCACCTGCC  Mouse  
ATTCGGGAGA TTTCCA CTACACTGCC  Rat  
-912
```

(B)  
```
CCCACAAAAC TTTCCA ACAGTTTCT  Mouse  
CCCACAAAAC TTTCCA AACTTGTTC  Rat  
-390
```

(C)  
```
TonE  Sox-9  GR  sp1  TonE  NFκB  AP2
-1 kb  -0.5 kb  + 1
```

(D)  
```
Fold (Luciferase activity)
0.0  0.2  0.4  0.6  0.8  1.0  1.2  1.4
```

(E)  
```
Vector  DN-TonEBP
```

(F)  
```
C  SiRNA
```

Unlabeled probe  Biotin labeled probe
```
330  400  500  330  500  mOsm/Kg
```

Binding complex
```
Wt probe  Mt probe  mOsm/Kg
330  500  330  500
```

Binding complex
TonEBP/OREBP is a regulator of nucleus pulposus cell function and survival in the intervertebral disc
Tsung-Ting Tsai, Keith G. Danielson, Asha Guttapalli, Erbil Oguz, Todd J. Albert, Irving M. Shapiro and Makarand V. Risbud

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