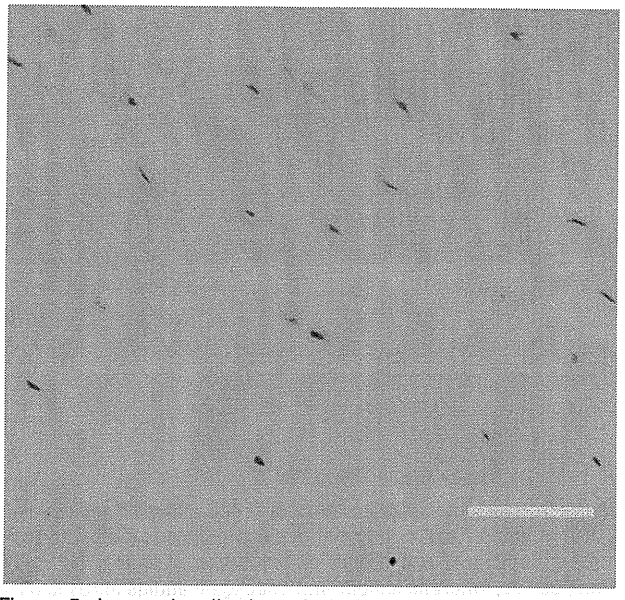

Figure 7. Immunolocalization of glypican-3 in coccygeal anulus fibrosus. Scale bar: 50 μm.

finding confirms some of the limitations of microarrays and corroborates the critical need to validate microarray data by a second methodology such as quantitative real-time RT-PCR.[27,28]

This is the first study reporting on the expression and localization of A2M, DSC2, NCAM1 (CD56), and CK18 in mature NP tissue of a non-notochordal species. Although the functional role of these proteins remains to be elucidated, the considerable differences in tissue distribution suggest that they may have specific physiologic functions in the NP. For instance, A2M is known as a potent protease inhibitor that also inhibits aggrecanases.[29] Therefore, a constitutively increased A2M expression in the NP, which is particularly rich in aggrecan, is of physiologic relevance. Desmocollins belong to a group of cadherins, and DSC2 is recognized as a membrane glycoprotein.[30] NCAM1 (CD56) is involved in contact-mediated cell interactions. Although this molecule is primarily expressed in cells of the nervous system, it has been identified in various tissues during development.[31,32] One major reason why we further examined the latter 2 molecules is that they represent cell surface molecules. In view of potential cell sorting or cell separation procedures, the expression of cell surface or cell adhesion proteins is of particular interest. Immunohistochemistry shows that both DSC2 and NCAM1 (CD56) are expressed in NP but not in AF cells, but a potential use of these proteins to identify NP cells requires further investigation. Given that DSC2 is expressed also in AC, it could, however, not separate NP from AC cells. Cytokeratin 18 has been identified in human notochordal cells during development.[33,34] Interestingly, remnants of cytokeratin-positive cells were also found in normal adult NP, although the expression of such simple epithelial cytokeratins has generally been associated with classic chordomas.[33,35]

Of the 5 genes that had shown significant expression differences between NP and AC in the rat only 2 demonstrated a similar pattern in the dog. In agreement with the rat results, both COMP and MGP expression were lower in NP than in AC also in the dog. In contrast, GPC3, which had been proposed as an NP marker in the rat, displayed no difference in the expression between NP and AC, but was enhanced in AF. Higher expression of GPC3 in AF compared to AC had already been observed in mature rats. Recently, it was reported that during human development, GPC3 was expressed in a highly tissue- and stage-specific manner.[36] Hence, the substantial discrepancy between rat and dog NP might be related to differences in the development of the NP tissues. Moreover, PTN also showed an opposite trend in the dog compared to the rat, with higher expression levels in AC than in NP and AF. Hence, findings from one species may or may not apply to another species and need to be confirmed for a specific molecule or situation. The observed differences may partly be related to the fact that rat discs are considered as notochordal, whereas discs from the beagle dogs are non-notochordal.[23]

Caudal discs are frequently used in animal models. Although similarities in biochemical and biomechanical properties have been found among species and between caudal and lumbar discs,[37,38] the limitations of the use of caudal discs need to be taken into consideration.[39] With respect to the genes and proteins investigated in this study, the general expression pattern was similar in lumbar and caudal discs. Nevertheless, certain variations such as the enhanced gene expression of NCAM1 (CD56) in the caudal NP, that was confirmed at the protein level, and of PTN and VIM in the caudal AF are evident.

In conclusion, this study reports on the expression of distinct molecules that have not been described previously in the IVD, in non-notochordal discs comparable with human discs. Tissue-specific differences in expression profiles may qualify some molecules as markers, although further studies will be required. In particular, considerable interspecies differences were noted when comparing rat and dog tissues, whereas the variations between disc levels (caudal *vs.* lumbar) were less significant. It might be speculated that the NP phenotype of the beagle as a chondrodystrophoid dog breed is more similar to the human than the NP of species whose discs do not naturally degenerate; however, a comparison with human IVD will be necessary to confirm this statement with respect to the expression profile of specific genes and proteins. Finally, while the present study only addressed normal disc gene expression profiles, there is also a need for comparing profiles between healthy and degenerated discs. Nevertheless, in view of the limited availability of healthy human IVD tissues, studies on species with similar characteristics – such as chondrodystrophoid dogs – largely contribute to a better understanding of the IVD and its relevant cell types. This

knowledge is of substantial value for the development of new treatment strategies for IVD diseases.

Key Points

- Phenotypic differences between dog nucleus pulposus and anulus fibrosus were investigated by large scale gene expression profiling.
- Variations were noted in relative expression levels between lumbar and coccygeal discs.
- Immunohistochemistry confirmed gene expression differences at the protein level.
- There were considerable divergences comparing rat and non-notochordal dog discs that are more similar to human discs.

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Osmolarity and Intracellular Calcium Regulate Aquaporin2 Expression Through TonEBP in Nucleus Pulposus Cells of the Intervertebral Disc

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ABSTRACT: The goal of this study was to examine the expression and regulation of aquaporin2 (AQP2), a tonicity-sensitive water channel in nucleus pulposus cells of the intervertebral disc. We found that AQP2 protein was expressed in vivo in both rat and human discs. We determined whether AQP2 promoter expression was regulated by osmolarity in a tonicity enhancer binding protein (TonEBP)-dependent manner. When TonEBP was suppressed under hypertonic conditions or overexpressed under isotonic conditions, AQP2 promoter activity was correspondingly inhibited or induced. The role of TonEBP in controlling AQP2 expression was confirmed using mouse embryonic fibroblasts (MEFs) derived from TonEBP-null mice. We studied whether calcium in addition to osmolarity played a role in regulation of AQP2 in nucleus pulposus cells. We also determined whether both TonEBP and calcineurin–nuclear factor of activated T cells (NFAT) signaling contributed to ionomycin, a calcium ionophore, mediated induction of AQP2. Co-transfection of AQP2 reporter with calcineurin (CnA/B) and/or NFAT1–4 vectors suggested that this pathway did not control AQP2 promoter activity in nucleus pulposus cells. These findings were also validated using MEFs from TonEBP, fibroblasts from CnAα- and CnAβ-null mice, and mutant TonE reporter constructs. Results of these studies suggest that, in nucleus pulposus cells, osmotic pressure and calcium modulate AQP2 expression through TonEBP and are independent of the calcineurin–NFAT pathway. Because calcium flux reflects a change in applied stress, the possibility exists that NFAT5/TonEBP modulate not just water balance in the disc but also accommodate applied biomechanical forces.
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Key words: intervertebral disc, nucleus pulposus, aquaporin2, tonicity enhancer binding protein, osmolarity, calcium, nuclear factor of activated T cells

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INTRODUCTION

THE INTERVERTEBRAL DISC is a specialized biomechanical structure that permits movement between adjacent vertebrae and accommodates applied compressive forces. It consists of an outer ligamentous annulus fibrosus that encloses a gel-like tissue, the nucleus pulposus. Whereas sparse, cells in the nucleus pulposus secrete a complex extracellular matrix that primarily contains collagen type II and the proteoglycan aggrecan. The numerous charged glycosaminoglycan side-chains of the aggrecan molecule interact with cations, thereby raising the osmolarity and the water content of the disc tissues.[1–4] The unique hydration properties of the nucleus pulposus promotes dynamic loading and unloading, permitting the spine to contain large shifts in biomechanical forces. However, the mechanisms by which these cells accommodate changes in osmolarity and fluid flow have received little attention.

One of the primary responses of disc cells to variations in local osmolarity is a change in regulatory cell volume. Disc cells and chondrocytes alike adapt to these osmotic shifts by remodeling their cytoskeleton and by catalyzing the transport of osmotically active molecules and water across the plasma membrane.[5–8] Water transport across the cell membrane is regulated by a large family of channel-forming proteins: the aquaporins (AQPs).[9] AQP2, an arginine vasopressin regulated channel, plays an important role in water reabsorption by connecting tubules and collecting ducts of the kidney.[10] When activated, phosphorylation of critical serine residues in AQP2 results in its translocation from cytoplasmic vesicles to the apical membrane. Intercalated with membrane proteins, AQP2 enhances water influx into the cell.[10] Recent studies have suggested that, in the kidney, expression of AQP2 is regulated by tonicity enhancer binding protein (TonEBP), also called nuclear factor of

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activated T cells (NFAT)5.[11–13] Studies by Li et al.[12] suggested that calcium ions with calcineurin–NFAT participate in regulation of AQP2 expression. Related to the functional importance of this system, Pritchard et al.[5] have documented the presence of calcium transients in disc cells exposed to osmotic stress. Whether the NFAT5 calcineurin signaling system serves to adapt nucleus pulposus cells to these transients has not been delineated.

The objective of this study was to determine the role of AQP2 in regulating the hydration status of cells of the intervertebral disc. Specifically, we asked the following question: do the nucleus pulposus cells express AQP2 and is their expression regulated by disc osmolarity and calcium? Results of this study clearly show that, in both the rat and the human, nucleus pulposus cells express AQP2 protein. Importantly, our data indicates that osmotic pressure and calcium modulate AQP2 expression through TonEBP and is independent of the calcineurin–NFAT signaling pathway. This finding lends credence to the view that, by regulating the hydration status of the disc, TonEBP maintains cell function in a hyperosmotic mechanically stressed environment.

MATERIALS AND METHODS

Reagents and plasmids

Rabbit polyclonal TonEBP antibody was a kind gift from Dr. H. Moo Kwon, University of Maryland. Wildtype (WT) AQP2, and mutant (MT) AQP2 luciferase reporters were provided by Dr. Feng Chen, Washington University, St. Louis, MO, USA.[12] Plasmids were kindly provided by Dr. Takashi Ito, Osaka University, Osaka, Japan (taurine transporter [TauT] [WT], TauT [MT] reporter),[14] Dr. Ben C. Ko, University of Hong Kong, China (FLAG-DN-TonEBP, FLAG-TonEBP, and FLAG-CMV2),[15] Dr. Gerald Crabtree, Stanford University (CnA and CnB), Dr. Jeffery Molkentin, Cincinnati Children's Hospital Medical Center (NFAT4 and pECE), and Dr. Tania Crotti, Harvard Institutes of Medicine (NFAT2). Plasmids for NFAT1 (11100) and CA-NFAT2 (11102), developed by Dr. Anjana Rao,[16] NFAT3 (10961), developed by Dr. Toren Finkel,[17] and 3xNFAT-Luc (17870), developed by Dr. Gerald Crabtree,[18] were obtained from Addgene (Cambridge, MA, USA). As an internal transfection control, vector pRL-TK (Promega) containing *Renilla reniformis* luciferase gene was used. The amount of transfected plasmid, the pretransfection period after seeding, and the post-transfection period before harvesting were optimized for rat nucleus pulposus cells using pSV β-galactosidase plasmid (Promega).[19]

Human tissue specimens

Human tissues were collected as surgical waste during spinal surgical procedures. In line with Thomas Jefferson University's Institutional Review Board guidelines, informed consent for sample collection was obtained for each patient. Assessment of the disease state was performed using the modified Thompson grading.[20]

Immunohistological studies

Freshly isolated rat spines or human discal tissues were immediately fixed in 4% wt/vol paraformaldehyde in PBS and embedded in paraffin. Transverse and coronal sections, 6–8 μm in thickness, were deparaffinized in xylene and rehydrated through graded ethanol. A few sections were stained with alcian blue, eosin, and hematoxylin. For localizing AQP2, sections were incubated with the anti-AQP2 antibody (Alamone Laboratories, Haifa, Israel, or Calbiochem) in 2% wt/vol BSA in PBS at a dilution of 1:200 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) for 10 min at room temperature or biotinylated goat anti-rabbit (1:200) for 45 min. Sections were incubated with a streptavidin/peroxidase complex for 5 min and washed with PBS, and color was developed using 3′-3-diaminobenzidine (Vecta Stain Universal Quick Kit; Vector Laboratories). AQP2-labeled human tissue sections were counterstained with hematoxylin. Preimmunne rabbit IgG (1:200) was used as an isotype control.

Isolation of rat nucleus pulposus cells

Nucleus pulposus cells were isolated from the rat spine using the method reported earlier[19,21] and approved by the Institutional Animal Care Committee of Thomas Jefferson University. Briefly, male Wistar rats, (250 g) were killed with CO_2, and lumbar intervertebral discs were removed from the spinal column. The gel-like nucleus pulposus was isolated, using a dissecting microscope, and treated with 0.1% wt/vol collagenase and 10 U/ml hyaluronidase for 4–6 h. This procedure partially digested the tissue and thereby enhanced the subsequent release of cells trapped in the dense matrix. The partially digested tissue was maintained as an explant in DMEM and 10% FBS supplemented with antibiotics. Nucleus pulposus cells migrated out of the explant after 1 wk. When confluent, the cells were lifted using a trypsin (0.25%) EDTA (1 mM) solution and subcultured in 10-cm dishes.

Knockout cells

TonEBP/NFAT5 wildtype and null mouse embryonic fibroblasts (MEFs; originally from Dr. Steffan N. Ho), were provided by Dr. Feng Chen, Washington University, St. Louis, MO, USA.[12] Primary medullary fibroblasts derived from CnAα-null, CnAβ-null, and CnA wildtype mice were provided by Dr. Jennifer Gooch, Emory University. These cells were used as control cells for some experiments.

Immunofluorescence microscopy

Cells were plated in flat-bottomed 96-well plates (5000 cells/well). After 24-h culture, cells were fixed with 4% vol/vol paraformaldehyde, permeabilized with 0.2% Triton-X 100 in PBS for 10 min, blocked with PBS containing 5% vol/vol FBS, and incubated with anti-AQP2 (1:200; BD Biosciences) antibody at 4°C overnight. As a negative control, cells were reacted with isotype IgG under similar

conditions. After washing, the cells were incubated with Alexa fluor-488–conjugated anti-mouse secondary antibody (Molecular Probes, St. Louis, MO, USA), at a dilution of 1:50 for 1 h at room temperature. Cells were washed and imaged using a laser scanning confocal microscope (Fluoview; Olympus).

Real-time RT-PCR analysis

At the end of treatment, total RNA was extracted from nucleus pulposus cells using RNAeasy mini columns (Qiagen). Before elution from the column, RNA was treated with RNase free DNase I. One hundred nanograms total RNA was used as template for real-time PCR analysis. Reactions were set up in microcapillary tubes using 1 μl RNA with 9 μl of a LightCycler FastStart DNA Master SYBR Green I mix (Roche Diagnostics, Indianapolis, IN, USA), to which gene-specific forward and reverse PCR primers were added (AQP2: NCBI NM_012909; forward: 5′-tgtcaatgctctccacaacaacgc-3′: 435–459 bp, reverse: 5′-aaacttgccagtgacaactgctgg-3′: 655–678 bp). Each set of samples included a template-free control. PCR reactions were performed in a LightCycler (Roche) according to the manufacturer's instructions. All the primers used were synthesized by Integrated DNA Technologies(Coralville, IA, USA).

Western blotting

Total cell lysates were resolved on 8% wt/vol or 10% wt/vol SDS-polyacrylamide gels. Proteins were transferred by electroblotting to PVDF membranes (Bio-Rad). The membranes were blocked with 5% wt/vol nonfat dry milk in TBST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% vol/vol Tween 20) and incubated overnight at 4°C in 3% wt/vol nonfat dry milk in TBST with antibodies against AQP2 (Alamone Laboratories or BD Biosciences, 1:1000), β-tubulin (1:1000; DSHB, Iowa City, IA). Immunolabeling was detected using the ECL reagent (Amersham Biosciences).

Transfections and dual luciferase assay

Nucleus pulposus cells or WT and null MEFs were transferred to 24-well plates at a density of 5.0–6.0 × 10^4 cells/well 1 day before transfection. LipofectAMINE 2000 (Invitrogen) or Transgater (America Pharma Source, Gaithersburg, MD, USA) was used as a transfection reagent. For each transfection, desired concentrations and combination of plasmids were premixed with the transfection reagent. In some experiments, 24 h after transfection, cells were cultured in hypertonic media (410–500 mosmol/kg) or treated with ionomycin (1 μM) and phorbol myrystate acetate (PMA) (100 ng) with or without FK506 (10 ng/ml) and cyclosporine A (1 μg/ml) or BAPTA-AM (10 μM). 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 analyses were carried out in triplicate.

Statistical analysis

All measurements were performed in triplicate; data are presented as mean ± SD. Differences between groups were analyzed by Student's *t*-test ($p < 0.05$).

RESULTS

Sagittal sections of the neonatal rat (Fig. 1A) and degenerate human discs (Figs. 1C and 1D) were stained with an antibody to AQP2 or isotype control (Fig. 1E). Some sections were also counterstained with H&E and alcian blue for morphology assessment (Fig. 1B). AQP2 is expressed by cells of the nucleus pulposus in rat and human discs (Figs. 1A, 1C, and 1D). In both cases, staining is localized to the plasma membrane. In human tissue, some cells showed an intense labeling, whereas others showed weaker AQP2 expression. Moreover, staining is also seen in the cytosol of human nucleus pulposus cells (Figs. 1C and 1D). Whereas some variability in staining is observed in the human tissue sections, this probably is caused by the degree of degeneration of the disc, inherent biological variations between individuals, and heterogeneity in cell populations within the tissue. There is weak expression of AQP2 protein in the inner annulus fibrosus cells of the rat disc (data not shown). In addition, we studied expression of AQP2 in cultured rat nucleus pulposus cells using immunofluorescence microscopy. Similar to the native tissues, nucleus pulposus in culture express AQP2 protein (Fig. 1F).

We probed expression levels of AQP2 in native rat disc tissues using Western blot analysis. Figure 1G indicates that nucleus pulposus tissue expresses a prominent 40- to 42-kDa band and a weaker 29-kDa AQP2 band, whereas only the 42-kDa AQP2 band was detectable in rat annulus fibrosus tissue. Moreover, the expression level of AQP2 in nucleus pulposus tissue is significantly higher than the annulus fibrosus as seen from densitometric analysis (Fig. 1H). Similar to native tissue, cultured rat disc cells express AQP2 protein and exhibit a similar pattern of expression (Fig. 1I).

We next examined the effect of osmolarity on expression of AQP2 in nucleus pulposus cells. When cells are cultured under hypertonic conditions (450 mosmol/kg), there is a time-dependent increase in AQP2 mRNA expression (Fig. 2A). Compared with isoosmotic expression, AQP2 mRNA increase is significantly different as early as 8 h and is further increased at 24 h. Hypertonicity resulted in a concomitant increase in AQP2 protein levels (Figs. 2B and 2C). To study the effect of tonicity on the regulation of AQP2 in nucleus pulposus cells, we used a 0.6-kb rat AQP2 luciferase wildtype reporter construct and a construct containing mutations in TonEBP and NFAT binding sites (Fig. 2D). Culture of nucleus pulposus cells in hypertonic media results in an osmolarity-dependent increase in activity of the wildtype AQP2 reporter (Fig. 2D). Predictably, hypertonicity failed to induce a rise in the activity of the mutant AQP2 construct. In a parallel study, we measured the effect of hyperosmolarity on TonEBP protein expression in nucleus pulposus cells. As expected, a robust

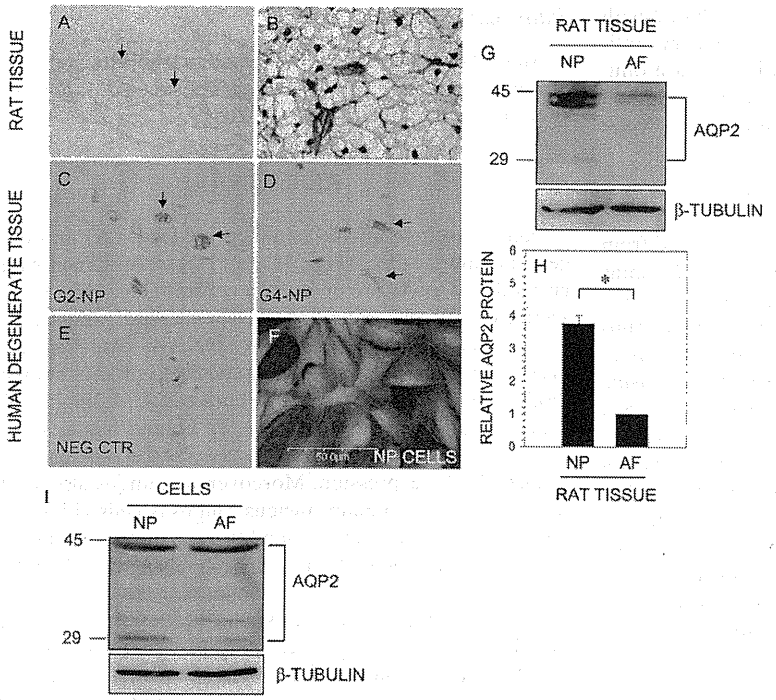

FIG. 1. AQP2 is expressed by the disc tissue. Sections of the intervertebral disc of neonatal rat (A and B) and human (C–E) stained with an antibody against AQP2. Sections were treated with anti-AQP2 antibody (A, C, and D), stained with H&E and alcian blue (B), or treated with isotype antibody (E). Note that nucleus pulposus (NP) cells express AQP2 protein (A, C, and D; arrows) Magnification, ×20 and ×40. (F) Immunofluorescent detection of AQP2 in cultured NP cells. Cells were treated with an antibody to AQP2, and cell nuclei were stained with propidium iodide (PI). NP cells express AQP2. Magnification, ×20. (G) Western blot analysis of AQP2 expression by NP and annulus fibrosus (AF) tissue. Note, strong expression of the 42-kDa AQP2 band in tissue extracts. (H) Multiple blots were quantified by densitometric analysis. The native NP tissue expressed higher AQP2 protein levels than AF tissue. (I) AQP2 expression by cultured NP and AF cells. Cellular proteins were separated by SDS-PAGE and analyzed by Western blot using an antibody to AQP2 and β-tubulin. Note the prominent expression of AQP2 by NP and AF cells.

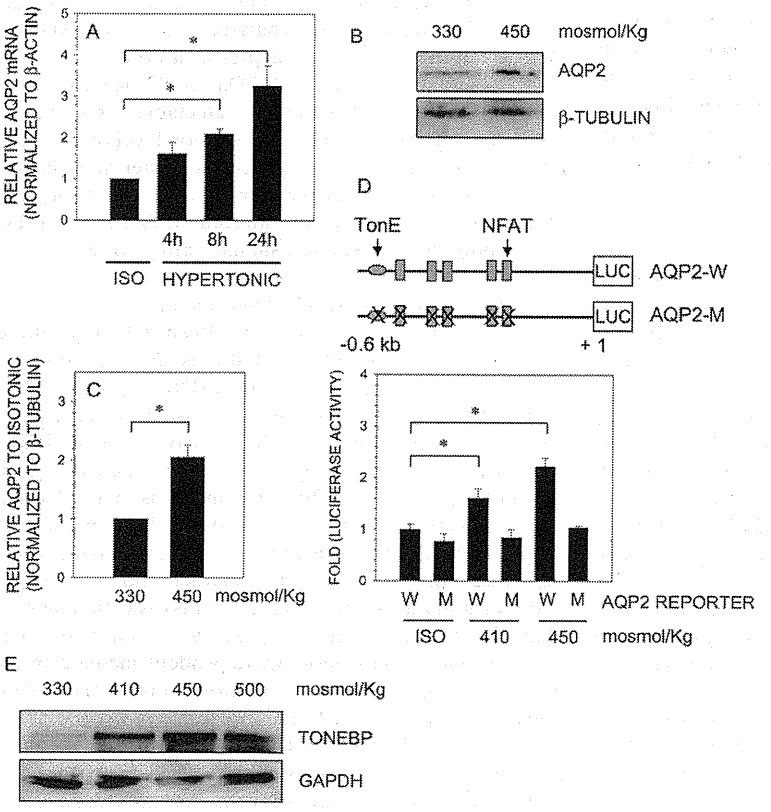

FIG. 2. Effect of osmolarity on AQP2 expression in nucleus pulposus cells. Cells were cultured in hypertonic medium (450 mosmol/kg) for 4–24 h, and AQP2 expression was analyzed. (A) Real-time RT-PCR analysis of AQP2 expression indicated a time-dependent increase in mRNA expression under hypertonic conditions. (B) Western blots analysis showed there was osmotic induction in AQP2 protein expression in NP cells. (C) Multiple Western blots of AQP2 quantified by densitometric analysis. Note NP cells exhibit induction in AQP2 levels under hypertonic conditions (D) Cartoon showing AQP2 promoter constructs used for transfections. Mutant construct (AQP2-M) contains point mutations in TonEBP and all NFAT binding motif, whereas wildtype (AQP2-W) construct contains native site. NP cells were transfected with wildtype and mutant AQP2 reporter constructs along with pRL-TK vector. Cells were cultured under isotonic or increasingly hypertonic (410–450 mosmol/kg) conditions for 24 h, and luciferase reporter activity was measured. NP cells showed an osmolarity-dependent increase in wildtype promoter activity. Mutant vector did not change its activity under hypertonic conditions. (E) Western blot analysis of the expression of TonEBP protein by NP cells at 330–500 mosmol/kg. An increase in medium osmolarity from isotonic to 500 mosmol/kg resulted in a robust increase in TonEBP protein. Quantitative data represents mean ± SD of three independent experiments ($n = 3$); *$p < 0.05$; ns = nonsignificant.

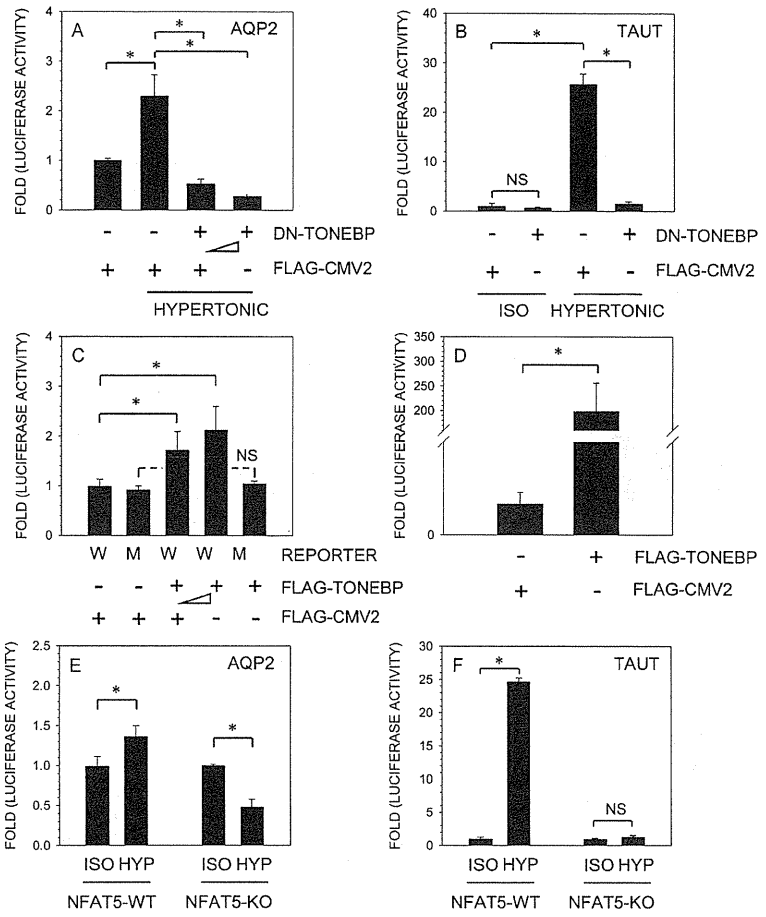

FIG. 3. TonEBP regulation of AQP2 promoter activity. AQP2 (A) or TauT (B) reporter constructs along with DN-TonEBP, or empty backbone FLAG-CMV2, were transfected into NP cells, and luciferase activity was measured. Expression of DN-TonEBP resulted in a complete suppression of hypertonicity-mediated induction in AQP2 and TauT promoter activity. When pTonEBP was co-expressed with AQP2 (C) or TauT (D) under isotonic conditions, there was a significant increase in activity of both the reporters in NP cells. Note, the activity of mutant AQP2 reporter was unaffected by TonEBP. NFAT5-null and wildtype MEFs were transfected with AQP2 (E) or TauT (F) reporter and cultured under isotonic or hypertonic conditions (500 mosmol/kg). Wildtype cells evidenced an increase in activity of both the reporters under hypertonic conditions. In contrast, in hypertonic media, null MEFs showed a significant suppression of AQP2 reporter activity, whereas TauT activity remained unchanged. Values shown are the mean ± SD of three independent experiments performed in triplicate, $*p < 0.05$.

osmolarity-dependent increase in TonEBP protein expression is evident (Fig. 2E).

To study whether TonEBP is needed for the induction of AQP2 promoter activity, nucleus pulposus cells were transiently co-transfected with plasmids encoding DN-TonEBP and full-length TonEBP. Figure 3 shows that forced expression of DN-TonEBP completely abolishes hypertonic induction of AQP2 (A) and TauT (B) promoter activity. When the medium is hypertonic, the level of AQP2 activity in cells transfected with DN-TonEBP is lower than expression under isotonic condition. A significant inhibitory effect of DN-TonEBP expression on AQP2 reporter activity is seen at a dose of 100 ng, which is further enhanced when the concentration of the DN-TonEBP is increased to 300 ng (Fig. 3A). On the other hand, overexpression of TonEBP under isotonic conditions, using pFLAG-TonEBP vector, results in a dose-dependent increase in AQP2 (Fig. 3C) and TauT (Fig. 3D) promoter activities. We used MEFs derived from TonEBP/NFAT5-null and wildtype mice to further validate the inductive effect of TonEBP on AQP2. Figure 3E shows that hypertonicity results in a small but significant increase in AQP2 promoter activity in wildtype cells. Interestingly, under hypertonic conditions, there is an ~50% decrease in AQP2 promoter activity in TonEBP-null cells. As expected, hypertonicity causes a significant increase (20- to 25-fold) in TauT reporter activation in wildtype cells, whereas in null MEFs, activity did not change significantly (Fig. 3F).

To ascertain whether calcineurin signaling plays role in AQP2 expression in nucleus pulposus cells, we treated cells with the calcium ionophore, ionomycin, together with PMA. This chemical cocktail activates calcineurin-mediated signaling. Figures 4A and 4B show that treatment with ionomycin results in increased AQP2 mRNA (Fig. 4A) and protein (Fig. 4B) expression. Surprisingly, inhibitors of calcineurin signaling, FK506 and cyclosporine A (CsA), did not diminish AQP2 protein levels (Fig. 4B). To confirm these findings, we transfected nucleus pulposus cells with wildtype or mutant AQP2 reporters and measured their activity after ionomycin treatment. Figures 4C and 4D show that ionomycin treatment results in an increase in wildtype (Fig. 4C) but not mutant (Fig. 4D) AQP2 promoter activity. Inclusion of FK506 and CsA did not block ionomycin mediated induction in AQP2 promoter activity. However, treatment with the calcium chelator BAPTA-AM completely suppressed inomycin-mediated induction in AQP2 reporter activity. To further validate these findings, we used NFAT5-null and wildtype MEFs and measured activities of AQP2

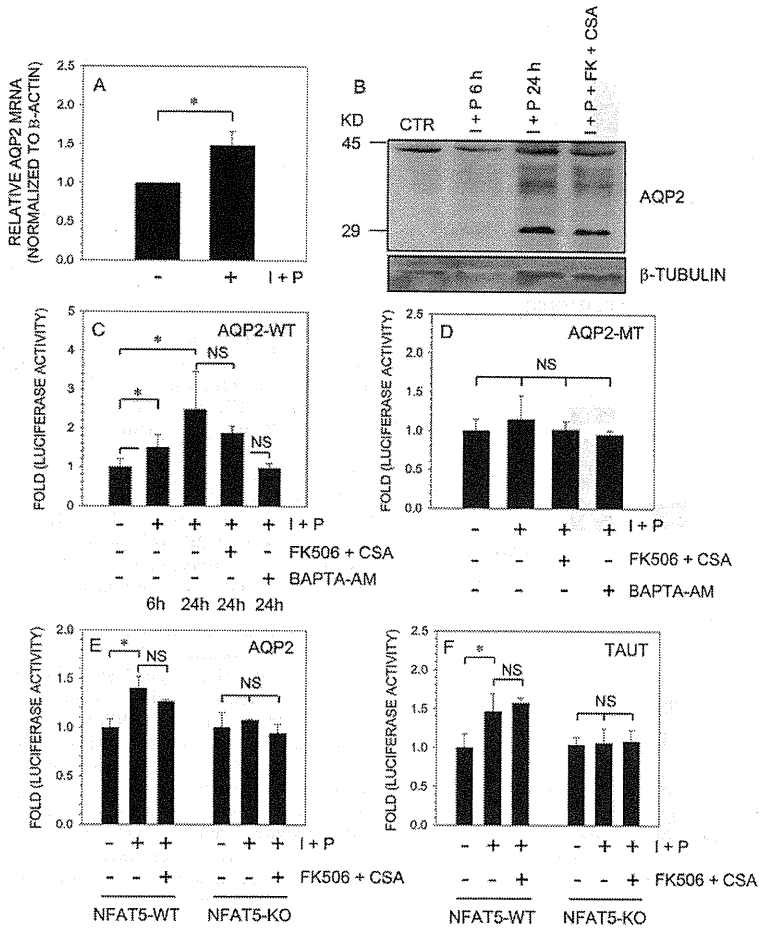

FIG. 4. Effect of calcium ions on AQP2 expression. NP cells were treated with the calcium ionophore, ionomycin (I; 1 μM), and PMA (P; 100 ng), and AQP2 expression was measured. (A) Ionomycin treatment resulted in small but significant increase in AQP2 mRNA expression. (B) After treatment, AQP2 levels were measured by Western blot analysis. Note, the increase in newly synthesized AQP2 protein (29 kDa) after ionomycin treatment. Addition of calcineurin inhibitors, FK506 (10 ng/ml) and cyclosporine A (CsA; 1 μg/ml), did not suppress synthesis of AQP2 protein. (C and D) NP cells were transfected with wildtype (C) or mutant (D) AQP2 reporter and treated with ionomycin and PMA with or without FK506 and CsA or BAPTA-AM (10 μM). Note treatment with ionomycin increased the activity of the wildtype (WT) but not the mutant (MT) AQP2 reporter. FK506 and CsA did not inhibit ionomycin-mediated increase in AQP2-WT promoter activity, whereas BAPTA-AM completely blocked the induction. The activity of AQP2 (E) or TauT (F) reporters was measured in NFAT5 wildtype (Wt) or knockout (KO) MEFs after ionomycin treatment. In contrast to the WT cells, the KO cells did not exhibit an increase in activity of both promoters after ionomycin treatment. FK506 and CsA did not block the inductive effect of ionomycin on either of the reporters in NFAT5-WT cells. Values shown are the mean ± SD of three independent experiments; $*p < 0.05$.

and TauT reporters after ionomycin treatment. Figure 4 shows that ionomycin treatment leads to an increase in both AQP2 (Fig. 4E) and TauT (Fig. 4F) reporter activities. Again, inclusion of FK506 and CsA did not affect promoter induction in wildtype cells. In contrast, in NFAT5-null cells, ionomycin did not in increase either AQP2 (Fig. 4E) or TauT (Fig. 4F) reporter activities.

To determine the role of calcineurin signaling in AQP2 expression, we co-transfected rat nucleus pulposus cells with plasmids expressing CnA and calcineurin B (CnB) along with an AQP2 reporter. Figure 5A shows that calcineurin overexpression had no measurable inductive effect on AQP2 promoter activity. We measured AQP2 reporter activity in NFAT5 wildtype and null cells after calcineurin overexpression. Again, calcineurin did not induce AQP2 reporter activity. To further evaluate whether calcineurin signaling was involved, we used primary fibroblasts derived from CnAα-null, CnAβ-null, and wildtype mice. Treatment of wildtype cells with ionomycin results in a small but significant increase in AQP2 reporter activity; again, FK506 and CsA fail to block this activation (Fig. 5C). Interestingly, in both CnAα-null and CnAβ-null fibroblasts, ionomycin treatment results in pronounced activation (2- to 3-fold) of the AQP2 reporter.

To study the relationship between AQP2 and NFAT signaling, we transfected nucleus pulposus cells with plasmids encoding NFAT1–4 and measured the activity of the AQP2 reporter. Figure 6A shows that co-expression of NFAT2, calcineurin, or both has no effect on AQP2 reporter activity. To confirm this observation, we performed a dose-response study using both NFAT2 and CA-NFAT2 expression vectors. Increasing concentration of either NFAT2 or CA-NFAT2 has no effect on AQP2 reporter activity in nucleus pulposus cells (Fig. 6B). Similarly, co-expression of NFAT1 (Fig. 6C), NFAT3 (Fig. 6D), or NFAT4 (Fig. 6E) with or without calcineurin plasmids has minimal effect on AQP2 reporter activation in nucleus pulposus cells. In a parallel experiment, we used NFAT5/TonEBP-null and wildtype MEFs and measured AQP2 reporter activity after transfection with CA-NFAT2. In both wildtype and null NFAT5 cells, CA-NFAT2 failed to increase AQP2 promoter activity (Fig. 6F). To confirm that transfected NFAT plasmids expressed functional proteins in nucleus pulposus cells, we measured activation of a NFAT responsive 3xNFAT reporter. Figure 6G shows that there is significant activation of 3xNFAT luciferase reporter when co-transfected with NFAT1 or CA-NFAT2, NFAT3, or NFAT4 expression plasmids.

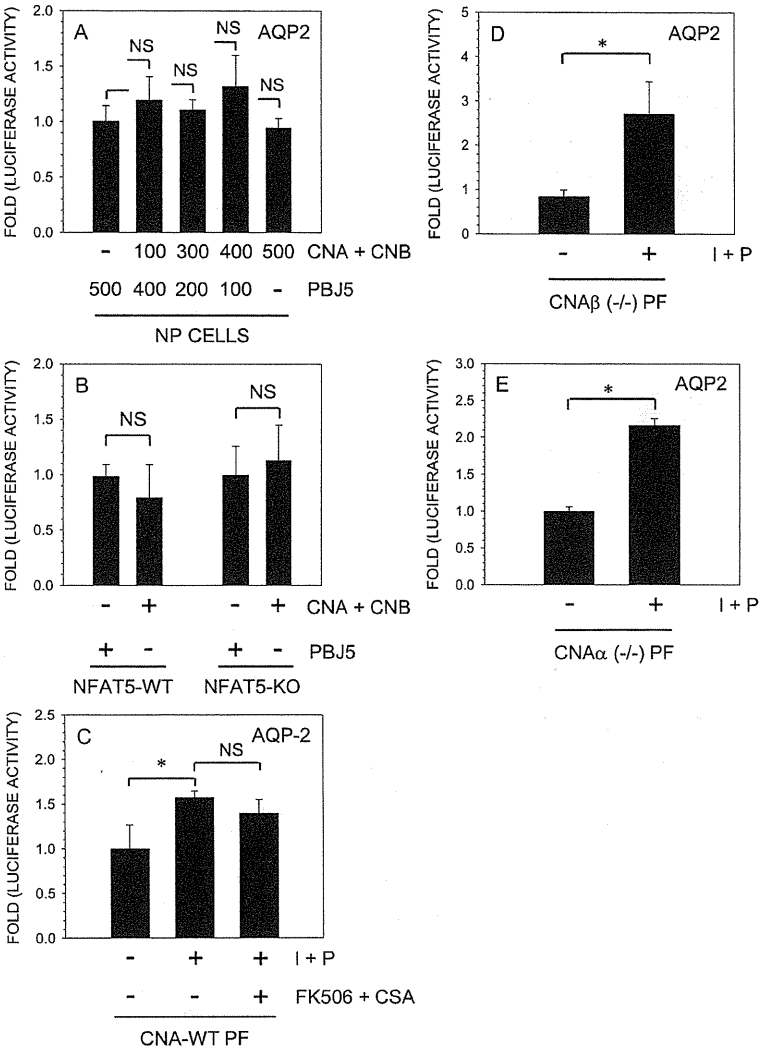

FIG. 5. Calcineurin signaling does not participate in regulation of AQP2 promoter activity in NP cells. The AQP2 reporter along with calcineurin A/B constructs or empty vector pBJ5 was transfected into NP cells (A) or NFAT5-null and wildtype MEFs (B), and luciferase activity was measured. The co-expression of calcineurin subunits did not increase AQP2 reporter activity in transfected cells. (C–E) Primary fibroblasts (PFs) derived from calcineurin wildtype (WT) or CnAα- or CnAβ-null ($^{-/-}$) mice were transfected with AQP2 reporter and treated with ionomycin and PMA with or without FK506 and CsA. Note, ionomycin significantly increased AQP2 reporter activity in both the CnAα- and CnAβ-null cells; a relatively small inductive effect was seen in wildtype cells that remained constant after addition of calcineurin inhibitors, FK506 and CsA. Values shown are the mean ± SD of three independent experiments performed in triplicate; $^{*}p < 0.05$

DISCUSSION

The experiments described in this study showed for the first time that AQP2, a tonicity-sensitive water channel protein, was expressed by nucleus pulposus cells that populate the hydrodynamically stressed, hyperosmolar microenvironment of the intervertebral disc. Moreover, AQP2 expression was regulated by TonEBP, a transcription factor that we have shown to promote aggrecan gene expression.[6] In addition, our studies showed that, aside from osmolarity, AQP2 activity in nucleus pulposus cells is dependent on intracellular calcium levels but independent of calcineurin–NFAT signaling pathways. This observation is particularly relevant to disc cell function, because calcium signaling is closely linked to the transduction of applied biomechanical forces. Accordingly, expression of TonEBP permits nucleus pulposus cells to autoregulate the osmotic environment by controlling water transport while at the same time permitting cells to accommodate to mechanical loading, a functional characteristic of the disc microenvironment.

Building on earlier observations that the cells in the nucleus pulposus exist in a unique microenvironment, we evaluated AQP expression in the discal tissues. At the mRNA and protein level, there was a robust expression of AQP2 in nucleus pulposus cells of both the neonatal and mature rat discs; the protein was also expressed by cells of the annulus fibrosus. This observation was not unexpected because the annulus, like the nucleus, is rich in proteoglycans. Moreover, because the annulus is under tension and mechanically stressed,[4] AQP2 would serve to enhance water transport in these cells. Surprisingly, we found that this protein was strongly expressed in degenerate human disc tissue. Possibly as a survival mechanism, disc cells may respond to the loss of matrix glycans that accompany tissue degradation by promoting their ability to transport water from the extracellular matrix to the cell interior. That AQP2 was expressed by the nucleus pulposus was in contrast to an earlier immunohistological study where it was reported that this protein was not expressed in the human disc.[22] Although this negative result may have been caused

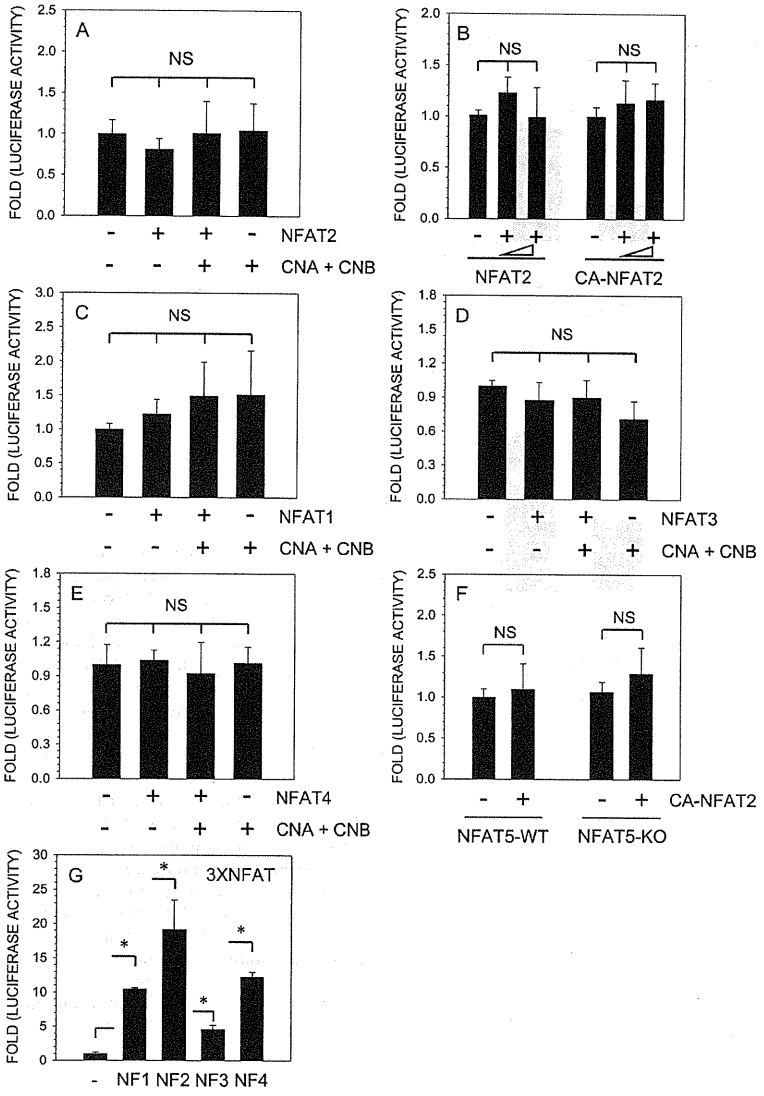

FIG. 6. AQP2 promoter activity is independent of NFAT signaling in NP cells. NP cells were transfected with NFAT vectors (NFAT2, -1, -3, -4) and/or CnA and CnB along with AQP2 reporter. (A) NFAT2 or calcineurin alone or together did not affect AQP2 reporter activity. (B) AQP2 reporter activity was not induced when co-transfected with increasing dose (200–400 ng) of either NFAT2 or CA-NFAT2 expression plasmid. NFAT1 (C), NFAT3 (D), or NFAT4 (E) alone or when added with calcineurin did not significantly change the AQP2 reporter activity in NP cells. (F) NFAT5 wildtype (WT) and null (KO) cells were transfected with AQP2 reporter with constitutively active (CA)-NFAT2 plasmid (100 ng). Note, AQP2 reporter activity in both NFAT5-WT and KO cells did not change significantly in the presence of CA-NFAT2. (G) NP cells were transfected with 3xNFAT luciferase construct with or without NFAT1 or CA-NFAT2 or NFAT3 or NFAT4, and reporter activity was measured. Co-expression of NFAT1–4 resulted in a significant increase in activity of 3xNFAT reporter plasmid indicating functionality of expressed proteins. Values shown are the mean ± SD of three independent experiments; $*p < 0.05$, ns = nonsignificant.

by an unreactive antibody, this study was able to show the expression of this protein in native rat tissue, human degenerative samples, and cells in culture using a plethora of analytical techniques. Moreover, microarray analysis by two independent groups have confirmed AQP2 mRNA expression in human nucleus pulposus and annulus fibrosus tissue (Dr. Sibylle Grad, AO Research Institute, and Dr. Helen Gruber, Carolinas Health Center, personal communications).

Gain and loss of function experiments were performed to learn whether hyperosmolarity induced transcriptional activation of AQP2 reporter activity and whether this activation was regulated by TonEBP. In hypertonic medium, suppression of TonEBP activity blocked induction of AQP2; pTonEBP enhanced AQP2 reporter expression under isotonic conditions. We used MEFs derived from TonEBP/NFAT5 wildtype and null mice to confirm these findings because of similarities in the response by nucleus pulposus cells and TonEBP wildtype MEFs to hypertonic stimulation. In hypertonic media, TonEBP-null MEFS failed to induce AQP2 promoter activity. Together, the results of these functional studies indicated that, in addition to regulating the taurine transporter (TauT), other osmolytes, and HSP-70,[6,7,14,23] TonEBP serves to adapt nucleus pulposus cells to the hypertonic conditions of the disc by controlling the expression of AQP2.

The mechanism of activation of TonEBP is not completely understood, especially whether it is mediated by direct phosphorylation of the protein.[13] There is also some evidence in T cells and kidney cells to indicate that regulation may be mediated by a phosphatase, calcineurin, which is activated by calcium ions.[24,12] The results of the studies described herein suggest that this mechanism may be more cell type specific. Treatment of nucleus pulposus cells with cyclosporine and FK506, agents that inhibit calcineurin signaling, failed to change the promoter activities

of the TonEBP target gene TauT. Further support of the notion that calcineurin signaling was not needed for TonEBP-dependent promoter activation was from studies performed on TonEBP/NFAT5-null MEFs. We noted that, after ionomycin treatment, the null cells failed to induce AQP2 and TauT reporter activity. Overexpression of calcineurin in NFAT5-null MEFs and nucleus pulposus cells did not elevate AQP2 reporter activity. Moreover, ionomycin treatment of primary fibroblasts derived from calcineurin (CnAα and CnAβ) null mice induced AQP2 reporter activity. Because of the similar response of CnA wildtype fibroblasts and nucleus pulposus cells to ionomycin treatment, fibroblasts from CnA-null mice were chosen as controls to monitor AQP2 promoter activity. These studies indicated that AQP2 activity was not dependent on calcineurin; in contrast, because ionomycin preferentially raises the intracellular calcium ion concentration, studies using BAPTA-AM, a calcium chelator, supported the notion that stimulation of AQP2 reporter activity was caused by a rise in the intracellular activity of this ion. From a functional viewpoint, the critical role of calcium ions in this transduction system is of considerable importance. Mechanical shear and loading induce calcium transients in cells of many skeletal tissues including the intervertebral disc.[5] Thus, processes that cause an increase in intracellular calcium level may well provide one mechanism by which mechanical forces influence not just membrane deformation but also signal changes in local osmotic conditions.

Whereas we have shown that ionomycin profoundly influences TonEBP-dependent promoter activity, we considered the possibility that other members of the NFAT family may be needed for AQP2 promoter activity. Results of one recent study suggested that NFATc1 or NFAT2 serves as a regulator of AQP2 transcription.[12] It is well documented that calcineurin-mediated dephosphorylation of SP motifs results in the nuclear import of NFAT and stimulation of transcription.[25] However, an increasing number of studies also suggest that inducible phosphorylation of the transactivation domain (TAD) of NFAT and other mechanisms may regulate its activity, lending support to a calcineurin-independent pathway.[26–29] To address this possibility, we overexpressed individual NFATs and determined AQP2 reporter activity. In line with previous studies,[12] we found that NFAT1, -3, and -4 did not increase AQP2 promoter activity. A lack of AQP2 induction after co-expression of constitutively active NFAT2 provided evidence that, unlike the kidney, AQP2 promoter activity is independent of NFAT2 activation in nucleus pulposus cells. That expressed NFATs were functional was confirmed by measuring activation of 3xNFAT luciferase reporter that contains three NFAT binding sites upstream of minimal interleukin (IL)-2 promoter. Based on these findings, we conclude that NFAT5/TonEBP is the major regulator of AQP2 promoter activity in cells of the nucleus pulposus.

In summary, cells of the nucleus pulposus are adapted to function in an environmental niche that has a limited oxygen supply and a high osmotic pressure. We showed that NFAT5/TonEBP regulates a critical water transport gene, AQP2. This finding, together with the observation that TonEBP governs the expression of osmolytes and osmolyte transporters, provides a new insight into mechanisms by which this transcription factor regulates the osmolarity of the nucleus pulposus cells. Analysis of other factors influencing AQP2 expression indicates that promoter activity is influenced by shifts in calcium levels. Because calcium flux reflects a change in applied stress, the possibility exists that NFAT5/TonEBP modulates not just water balance in the disc but also accommodates functional biomechanical forces.

ACKNOWLEDGMENTS

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Activation of TonEBP by Calcium Controls β1,3-Glucuronosyltransferase-I Expression, a Key Regulator of Glycosaminoglycan Synthesis in Cells of the Intervertebral Disc*

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The goal of this investigation was to study the expression and regulation of β1,3-Glucuronosyltransferase-I (GlcAT-I), a key enzyme regulating GAG synthesis in cells of the intervertebral disc. There was a robust expression of GlcAT-I in the nucleus pulposus *in vivo*. Treatment with the calcium ionophore ionomycin resulted in increased GlcAT-I expression, whereas GlcAT-I promoter constructs lacking TonE site or a mutant TonE were unresponsive to the ionophore. Experiments using TonEBP and DN-TonEBP constructs showed that TonEBP positively regulated GlcAT-I promoter activity. ChIP analysis confirmed binding of TonEBP to the promoter. We further validated the role of TonEBP in controlling GlcAT-I expression using mouse embryo fibroblasts from TonEBP null mice. GlcAT-I promoter activity in null cells was significantly lower than the wild type cells. In contrast to wild type cells, treatment with ionomycin failed to increase GlcAT-I promoter activity in null cells. We then investigated if calcineurin (Cn)-NFAT signaling played a regulatory role in GlcAT-I expression. Inhibition of Cn following ionomycin treatment did not block GlcAT-I and *tauT*, a TonEBP-responsive reporter activity. GlcAT-I promoter activity was suppressed by co-expression of Cn, NFAT2, NFAT3, and NFAT4. Moreover, following ionomycin treatment, fibroblasts from CnAα and CnAβ null mice exhibited robust induction in GlcAT-I promoter activity compared with wild type cells. Results of these studies demonstrate that calcium regulates GlcAT-I expression in cells of the nucleus pulposus through a signaling network comprising both activator and suppressor molecules. The results suggest that by controlling both GAG and aggrecan synthesis, disc cells can autoregulate their osmotic environment and accommodate mechanical loading.

The intervertebral disc is a specialized structure that permits rotation as well as flexure and extension of the human spine. It consists of an outer ligament, the annulus fibrosus, that encloses a gel-like tissue, the nucleus pulposus. Although sparse, cells in the nucleus pulposus secrete a complex extracellular matrix that contains fibrillar collagens and the proteoglycan aggrecan. Glycosoaminoglycan (GAG)[2] components of the aggrecan molecule provide a robust hydrodynamic system that serves to accommodate applied biomechanical forces (1, 2). Surprisingly, although the importance of aggrecan secretion and function has been discussed by many investigators, mechanisms of control of GAG synthesis are poorly understood.

In the nucleus pulposus, the principle GAG is chondroitin sulfate. Structurally, this molecule is a heteropolysaccharide containing repeating units of *N*-acetylgalactosamine linked to glucuronic acid. In its fully sulfated form, the molecule exhibits a high charge density, and when hydrated, it assumes a linear configuration. Bound to the aggrecan core protein and associated with hyaluronic acid, the chondroitin sulfate chains form a giant polydispersed supramolecular structure. The high osmotic pressure of the aggregate contains the forces applied to the spine (3).

In an earlier study, we showed that nucleus pulposus cells responded to changes in osmotic pressure by up-regulating the transcription factor, TonEBP (tonicity enhancer-binding protein) (4). When expressed, this protein modulated aggrecan expression; whether it can regulate chondroitin chain synthesis has not been determined. It is noteworthy that other workers have shown that galactose-β1,3-glucuronysltransferase- 1 (GlcAT-I) activity was required for GAG chain synthesis (5); hence, there is the possibility that this enzyme serves as the rate-limiting step in GAG synthesis for chondrocytes and possibly other cell types (6, 7). Related to this point, it is now known that IL-1β suppressed GAG biosynthesis by down-regulating GlcAT-I expression and activity (8). A second factor regulating aggrecan synthesis is the intracellular calcium concentration (9, 10). In smooth muscle and Sertoli cells, calcium channel blockers have been shown to alter proteoglycan synthesis (11, 12). Furthermore, using *p*-nitrophenyl-β-D-xyloside as an exogenous primer for chain initiation, it was demon-

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[2] The abbreviations used are: GAG, glycosoaminoglycan; GlcAT-I, β1,3-glucuronosyltransferase-I; NFAT, nuclear factor of activated T cells; Cn, calcineurin; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester; PMA, phorbol 12-myristate 13-acetate; DN, dominant negative; CA, constitutively active; MEF, mouse embryo fibroblast; PBS, phosphate-buffered saline; ChIP, chromatin immunoprecipitation; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase.

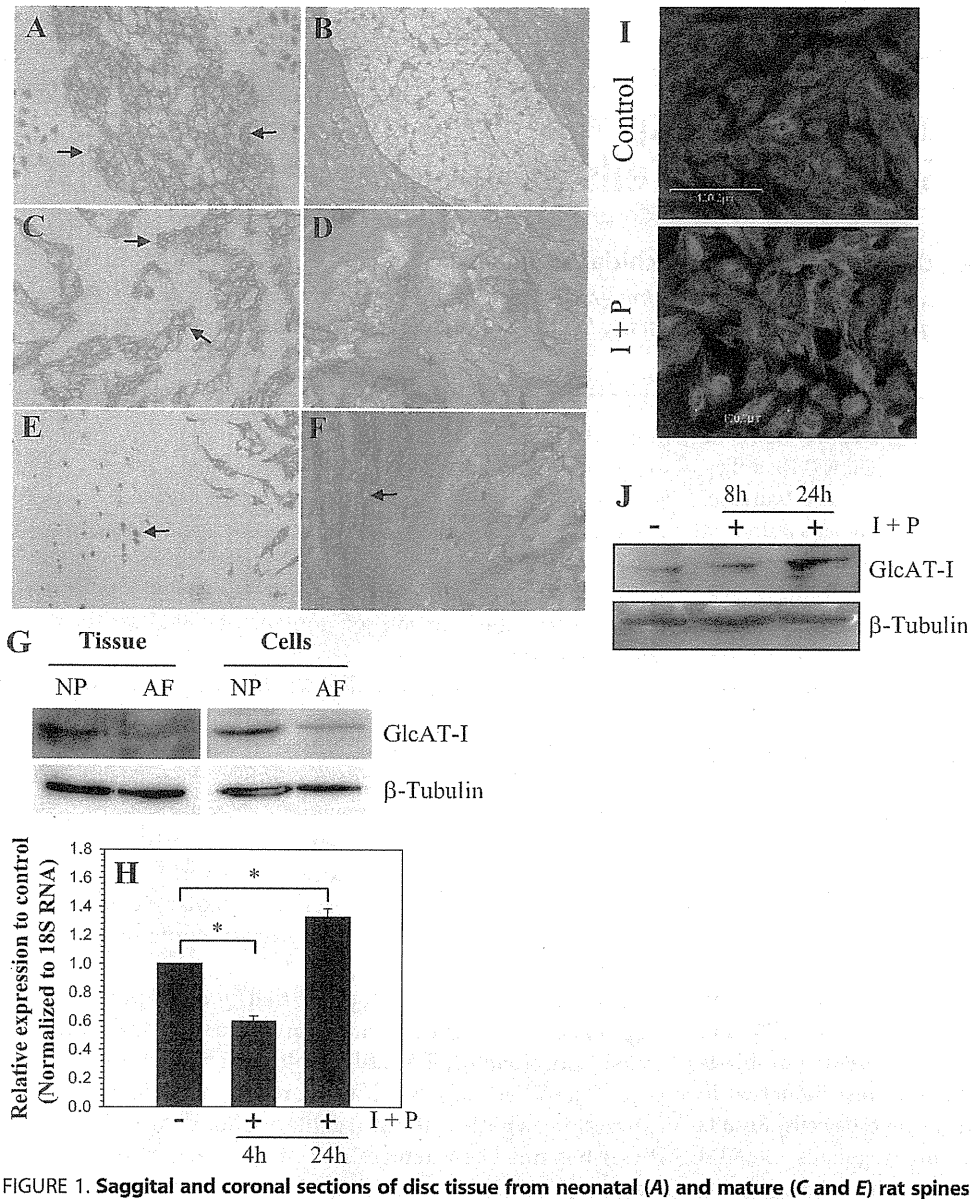

FIGURE 1. **Saggital and coronal sections of disc tissue from neonatal (*A*) and mature (*C* and *E*) rat spines stained with an antibody against GlcAT-I or stained with hematoxylin and eosin and Alcian blue (*B*, *D*, and *F*).** Note that nucleus pulposus cells in the neonatal (*A*) as well as skeletally mature disc cells (*C* and *E*) express GlcAT-I protein; much of the staining is localized to the cytosol and plasma membrane (*A* and *C*; *arrows*). Furthermore, inner annulus fibrosus cells localized in amorphous Alcian blue-positive matrix (*F*; *arrow*) express GlcAT-I protein (*E*; *arrow*). Isotype and secondary antibody controls were negative (not shown). Magnification was ×20. *G*, Western blot analysis of GlcAT-I expression by nucleus pulposus (*NP*) and annulus fibrosus (*AF*) tissue and cultured cells. Note, the expression of the 43-kDa GlcAT-I band in tissue extracts. The native nucleus pulposus tissue and cells in culture expressed higher GlcAT-I protein levels than the annulus tissue and cells. *H*, real time reverse transcription-PCR analysis of GlcAT-I expression by cells treated with ionomycin (1 μM) and PMA (100 ng) (*I* + *P*) for 4–24 h. There was a time-dependent change in mRNA expression following treatment. At 4 h, GlcAT-I expression was suppressed; at 24 h, there was increased expression of the gene. *I*, immunofluorescent analysis of nucleus pulposus cells treated with ionomycin and PMA. Cells showed increased GlcAT-I expression 24 h after the treatment. *J*, Western blot of nucleus pulposus cells treated with ionomycin and PMA. Note the increased GlcAT-I expression 24 h after treatment.

strated that GAG synthesis was inhibited by calcium blocking agents (12). Since these agents affected both chondroitin sulfate and heparin sulfate, the results suggested that calcium ions controlled a common early step in the GAG biosynthetic pathway. In a recent study, calcium was shown to control GlcAT-I expression through Sp1 transcription factor (13).

The major objective of the investigation was to examine the regulation of GlcAT-I expression by nucleus pulposus cells of the intervertebral disc. We show for the first time that TonEBP regulates GlcAT-I expression and that regulation is dependent on intracellular calcium ions. We also demonstrate that calcium-dependent calcineurin (Cn)-NFAT signaling serves as a negative regulator of GlcAT-I expression in these cells. From this perspective, by controlling GAG as well as aggrecan synthesis, TonEBP permits nucleus pulposus cells to autoregulate the osmotic environment of the disc.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—Rabbit polyclonal TonEBP antibody was a kind gift from Dr. H. Moo Kwon (University of Maryland). TonEBP/NFAT5 wild type and null MEFs (originally from Dr. Steffan N. Ho), were provided by Dr. Feng Chen (Washington University, St. Louis, MO). Primary kidney medullary fibroblasts derived from CnAα null, CnAβ null, and CnA wild type mice were provided by Dr. Jennifer Gooch (Emory University). Plasmids were kindly provided by Dr. Takashi Ito (Osaka University, Japan) (*tauT* (taurine transporter) wild type (WT) and mutant (MT) reporter) (14) and Dr. Ben C. Ko (University of Hong Kong) (FLAG-DN-TonEBP, FLAG-TonEBP, and FLAG-CMV2) (15). DN-TonEBP contains amino acids 157–581 of human TonEBP (from clone KIAA0827). Dr. Gerald Crabtree (Stanford University) provided catalytic subunit (CnA) and regulatory subunit (CnB). Dr. Jeffery Molkentin (Cincinnati Children's Hospital Medical Center) supplied NFAT4, pECE, and Dr. Gerald Thiel (University of Saarland Medical Center, Germany) supplied DN-Sp1. Plasmids for NFAT1 (catalogue number 11100) and constitutively active NFAT2 (CA-NFAT2) (catalog number 11102) with key serine residues changed to alanine to prevent phosphorylation developed by Dr. Anjana Rao (16), plasmid for NFAT3 (catalog number 10961) developed by Dr. Toren Finkel (17), and plasmid for 3×NFAT-Luc (catalog number 17870) developed by Dr. Gerald Crabtree (18) were obtained from Addgene. 3×NFAT-Luc contains three NFAT binding sites upstream of the minimal interleukin-2 promoter and used to measure calcineurin-dependent NFAT activation. As an internal transfection control, vector pRL-TK (Promega)

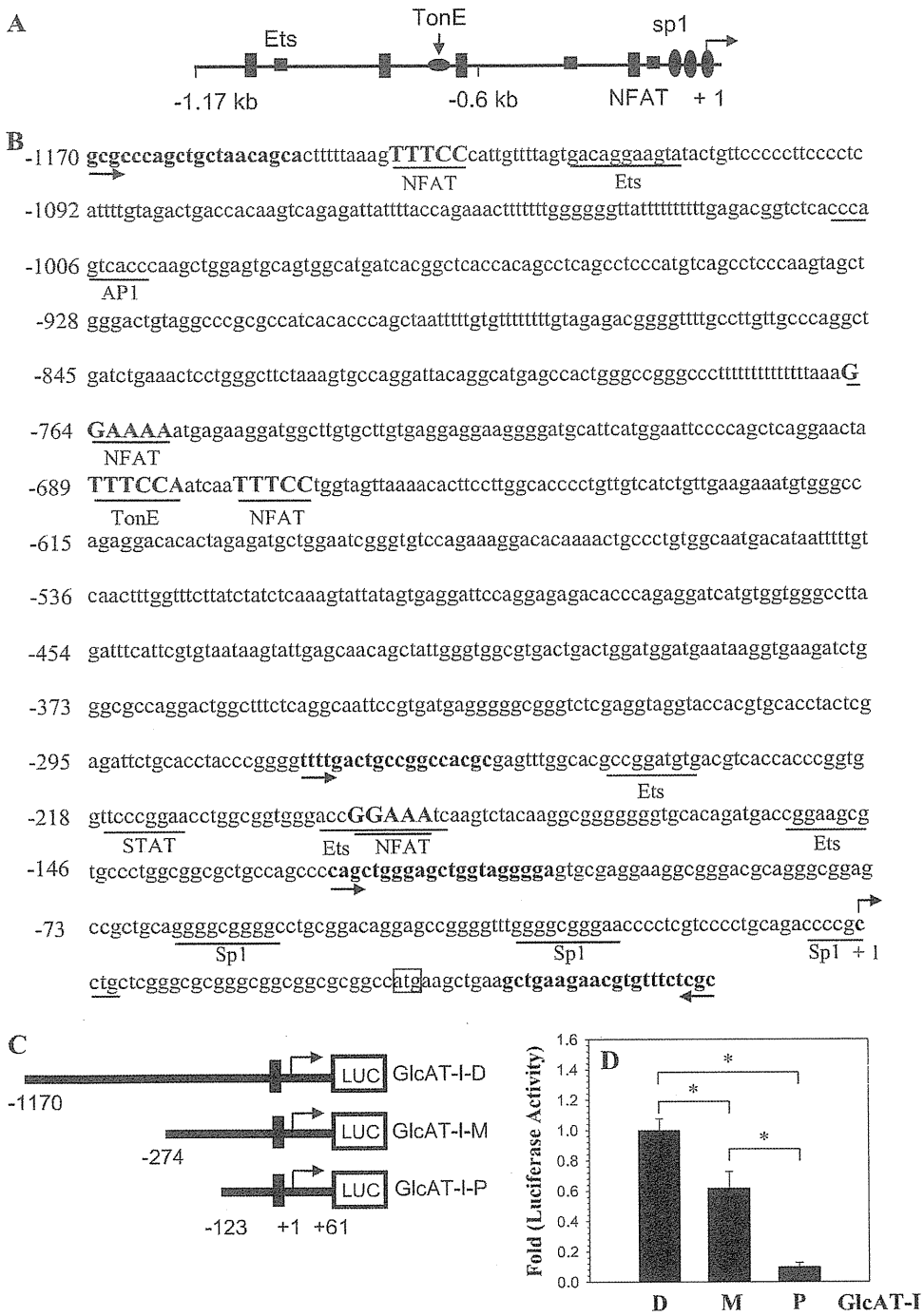

FIGURE 2. **GlcAT-I promoter contains TonEBP and NFAT binding motifs.** *A*, promoter organization of the human GlcAT-I gene. The transcription start site is marked as *+1*. The TonE site is shown as a *flattened circle*, Spls are indicted as *ovals*, and the NFAT binding motifs are shown as *rectangles*. *B*, DNA sequence of the promoter region of the human GlcAT-I gene. TonE (TTTCCA) and NFAT (TTTCC or GGAAA) consensus sequences are marked in *boldface type* and *underlined*. The *arrows* indicate the starting location of the primers used to generate promoter constructs. The transcription start site is marked as *+1*; *ATG* marks the translation start site. Spl sites are *underlined* and lie within first 100 bases. *C*, schematic diagram showing a map of successive PCR-generated 5′ deletion constructs of the human GlcAT-I promoter. The GlcAT-I-D construct consists of a 1,231-bp fragment containing 1,170 bp of the upstream GlcAT-I promoter sequence linked to 61 bp of exon 1 (*i.e.* −1170/+61), whereas GlcAT-I-M and GlcAT-I-P constructs contain a 335-bp fragment (−274/+61) and a 184-bp fragment (−123/+61), respectively. *D*, basal activities of GlcAT-I promoter constructs relative to full-length construct GlcAT-I-D in nucleus pulposus cells. Cells showed maximal luciferase activity for the GlcAT-I-D construct, whereas the shortest construct, GlcAT-I-P, showed minimal activity. Values shown are mean ± S.D. of three independent experiments. *, $p < 0.05$.

containing the *Renilla reniformis* luciferase gene was used. The amount of transfected plasmid, the pretransfection period after seeding, and the post-transfection period before harvesting have been optimized for rat nucleus pulposus cells using pSV β-galactosidase plasmid (Promega) (19).

Immunohistological Studies—Freshly isolated discs were immediately fixed in 4% paraformaldehyde in PBS and then embedded in paraffin. Transverse and coronal sections, 6–8 μm in thickness, were deparaffinized in xylene, rehydrated through graded ethanol, and stained with Alcian blue, eosin, and hematoxylin. For localizing GlcAT-I, sections were incubated with the anti-GlcAT-I antibody (Novus) 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) for 10 min at room temperature. Sections were incubated with a streptavidin-peroxidase complex for 5 min and washed with PBS, and color was developed using 3′,3-diaminobenzidine (Vecta Stain Universal Quick Kit; Vector Laboratories).

Isolation of Nucleus Pulposus Cells and Treatments of Cells—Rat nucleus pulposus cells were isolated using a method reported earlier by Risbud *et al.* (19). Nucleus pulposus cells and MEFs were maintained in Dulbecco's modified Eagle's medium and 10% fetal bovine serum supplemented with antibiotics. In some experiments, cells were treated with 1 μM ionomycin and PMA (100 ng/ml) with or without FK505 (10 ng/ml) and cyclosporine A (1 μg/ml) or BAPTA-AM (10 μM) or bisanthracycline (WP631; 50–100 nM).

Real Time RT-PCR Analysis—Following treatment, total RNA was extracted from nucleus pulposus cells using RNAeasy minicolumns (Qiagen). Before elution from the column, RNA was treated with RNase-free DNase I. 100 ng of total RNA was used as template for real time PCR analysis. Reactions were set up in microcapillary tubes using 1 μl of RNA with 9 μl of a LightCycler FastStart DNA Master SYBR Green I mix (Roche Applied Science) to which gene-specific forward and reverse

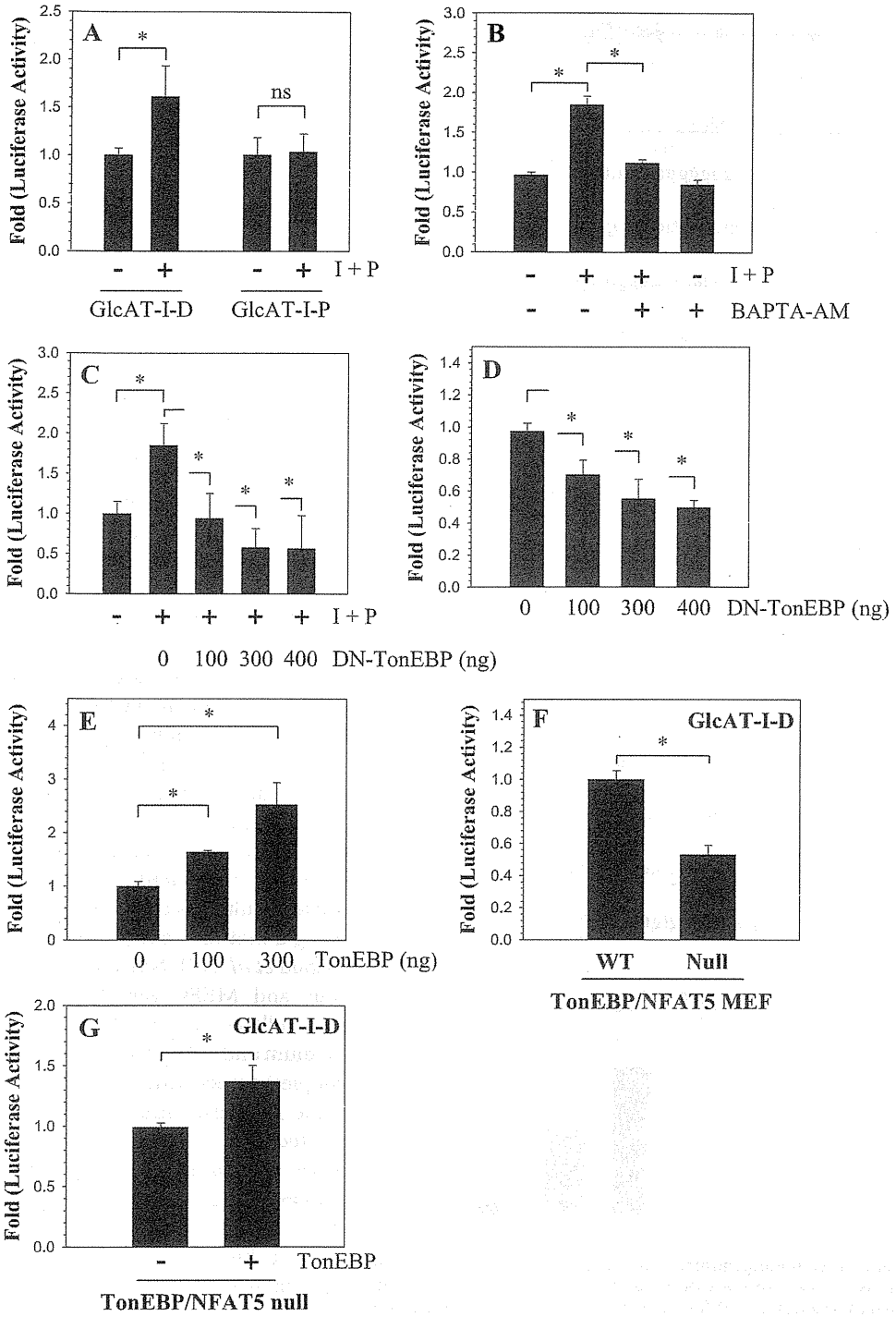

FIGURE 3. **Calcium regulates GlcAT-I promoter activity through TonEBP.** *A*, GlcAT-I-D or GlcAT-I-P reporter activity measured following ionomycin and PMA (*I* + *P*) treatment. Treatment resulted in induction in GlcAT-I-D but not GlcAT-I-P reporter activity. *B*, effect of BAPTA-AM (10 μM) on GlcAT-I-D reporter activity. The calcium chelator completely blocks ionomycin-mediated activity but not basal activity of the GlcAT-I-D reporter in nucleus pulposus cells. *C* and *D*, effect of ionomycin treatment on cells transfected with GlcAT-I-D reporter construct along with DN-TonEBP or empty backbone FLAG- CMV2. Note that the expression of DN-TonEBP resulted in a complete suppression of ionomycin-mediated induction in GlcAT-I promoter activity. In addition, when TonEBP function was blocked, there was suppression of basal GlcAT-I-D activity. *E*, effect of TonEBP on GlcAT-I promoter activity. When pTonEBP was co-expressed, there was a dose-dependent increase in GlcAT-I reporter activity. *F*, GlcAT- I promoter activity of TonEBP/NFAT5 null and wild type cells. Null cells evidenced decreased basal activity of reporter compared with wild type cells. *G*, effect of co-expression of TonEBP and GlcAT-D in null cells. When co-expressed, TonEBP increased GlcAT-I-D reporter activity. Values shown are mean ± S.D. of three independent experiments performed in triplicate. *, $p < 0.05$.

PCR primers were added (GlcAT-I (NCBI number NM_001128184), forward (5′-atgcccagtttgatgctactg-cac-3′) and reverse (5′-tgttcctcct-gcttcatcttcggt-3′)). Each set of samples included a template-free control. PCRs were performed in a LightCycler (Roche Applied Science) according to the manufacturer's instructions. All the primers used were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Immunofluorescence Microscopy—Cells were plated in flat bottom 96-well plates (5 × 103/well) and treated with ionomycin for 6–24 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% fetal bovine serum, and incubated with antibodies against GlcAT-I (1:200) (Novus), TonEBP (1:200) (Calbiochem), NFAT-2 (1:200; Abcam), NFAT-3 (1:100; Cell Signaling), or NFAT-4 (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4 °C overnight. As a negative control, cells were reacted with isotype IgG under similar conditions. After washing, the cells were incubated with Alexa Fluor-488-conjugated anti-mouse secondary antibody (Invitrogen) at a dilution of 1:50 and 10 μM propidium iodide for 1 h at room temperature. Cells were imaged using a laser-scanning confocal microscope (Olympus Fluoview).

Nuclear Protein Extraction and Western Blotting—Cells were placed on ice immediately following treatment and washed with ice-cold Hanks' balanced saline solution. Nuclear and cytosolic proteins were prepared using the CellLytic NuCLEAR extraction kit (Sigma). All of the wash buffers and final resuspension buffer included 1× protease inhibitor mixture (Pierce), NaF (5 mM), and Na_3VO_4 (200 μM). Nuclear or total cell proteins were resolved on 8–12% SDS-polyacrylamide gels and transferred by electroblotting to nitrocellulose membranes (Bio-Rad). The membranes

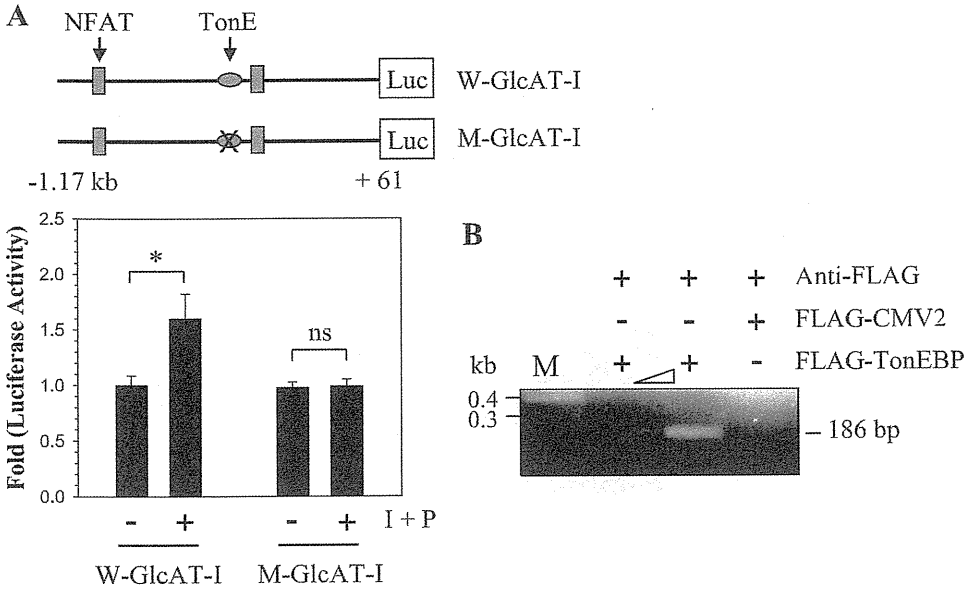

FIGURE 4. **Ionomycin mediated induction of GlcAT-I promoter activity requires TonEBP binding to TonE.** Effect of a 4-bp mutation introduced into the TonE motif of the GlcAT-I-D reporter plasmid. Nucleus pulposus cells were transfected with wild type GlcAT-I-D (*W-GlcAT-I*) or mutant GlcAT-I-D (*M-GlcAT-I*) reporter plasmids, and the induction of the luciferase activity was determined following ionomycin treatment. Treatment caused an induction of wild type reporter activity, whereas the mutant reporter failed to increase activity. Values shown are mean ± S.D. of three independent experiments. *, $p < 0.05$. *B*, interaction of TonEBP with the GlcAT-I promoter measured using a chromatin immunoprecipitation assay. COS7 cells were transfected with GlcAT-I-D along with either FLAG-TonEBP or FLAG-CMV2 empty vector. PCR amplification was performed using primer pairs that encompass TonE sequences of the GlcAT-I promoter. The use of anti-FLAG antibody resulted in generation of a PCR amplicon containing TonE only when FLAG-TonEBP was present. The addition of increasing amounts of FLAG-TonEBP vector evidenced enhanced binding to TonE. Pull-down using anti-FLAG antibody did not result in the formation of a PCR product when cells received empty FLAG-CMV2 vector.

were blocked with 5% nonfat 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% nonfat dry milk in TBST with the anti-GlcAT-I (1:500; Novus) or anti-TonEBP antibody (1:3,000; from Dr. Kwon). Immunolabeling was detected using the ECL reagent (Amersham Biosciences).

Generation of GlcAT-I Reporter and Deletion Constructs—PCR amplification using genomic DNA of a 1,231-bp fragment containing 1,170 bp of the upstream promoter sequence linked to 61 bp of exon 1 (*i.e.* −1170 to +61) of the human GlcAT-I gene was performed using the following primers (forward, 5′ CTAGCTAGCGCGCCCAGCTGCTAACAGCA-3′ (NheI site underlined); reverse, 5′-CCCAAGCTTGCGAGAAACACGT-TCTTCAGC-3′ (HindIII site underlined)) with the addition of GC buffer 1 and LA Taq polymerase (Takara Mirus Bio). Similarly, to generate successive 5′ deletions, promoter fragments of 335 bp (−274 to +61) and 184 bp (−123 to +61) were PCR-amplified using specific forward primers and the same reverse primer. The resulting DNA fragments were subcloned into pCR2.1 TA vector (Invitrogen), isolated by restriction digestion with NheI and HindIII, and ligated into the luciferase basic expression vector, pGL3 (Promega). The identity of each GlcAT-I promoter sequence was confirmed by sequencing.

Site-directed Mutagenesis of TonE—GlcAT-I-D reporter plasmid was used to mutate the TonE site (TTTCCA to TAAAAA). Mutants were generated using the QuikChange II XL site-directed mutagenesis kit (Stratagene), using forward and reverse primer pair containing the desired mutation, following the manufacturer's instructions. The mutation was verified by sequencing.

Chromatin Immunoprecipitation (ChIP) Assay—COS7 cells were transfected with GlcAT-I-D along with either FLAG-TonEBP (increasing amounts) or FLAG-CMV2 vector and cultured for 48 h. ChIP analysis was performed as described before (20). Cross-linked and fragmented lysates were immunoprecipitated with monoclonal anti-FLAG M2 antibody (Sigma). PCR analysis to identify the coprecipitated TonE fragment in GlcAT-I promoter was performed using the following primer sequences: forward, 5′-GGAAAAATGAGAAG-GATGGCTTG-3′; reverse, 5′-TGGA-CACCCGATTCCAGCATCTCTA-3′.

Transfections and Dual Luciferase Assay—Cells were transferred to 24-well plates at a density of 6×10^4 cells/well 1 day before transfection. To investigate the effect of TonEBP overexpression on GlcAT-I promoter activity, cells were cotransfected with 100–500 ng of pTonEBP or DN-TonEBP or backbone vector pcDNA3.1 with 250 ng of GlcAT-I reporter and 250 ng of pRL-TK plasmid. In some experiments, cells were transfected with 500 ng of GlcAT-I reporter plasmids with 500 ng of pRL-TK plasmid. Lipofectamine 2000 (Invitrogen) was used as a transfection reagent. For each transfection, plasmids were premixed with the transfection reagent. For measuring the effect of ionomycin on GlcAT-I reporter activity, 24 h after transfection, the cells in some wells were ionomycin-treated with or without inhibitors FK506 and CsA or BAPTA-AM. 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. Transfection efficiency for rat nucleus pulposus cells was about 50–60%, whereas for fibroblasts, it was close to 90%. Quantification of luciferase activities and calculation of relative ratios were carried out using a luminometer (TD-20/20; Turner Designs, CA). At least three independent transfections were performed, and all analyses were carried out in triplicate.

Statistical Analysis—All measurements were performed in triplicate; data are presented as mean ± S.D. Differences between groups were analyzed by Student's *t* test. *, $p < 0.05$.

RESULTS

Saggital sections of the neonatal rat (Fig. 1, *A* and *B*) and skeletally mature rat discs (Fig. 1, *C–F*) were stained with an antibody to GlcAT-I (Fig. 1, *A*, *C*, and *E*) or counterstained with hematoxylin and eosin and Alcian blue for morphology assessment (Fig. 1, *B*, *D*, and *F*). GlcAT-I is expressed by cells of the nucleus pulposus, annulus fibrosus, and cartilaginous end plate

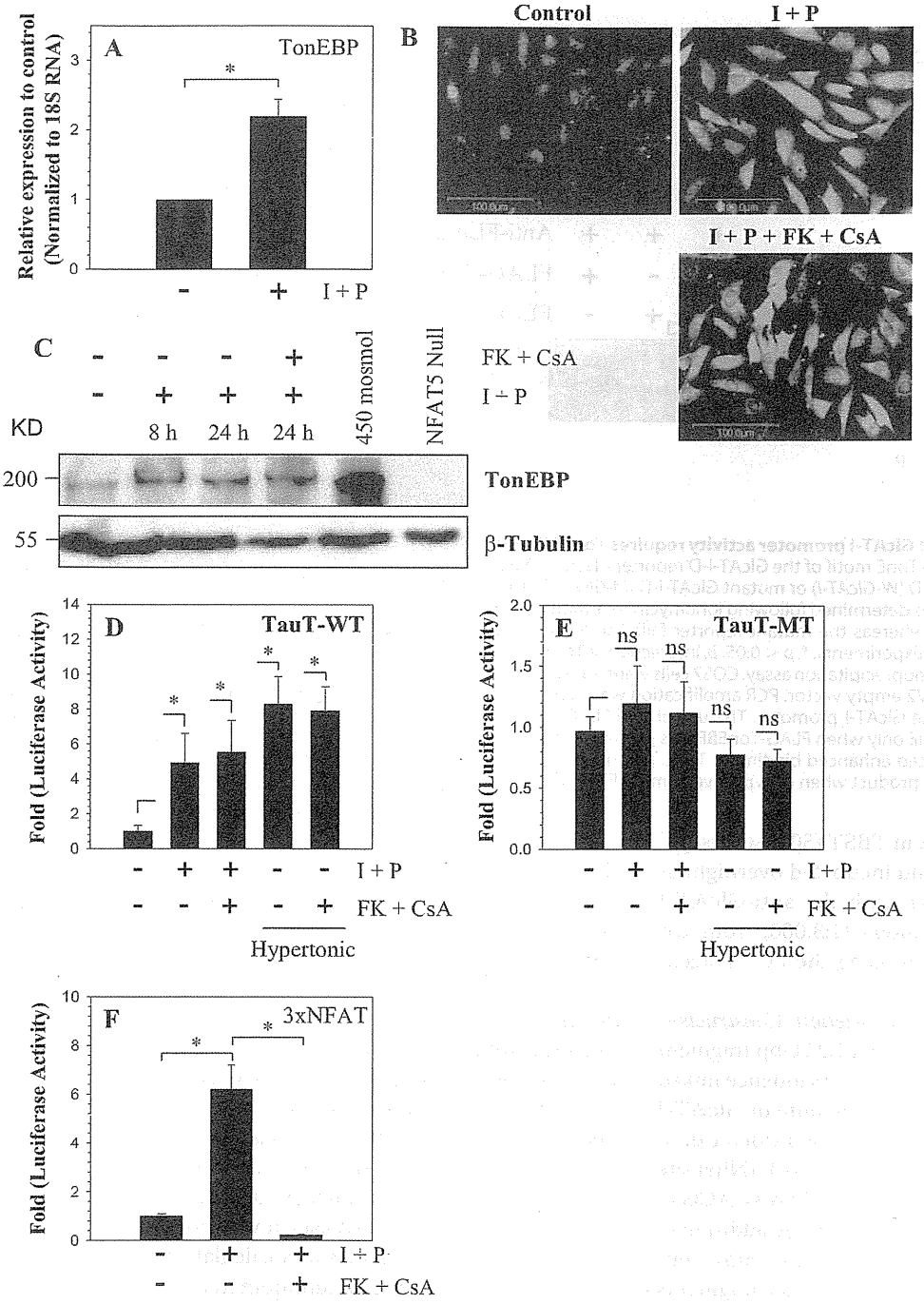

FIGURE 5. **Effect of calcium ions on TonEBP expression.** *A*, nucleus pulposus cells were treated with the calcium ionophore ionomycin (*I*; 1 μM), along with PMA (*P*; 100 ng), and TonEBP expression was measured. Ionomycin treatment resulted in significant increase in TonEBP mRNA expression. *B* and *C*, immunofluorescence and Western blot analysis of cells as treated in *A*. note the increase in TonEBP protein after ionomycin treatment. The addition of Cn inhibitors FK506 (10 ng/ml) and cyclosporine A (*CsA*; 1 μg/ml) did not suppress synthesis of TonEBP protein. When nucleus pulposus cells were cultured under hyperosmotic conditions (450 mosmol/kg), there was high induction in TonEBP, whereas TonEBP protein was undetectable in TonEBP null cells. *D* and *E*, reporter activity of nucleus pulposus cells transfected with wild type (*D*) or mutant (*E*) *tauT* reporter and treated with ionomycin and PMA with or without FK506 and cyclosporine. Note that treatment with ionomycin increased the activity of the WT but not the TonE-MT *tauT* reporter. FK506 and CsA did not inhibit ionomycin-mediated increase in *tauT*-WT promoter activity. As expected under hyperosmotic conditions, there is a robust induction in activity of *tauT*-WT but not *tauT*-MT reporter. *F*, induction of the activity of 3×NFAT reporter in NP cells following ionomycin and PMA treatment. The reporter is highly induced, whereas the addition of FK506 and cyclosporine completely blocks activation, indicating a requirement for Cn in this process. Values shown are mean ± S.D. of three independent experiments performed in triplicate. *, $p < 0.05$.

in rat disc (Fig. 1, *A*, *C*, and *E*). In all cases, staining is localized to the cytosol (Fig. 1, *A* and *C*). Expression of GlcAT-I in native disc tissues and cultured cells was studied using Western blot analysis. Fig. 1*G* indicates that nucleus pulposus tissue expresses a prominent 43-kDa GlcAT-I band. Moreover, the expression level of GlcAT-I in nucleus pulposus tissue is higher than in the annulus fibrosus (Fig. 1*G*). Similar to native tissue, cultured rat disc cells exhibit a similar pattern of expression (Fig. 1*G*). To explore the premise that intracellular calcium regulated GlcAT-I expression, nucleus pulposus cells were treated with ionomycin, a calcium ionophore, along with PMA, and expression of GlcAT-I was analyzed. Fig. 1*H* shows that treatment with ionomycin for 24 h results in increased GlcAT-I mRNA levels in nucleus pulposus cells. In addition, we studied the expression of GlcAT-I in nucleus pulposus cells using immunofluorescence microscopy and Western blot analysis. Ionomycin treatment results in increased GlcAT-I protein expression (Fig. 1, *I* and *J*); the increase is pronounced 24 h after treatment (Fig. 1*J*).

To investigate the regulation of expression, we analyzed the 1.17-kb promoter sequence of human GlcAT-I and measured the activity of different size promoter fragments. Analysis revealed that the GlcAT-I promoter contains four NFAT (GGGAA or TTTCC) as well as a conserved TonE (TTTCCA) motif at −684 bp (Fig. 2, *A* and *B*). To analyze promoter function, we generated luciferase reporter constructs containing a −1170/+61 bp (pGlcAT-I-D), a −274/+61 bp (pGlcAT-I-M), and a −123/+61 bp (pGlcAT-I-P) fragment of the human GlcAT-I promoter (Fig. 2*C*). We measured the basal activity of all three fragments in nucleus pulposus cells. Fig. 2*D* shows that the −1170/+61 bp fragment has maximal basal activity, whereas the −123/+61 bp fragment exhibits the least activity.

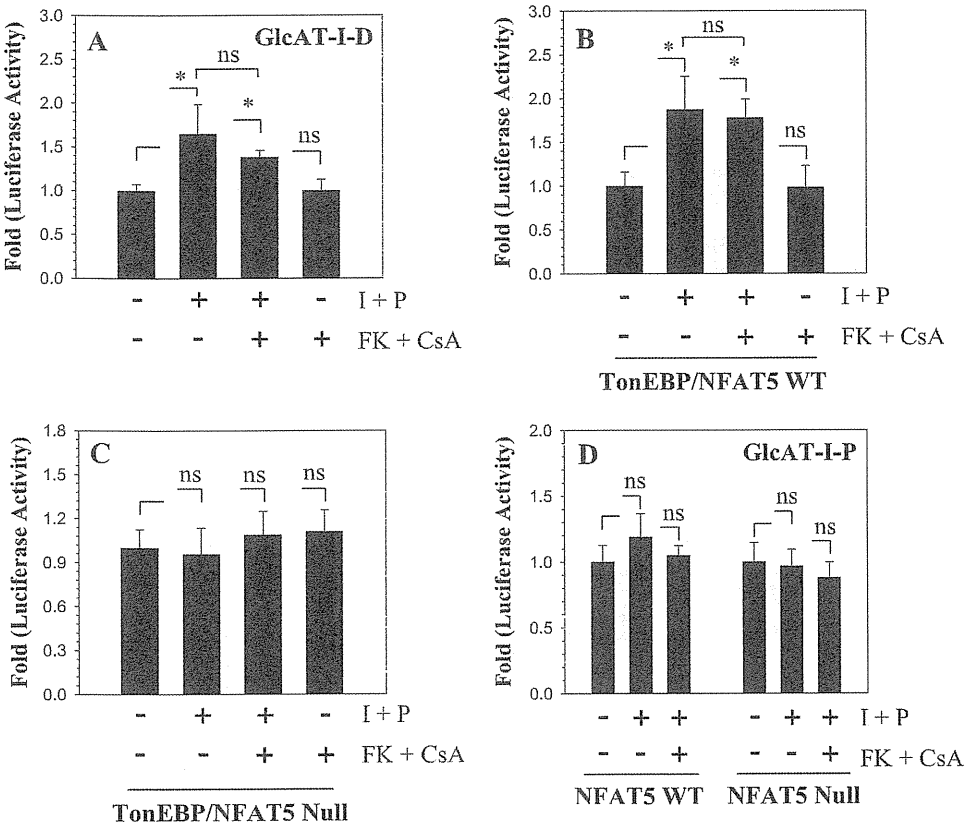

FIGURE 6. **Ionomycin mediates GlcAT-I promoter activation, although TonEBP is independent of calcineurin.** Nucleus pulposus cells (*A*), TonEBP/NFAT5 wild type (*B*), and TonEBP/NFAT5 null (*C*) MEFs were transfected with GlcAT-I-D reporter, and activity was measured following treatment with ionomycin with or without FK506 and cyclosporine A. Unlike TonEBP/NFAT5, null MEFs GlcAT-I-D reporter activity was induced in both nucleus pulposus and NFAT5 wild type cells. Calcineurin inhibitors did not suppress ionomycin-induced or basal activity of GlcAT-I reporter in any of the cell types. *D*, effect of ionomycin treatment on GlcAT-I-P reporter activity. The reporter was nonresponsive to ionomycin treatment in both TonEBP/NFAT5 wild type and null MEFs. Values shown are mean ± S.D. of three independent experiments performed in triplicate. *, $p < 0.05$.

We next examined the effect of ionomycin treatment on GlcAT-I promoter activity in nucleus pulposus cells. When cells are treated with ionomycin and PMA, there is an increase in activity of −1170/+61 bp GlcAT-I promoter (pGlcAT-I-P); the shortest promoter fragment (pGlcAT-I-P), lacking TonE and NFAT sites, did not show any change in activity (Fig. 3*A*). We then determined if ionomycin-mediated activation of GlcAT-I required calcium ions. When cells were treated with the calcium chelator BAPTA-AM along with ionomycin, there was complete inhibition of GlcAT-I-D reporter activation (Fig. 3*B*). To investigate if TonEBP participated in ionomycin-mediated induction of GlcAT-I promoter activity, nucleus pulposus cells were transiently co-transfected with plasmids encoding DN-TonEBP or full-length TonEBP. Fig. 3*C* shows that forced expression of DN-TonEBP completely abolishes ionomycin induction of GlcAT-I promoter activity. Moreover, expression of DN-TonEBP also suppresses the basal activity of the GlcAT-I-D promoter fragment (Fig. 3*D*). A significant inhibitory effect of DN-TonEBP expression on GlcAT-I reporter activity is seen at a dose of 100 ng, which is further enhanced when the concentration of the DN-TonEBP is increased to 400 ng (Fig. 3*D*). On the other hand, overexpression of TonEBP using the pFLAG-TonEBP vector results in a dose-dependent increase in GlcAt-I-D promoter activity in the absence of ionomycin (Fig. 3*E*). We used MEFs derived from TonEBP/NFAT5 null and wild type mice to further validate the role of TonEBP in regulation of the GlcAT-I promoter. Fig. 3*F* shows that the basal GlcAT-I promoter activity in null cells is 50% lower than in the wild type cells. Moreover, co-transfection with TonEBP expression vector in null cells results in an increase in basal GlcAT-I promoter activity (Fig. 3*G*).

We then evaluated whether GlcAT-I promoter activity required that TonEBP be bound to TonE. For this purpose, we introduced a 4-base pair mutation in the TonE site of the GlcAT-I-D reporter plasmid (TTTCCA to TAAAAA). Following ionomycin treatment, nucleus pulposus cells transfected with wild type reporter plasmid evidence induction of activity (Fig. 4*A*). In contrast, when a mutant plasmid is used, induction of the reporter is completely blocked (Fig. 4*A*). Moreover, wild type promoter also exhibited a significantly higher basal activity than the TonE mutant reporter (not shown). We used the ChIP assay to evaluate the interaction of TonEBP protein with the TonE motif in the GlcAT-I promoter (Fig. 4*B*). TonEBP associated with the TonE motif under basal conditions. Binding of TonEBP to TonE was proportional to the amount of transfected FLAG-TonEBP vector. When an empty FLAG vector was co-transfected in place of FLAG-TonEBP, PCR analysis indicated that the amplicon is not formed. This was confirmed by cloning and sequencing of the PCR product.

The mechanism of TonEBP activation by ionomycin in nucleus pulposus cells was investigated. Fig. 5*A* shows that ionomycin treatment results in increased TonEBP mRNA expression. Moreover, both immunofluorescence and Western blot analysis indicate that there is a concomitant increase in TonEBP protein expression (Fig. 5, *B* and *C*); the increase in protein level is evident as early as 8 h, and it remains high at 24 h (Fig. 5*C*). We explored the possibility that activation of Cn, a calcium-dependent phosphatase that mediates NFATc signaling, was responsible for TonEBP induction. Treatment of nucleus pulposus cells with Cn inhibitors, FK506 and cyclosporine A, in the presence of ionomycin did not block induction in TonEBP protein levels (Fig. 5, *B* and *C*). In addition, we measured the promoter activity of the TonEBP target gene, *tauT*. Significant activation in *tauT* reporter activity is evident following ionomycin treatment; simultaneous treatment with Cn inhibitors fails to suppress induction (Fig. 5*D*). In contrast to

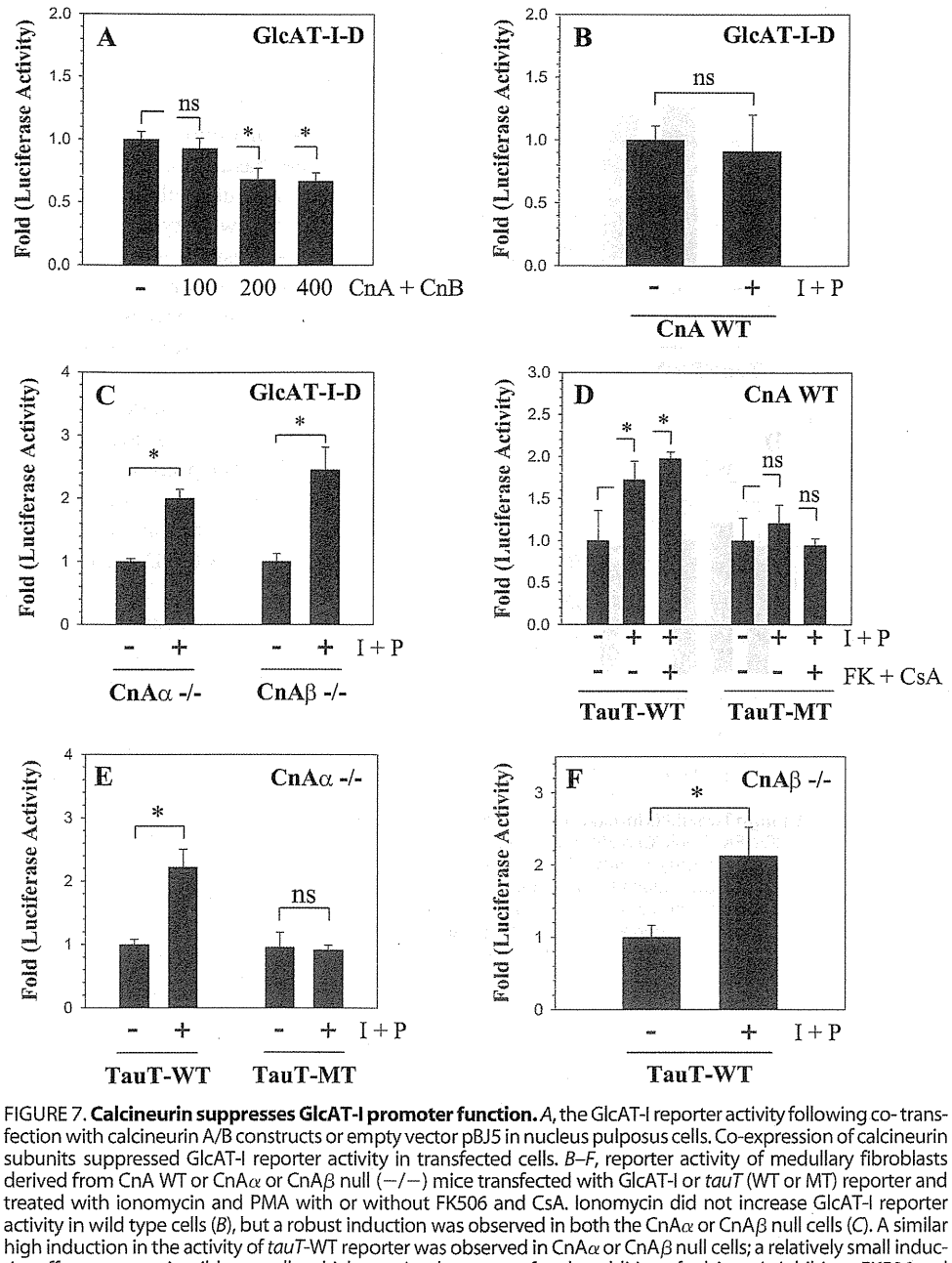

FIGURE 7. **Calcineurin suppresses GlcAT-I promoter function.** *A*, the GlcAT-I reporter activity following co-transfection with calcineurin A/B constructs or empty vector pBJ5 in nucleus pulposus cells. Co-expression of calcineurin subunits suppressed GlcAT-I reporter activity in transfected cells. *B–F*, reporter activity of medullary fibroblasts derived from CnA WT or CnAα or CnAβ null (−/−) mice transfected with GlcAT-I or *tauT* (WT or MT) reporter and treated with ionomycin and PMA with or without FK506 and CsA. Ionomycin did not increase GlcAT-I reporter activity in wild type cells (*B*), but a robust induction was observed in both the CnAα or CnAβ null cells (*C*). A similar high induction in the activity of *tauT*-WT reporter was observed in CnAα or CnAβ null cells; a relatively small inductive effect was seen in wild type cells, which remained constant after the addition of calcineurin inhibitors FK506 and CsA. Neither wild type (*D*) nor the null cells (*E* and *F*) displayed induction in *tauT*-MT reporter activity. Values shown are mean ± S.D. of three independent experiments performed in triplicate. *, $p < 0.05$.

the wild-type *tauT* reporter, activity of mutant *tauT* construct is unaffected by ionomycin (Fig. 5*E*). To confirm that ionomycin promotes Cn-NFAT signaling in nucleus pulposus cells, we measured the activity of 3×NFAT reporter. Fig. 5*F* shows that ionomycin treatment significantly up-regulates 3×NFAT reporter activity, which is completely blocked by the addition of Cn inhibitors FK506 and cyclosporine A.

To ascertain if Cn signaling together with TonEBP plays a role in GlcAT-I expression, we treated nucleus pulposus cells with ionomycin and PMA together with FK506 and cyclosporine A. Fig. 6*A* shows that ionomycin treatment results in an increase in GlcAT-I promoter activity. The presence of FK506 and cyclosporine did not block ionomycin-dependent induction in promoter activity. To further validate these findings, we used NFAT5 null and wild type MEFs and measured activities of GlcAT-I reporters following ionomycin treatment. Fig. 6*B* shows that ionomycin causes an increase in GlcAT-I reporter activity. Again, inclusion of FK506 and cyclosporine did not affect promoter induction in wild type MEFs. In contrast, in NFAT5 null MEFs, ionomycin did not alter GlcAT-I reporter activity (Fig. 6*C*). Moreover, neither wild type nor null cells exhibit an increase in GlcAT-I-P reporter (Fig. 6*D*).

The contribution of Cn signaling in GlcAT-I expression was studied by co-transfecting nucleus pulposus cells with plasmids expressing CnA and CnB along with GlcAT-I-D reporter. Fig. 7*A* shows that nucleus pulposus cells receiving 200 ng or more of Cn plasmids display suppression in GlcAT-I reporter activity. To further explore if Cn signaling played a regulatory role, we used primary medullary fibroblasts derived from CnAα null, CnAβ null, and wild type mice. Treatment of wild type fibroblasts with ionomycin results in little change in GlcAT-I reporter activity (Fig. 7*B*). Interestingly, in both CnAα null and CnAβ null cells, ionomycin treatment enhances activation (2-3-fold) of the GlcAT-I-D reporter (Fig. 7*C*). We then measured the activity of both wild type (containing the WT TonE motif) and mutant *tauT* reporter (mutant TonE motif) in these cells. Wild type cells show a small increase in the activity of the WT-*tauT* reporter; activity is unaffected by the calcineurin inhibitors (Fig. 7*D*). A significant induction in *tauT* reporter is also seen in both CnAα (Fig. 7*E*) and CnAβ (Fig. 7*F*) null cells. Mutant *tauT* reporter is not induced in either the wild type, CnAα, or CnAβ null fibroblasts.

To investigate the relationship between GlcAT-I and NFAT signaling, we transfected nucleus pulposus cells with plasmids encoding NFAT1–4 and measured the activity of the GlcAT-I-D reporter. Fig. 8*A* shows that co-expression of NFAT1 alone has no effect on GlcAT-I reporter activity. However, when Cn (CnA and CnB) was co-expressed with or without NFAT1, there is a significant suppression in reporter activity. Unlike NFAT1, co-expression of CA-NFAT2 (Fig. 8*B*), NFAT3 (Fig. 8*C*), or NFAT4 (Fig.