Calcineurin mediates bladder wall remodeling secondary to partial outlet obstruction

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Calcineurin mediates bladder wall remodeling secondary to partial outlet obstruction. Am J Physiol Renal Physiol 301: F813–F822, 2011. First published July 20, 2011; doi:10.1152/ajprenal.00586.2010.—We hypothesized that the calcineurin-nuclear factor of activated T-cells (NFAT) pathway is activated following partial bladder outlet obstruction (pBOO), which would allow for pharmacologic treatment to prevent the ensuing bladder wall hypertrophy. Using a model of pBOO in male mice, we were able to demonstrate increased nuclear importation of the transcription factors NFAT and myocyte enhancer factor 2 both of which are under control of calcineurin in both the whole bladder wall as well as the urothelium. We further confirmed that this pathway was activated using transgenic mice containing an NFAT-luciferase reporter construct. Mice were randomized following pBOO to treatment with or without cyclosporine A (CsA), a known inhibitor of calcineurin. The bladder-to-body mass ratio (mg bladder wt/g body wt) of 0.95 ± 0.03 in shams increased to 3.1 ± 0.35 following pBOO, and it dropped back to 1.7 ± 0.22 in the CsA+ group (P < 0.001). Luciferase values (RLU) of 1,130 ± 133 in shams increased to 2,010 ± 474 following pBOO and were suppressed to 562 ± 177 in the CsA+ group (P < 0.05). The myosin heavy chain mRNA (A/B) isoform ratio of 0.07 ± 0.03 in shams increased to 1.04 ± 0.19 following pBOO but it diminished to 0.24 ± 0.1 in the CsA+ group (P < 0.001). In vitro whole organ physiology studies demonstrated improved responses in those bladders from mice treated with CsA. The mRNAs for all four known calcineurin-responsive NFAT isoforms are expressed in the bladder wall, although NFATc3 and NFATc4 predominate. Both NFATc3 and NFATc4 are expressed in urothelial as well as smooth muscle cells. We conclude that pBOO activates the calcineurin-NFAT pathway and that CsA treatment decreased bladder hypertrophy, shifted the pattern of myosin isoform mRNA expression back toward that seen in normal controls, and resulted in improved in vitro whole organ performance.

bladder hypertrophy; cyclosporine A; NFAT; MEF-2

IN RESPONSE TO PARTIAL BLADDER outlet obstruction (pBOO), the human bladder can adapt by undergoing compensatory hypertrophy (17, 28, 32), which can be defined as a bladder that has developed increased mass, wall thickness, and can generate greater pressures to overcome the increased outlet resistance with a nonexistent or minimal postvoid residual urine. However, when the demands outstrip this adaptive response, the bladder develops a less efficient contractile performance characterized by increased urinary frequency, diminished voided volumes, and markedly elevated postvoid residuals (28). Ultimately, fibrosis sets in, resulting in rising storage pressures that can lead to renal compromise.

The molecular mechanisms that account for resulting bladder dysfunction are not well-understood. Su et al. (27) showed no changes in peak force or myosin light chain (MLC) phosphorylation, but a 10-fold drop in shortening velocity and an increase in basal MLC phosphorylation at rest using a rabbit model of pBOO. This drop in velocity of contraction was highly correlated with increased expression of the slower myosin heavy chain SM-A isoform. However, other factors contribute to this complex pathophysiology including alterations in innervations, hypoxia, mitochondrial energetics, as well as a prominent increase in the deposition of extracellular matrix proteins.

The mRNA and protein expression of a key regulator of intracellular calcium homeostasis, the sarcoplasmic endoplasmic reticulum calcium ATPase, are downregulated following pBOO, suggesting that cytosolic calcium homeostasis is also disrupted (26). Cytosolic calcium has other roles besides the initiation of contractility; the discharge of intracellular calcium stores has been tied to cellular hypertrophy (7, 12, 22, 24, 29). Steers et al. (25) demonstrated that increases in bladder mass after pBOO were blocked by the administration of diltiazem, which suggested a major role for cell calcium in the hypertrophic response. However, at that time, the “downstream” mechanisms for this effect were not apparent. Today, the calcineurin pathway is understood to play a role in transducing subtle shifts in basal cytosolic calcium levels into changes in gene expression across a variety of biological systems in response to pathologic stimuli (9) or development (13). Calcineurin is a phosphatase that when activated by slow and sustained changes in cytosolic calcium serves to dephosphorylate the nuclear factor of activated T-cells (NFAT) (14). In addition, calcineurin can activate the transcription factor myocyte enhancer factor 2 (MEF2), although this particular transcription factor is also under the control of histone deacetylases and the CamII kinase pathway (31). Following dephosphorylation, NFAT crosses the nuclear membrane to act as a transcription factor. This pathway can be inhibited by cyclosporine A (CsA), which blocks the phosphatase activity of the active calcineurin-calmodulin-Ca2+ complex. We hypothesized that partial outlet obstruction is associated with activation of the calcineurin pathway, an important (but not the only) step leading to bladder wall hypertrophy.

MATERIALS AND METHODS

pBOO and sham controls. All work was carried out in 8-wk-old male mice which were anesthetized with isoflurane and underwent microsurgical creation of pBOO as described by Austin et al. (1).
Briefly, under isoflurane anesthesia administered by nose cone, mice underwent a 1-cm laparotomy to expose the bladder. The ureters were identified, and under ×4.5 optical magnification, a 8–0 Prolene suture was passed around the bladder neck, above the prostate and below the ureters. The Prolene suture was tied down over a 22-gauge angiocatheter, and the abdomen was closed in two layers of 7–0 Maxon. Experiments to perform the gel shift assays, physiology, and histology were performed using a wild-type Swiss Webster strain obtained from Jackson Labs. An additional set of experiments was done using male transgenic mice containing an NFAT-luciferase reporter construct (30) that were selected from an established breeding colony (a founding breeder pair was obtained from Dr. Jeffrey Molkentin with an MTA from the University of Cincinnati).

Mice entered into the CsA drug trials were randomized to receive an infusion of 0.25 μl/h of CsA via Alzet pumps (model 2004, Durect, Cupertino, CA; CsA+) or no treatment (CsA−) for a period of 2 wk. This flow rate corresponds to a dose of 40 μg of MEF2b (Ab 33540, Abcam, Cambridge, MA), NFATc3, or NFATc4 (SC 8405 and 1153, Santa Cruz Biotechnology, Santa Cruz, CA) antibody and incubated for 10 min on ice followed by 10 min at room temperature before loading onto the gel. Upon completion of the binding reaction, all samples were run on a precast 5% TBE gel (Biorad, Richmond, CA) at 80 V at 4°C in the dark for ~1.5 h. Gels were scanned in the Odyssey Infrared Imaging System (Li-Cor).

Luciferase assay. Twenty microliters of each protein supernatant were added to a clear bottom 96-well assay plate and 50 μl of luciferase assay reagent (E1501, Promega) were added to each well by the automated luminometer. The resulting luminescence was recorded using a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA). All samples were run in duplicate and data were expressed as relative luciferase units per microgram of protein. Reverse transcription-polymerase chain reaction. Myosin heavy chain isoform analysis was performed by PCR as described by Austin et al. (1) using 1 μg total RNA as a starting point to generate cDNAs. The primers for the MHC isoforms SM-A and SM-B were upper 5′-CACTGCCGCCAATGATGAAC-3′; lower 5′-GCCGGGCTTCTCCTGTCGCTCC-3′; NFATc2 upper 5′-CACGCCGCC- GCTCTGCTGTTCTCA-3′, lower 5′-GGTGCCTTCCCGTCCT- CATAGTG-3′; NFATc3 upper 5′-CCGGGCTTCTCCTGTCGCT- 3′, lower 5′-CGGCTGAGGATGATGGTGAA-3′; NFATc4 upper 5′-CTTTGGCCCTGAGCTGGACTTCTC-3′, lower 5′-TAGCCTG- GCCGCCACTTCAATGGG-3′. The 18S primers were purchased from Ambion (cat. no. AM1717) Quantum RNA Classic II 18S and were used according to the manufacturer’s directions. All PCR products were confirmed by sequencing analysis and compared with known sequences using the BLAST program. Chromatin immunoprecipitation. We used the Imprint Chromatin Immunoprecipitation Kit (CHP1, Sigma, St. Louis, MO) to immunoprecipitate and purify DNA bound by the transcription factors of interest. Bladders subjected to pBOO and sham controls were used and the tissue samples were prepared as per the manufacturer’s protocol. Formalin fixation covalently linked transcription factors to their respective DNA sequences. Cross-linked DNA was isolated, subjected to ultrasonic shearing, and applied to stripwells containing the conjugated antibody against the transcription factor of interest using the kit protocol. The antibodies used were the mouse monoclonal to NFATc3 (Santa Cruz Biotechnology sc-8405) and the rabbit polyclonal to NFATc4 (Santa Cruz Biotechnology sc-13036). After the wells were washed, bound DNA fragments were released as per the manufacturer’s instructions and purified using GenElute Binding Columns provided. This purified DNA was used in a random priming PCR to amplify the immunoprecipitated DNA targets using Affymetrix Protocol; following this amplification, these DNA targets were fragmented and labeled using the Affymetrix protocol.

The labeled DNA for these CHIP assays was hybridized to Affymetrix genechips (Mouse promoter 1.0R arrays–900890) in two batches of control and pBOO samples. All files from this mouse promoter array 1.0R were processed by the CHOP bioinformatics core batches of control and pBOO samples. All files from this mouse promoter array 1.0R were processed by the CHOP bioinformatics core

EMSA. The Odyssey Infrared EMSA kit from Li-Cor (Lincoln, NE) was used for all gel shift assays. All binding reactions were performed at room temperature in the dark for 20 min in a volume containing 5 μg nuclear protein and 1 μl of an IR-labeled oligonucleotide probe. Total volume was adjusted to 20 μl with binding buffer. Using custom-made IR-labeled primers and 30-bp segments containing the NFAT or MEF-2 consensus binding sequences, we were able to create specific IR oligonucleotide probes using PCR. Using identical but nonlabeled primers, we also synthesized a “cold” non-IR probes for the competition reaction. To further confirm the identity of the shifted bands, nuclear protein extracts were also preincubated for 10 min with the IR-labeled oligonucleotide probes and a 1 μl of MEF2b (Ab 33540, Abcam, Cambridge, MA), NFATc3, or NFATc4 (SC 8405 and 1153, Santa Cruz Biotechnology, Santa Cruz, CA) antibody and incubated for 10 min on ice followed by 10 min at room temperature before loading onto the gel. Upon completion of the binding reaction, all samples were run on a precast 5% TBE gel (Biorad, Richmond, CA) at 80 V at 4°C in the dark for ~1.5 h. Gels were scanned in the Odyssey Infrared Imaging System (Li-Cor).

Real-time PCR. CHIP findings were confirmed for three promoters of interest using real-time PCR. cDNA was produced using 0.5 μg RNA with a commercially available kit (High Capacity cDNA Re-

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verse Transcription, Applied Biosystems). Amplification reactions contained 25 μg cDNA, 1 μl of TaqMan Gene Expression Assay Mix containing forward and reverse PCR primer and TaqMan probe, and 10 μl of TaqMan Fast Universal PCR Master Mix (Applied Biosystems). Fluorescent-labeled primer pairs for ctgf, voltage-gated potassium channel subunit (KCN5), BK α-subunit (KCNMA), and 18S were obtained from Applied Biosystems. PCR was performed using the Applied Biosystems 7500 Real-Time PCR system according to conditions suggested by the manufacturer and analyzed using the Applied Biosystems 7500 software. The relative quantity of the respective mRNAs was calculated by comparing the cycle number required to reach the exponential phase of amplification against the 18S RNA, which served as the housekeeping genes.

In vitro whole bladder physiology. For in vitro whole bladder cystometry, the bladders were canulated with a 26-gauge needle inserted at the urethral stump and secured in place with a 5–0 Vicryl water-tied suture around the bladder neck; the ureters were ligated with 7-0 Vicryl sutures to prevent leakage (1, 18). The needle was mounted onto a three-way stopcock connected to a pressure transducer and a microperfusion pump (KD Scientific, Holliston, MA). The bladder was immersed into a 5-ml organ chamber containing Tyrode’s solution (in mM: 127 NaCl, 2.7 KCl, 1.8 CaCl2, 0.5 MgCl2, 23.8 NaHCO3, 0.4 NaHPO4, and 5.6 glucose at 37°C) and bubbled with 95% O2-5% CO2 gas. Pressure transducer output was directed to

\[
\text{NFAT} \rightarrow \text{Probe} \rightarrow \text{SS} \rightarrow \text{MEF-2} \rightarrow \text{Probe} \rightarrow \text{Lane 1} \rightarrow \text{Lane 2} \rightarrow \text{Lane 3} \rightarrow \text{Lane 4} \rightarrow \text{NFATc4 antibody).
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Fig. 1. Gel shift assays demonstrating the increase in nuclear factor of activated T-cells (NFAT) and myocyte enhancing factor 2 (MEF-2) nuclear translocation following partial bladder outlet obstruction (pBOO) in nuclear protein isolates from the whole bladder wall. There is a clear shift of these oligonucleotide probes in the pBOO samples compared with the nonoperated (Ctl) and sham (SC) controls. Lane 1 shows the oligonucleotide probe run in the absence of any nuclear protein in the binding assay, and lane 2 demonstrates the loss of signal in the presence of a saturating excess of nonlabeled oligonucleotide probe. Lane 3 demonstrates that with antibody addition, there is a supershift of the MEF-oligonucleotide complex. With NFAT, there was a loss of the oligonucleotide signal when antibody against NFATc3 was added. A similar result was obtained when antibody against NFATc2 was added as shown in the bottom panel (lane 1 = no protein; lane 2 = pBOO; lane 3 = cold probe; and lane 4 = NFATc4 antibody).

Statistical analysis. Results are expressed as means ± SD. Statistical analyses are performed using the ANOVA and Newman-Keuls post hoc analysis with significance set at \( P < 0.05 \).

RESULTS

Using gel shift assays to probe nuclear protein isolates from the entire bladder wall, we observed a substantial rise in the nuclear importation of the two transcription factors that are under the control of calcineurin, NFAT and MEF-2 (Fig. 1). With the application of a saturating dose of “cold” (non-IR-labeled oligonucleotide probe), the gel shift signal was lost implying that the band shown corresponded to the transcription factor of interest. This was also confirmed by the application of an anti-MEF-2 antibody that resulted in a supershift as the antibody complexed with the transcription factor to form a higher molecular weight complex (Fig. 1B). In contrast, the application of the anti-NFATc3 and NFATc4 antibodies led to a loss of the signal similar to that we reported in our prior experience (Fig. 1A) with these antibodies (5, 18). We suspect that in bladder smooth muscle, this antibody recognizes an epitope near the oligonucleotide binding site that results in its displacement, and hence the loss of signal. We also demonstrated a substantial nuclear translocation of both NFAT and MEF-2 within the urothelium (Fig. 2), which supports the concept that the calcineurin pathway is activated by pBOO within this specific cell population of the bladder wall.

Early on in these studies, we used the NFAT-luciferase transgenic mouse as a second independent and quantifiable means of demonstrating NFAT translocation to the nucleus. Within this model, we demonstrated a rise in luciferase activity with pBOO over control and sham bladders that persisted over 1, 2, and 4 wk of obstruction (Fig. 3). Based on the results of this time course study, we the opt to carry out the trial of CsA over a 2-wk time course. Following pBOO with the randomized administration of an ALZET osmotic pump containing either CsA or saline, we demonstrated that the bladder/body mass index dropped substantially in the CsA treatment.
group (Fig. 4A). Survival in our series was 80% and was not affected by the application of CsA. Baseline luciferase activity in sham-operated mice (1,130 ± 133 RLU) increased to 2,010 ± 474 RLU following pBOO in the absence of CsA (P < 0.05; Fig. 4B). However, following pBOO and with the administration of CsA, luciferase levels declined to 562 ± 177 RLU (P < 0.05). This value was below that seen for both vehicle-treated pBOO and sham groups, which suggested that the dose of CsA we administered by Alzet pump was within a therapeutic range.

A myosin heavy chain SM-A/B ratio of 0.07 ± 0.03 was seen in sham bladders, and it increased to 1.04 ± 0.19 follow-

Fig. 3. Following pBOO, there was a statistically significant rise in the relative light units (RLU)/μg of protein expressed over a 1-, 2-, and 4-wk time frame over that seen in control and sham control bladder (+P < 0.05 vs. control and sham groups). The columns represent means ± SD obtained from 8–9 individual bladders in each group. The difference between control and sham controls was not significant.

Fig. 4. A: with pBOO, there was a statistically significant rise in the RLU/μg of protein expressed over that seen in control and sham control bladder that reverted back toward normal with the administration of cyclosporine A (CsA). B: a similar trend was seen when comparing the bladder body mass index (BBMI; defined as bladder mass in mg/animal wt in g). The columns represent means ± SD obtained from 8–9 individual bladders in each group. +Significant difference (P < 0.05) when comparing the pBOO group with the control and sham control groups. *Significant difference (P < 0.05) when comparing the pBOO group with the pBOO group treated with CsA.

Fig. 5. Pattern of myosin heavy chain mRNA expression is shifted from the fast B isoform, which predominates in normal bladder, to the slower A isoform, which increases after partial outlet obstruction. Following pBOO in the presence of CsA, the isoforms shift back toward the more favorable B isoform found in normal bladder. The graphs represent means ± SD obtained from 8–9 individual bladders in each group. The differences between the CsA(−) and CsA(+) groups are significant (P < 0.05) as are the differences between the CsA(−) vs. the sham and control groups (P < 0.01).
tion with electrical field stimulation or KCL (Fig. 6). With the administration of CsA, the bladder’s ability to generate pressure was improved, although not equal to that observed in nonoperated or sham control mice. The administration of CsA improved the bladder’s response to KCL stimulation following pBOO ($P < 0.05$; Fig. 6). While the administration of CsA improved the bladder’s response to electrical field stimulation following pBOO, this difference did not quite achieve statistical significance ($P = 0.054$; Fig. 6).

There are currently five isoforms of NFAT that have been described, and we sought to determine which isoforms were expressed in the murine bladder. By RT-PCR, we were able to demonstrate that the four calcineurin-responsive c1-c4 isoforms are all expressed in the bladder wall (Fig. 7). NFATc3 and NFATc4 were more highly expressed than NFATc1 and NFATc2 mRNA ($P < 0.05$). The expression of NFATc4 exceeded that of NFATc3 ($P < 0.05$). The expression of the NFATc1,3,4 isoforms declined in the presence of pBOO, but the decline was only significant for NFATc4 ($P < 0.05$). NFATc2 mRNA could only be faintly detected when run in the presence of much lower 18S primer concentrations that are very consistent with a low level of expression in the bladder (Fig. 7, inset). Sequencing of the PCR products and comparison with the known cDNA sequences in the National Library of Medicine Gene Bank using the BLAST query revealed a greater than 99% homology with the expected DNA sequences for these isoforms.

The CHIP on CHIP data revealed 269 promoter binding sites for NFATc3 and 43 sites for NFATc4. In this current analysis, we choose to focus on confirming two of these genes. The strongest CHIP signal for NFATc4 was for the connective tissue growth factor (Ctgf) promoter; Ctgf is a cytokine that stimulates the fibrotic response that is such a prominent feature of pBOO. Our analysis of the CHIP on CHIP data for NFATc3 also revealed strong binding to the promoter region of the voltage-gated potassium channel $\alpha$-subunit (KCNS2). We then confirmed the relevance of these CHIP findings by assessing the change in mRNA expression following pBOO in the presence and absence of CsA for these two genes. Real-time PCR revealed a substantial rise in mRNA expression for Ctgf following pBOO (Fig. 8, top) over that seen in control and sham tissues ($P < 0.05$). With the application of CsA, there was a statistically significant decline in the mRNA expression for Ctgf. qPCR for the potassium channel $\alpha$-subunit (KCNS2) revealed a significant rise in mRNA expression following pBOO that was reduced by the administration of CsA ($P < 0.05$; Fig. 8, bottom).

Neither NFATc3 nor NFATc4 showed binding to the promoter for the BK channel $\alpha$-subunit that was suggested by Layne et al. (19) to be under control of NFATc3, although they

Fig. 6. In vitro whole bladder physiology showing the responses to electrical field stimulation during saline infusion. Once the optimal volume for maximal pressure generation was reached, the Tyrode buffer was exchanged for a high-K$^+$ Tyrode buffer, and a final pressure generated was recorded. The graphs represent means ± SD of the maximal pressure obtained from 10 individual bladders in each group in response to KCl and electrical field stimulation. The final peak in each tracing represents the response to KCl. There was no difference between the control or sham groups. However, pBOO resulted in a significant drop in pressure in the absence of CsA (CsA−) when compared with control and sham groups ($P < 0.05$). *Significant difference ($P < 0.05$) when comparing the pBOO group with the control and sham control groups. #Significant difference ($P < 0.05$) when comparing the pBOO group with the pBOO group treated with CsA. For the responses to electrical field stimulation, the differences (*) between the CsA− and CsA+ groups approached significance ($P = 0.054$). Representative tracings are shown for all 4 groups.
acknowledged in their paper that this effect might be due to either a direct action of NFAT on the promoter or as a result of a secondary interaction. Although we did not identify NFATc3 binding to the BK channel promoter in our CHIP experiments, we opted to also study the impact of CsA administration on the mRNA expression of this ion channel subunit. We demonstrated that following pBOO BK channel subunit mRNA expression remained unchanged, and its expression was not altered by the administration of CsA (Fig. 8, bottom).

Immunohistochemical staining for NFATc3 and NFATc4 revealed that these isoforms were expressed in both the smooth muscle bundles (Fig. 9) as well as the urothelium (Fig. 10) as shown by the colocalization images; these findings correlate with the data from the gel shift assays shown in Figs. 1 and 2. The specificity of these antibodies was shown by the absence of signal noted when the sections were imaged in the presence of secondary antibody with the absence of the primary antibody (Fig. 9). We showed the images for the bladders subjected to pBOO; similar images were noted for control and sham bladder tissues.

DISCUSSION

In our initial work looking at the potential role for calcineurin activity in rabbit bladder wall remodeling following pBOO, atrial natriuretic factor (ANF) mRNA expression served as a surrogate marker for activation of this pathway (8). Early on, we sought to characterize the calcineurin pathway in our model of murine pBOO, but ANF mRNA is not expressed in obstructed murine bladders. Using gel shift assays, we demonstrated that two of the transcription factors under calcineurin control were upregulated following pBOO relative to the expression seen in sham control and control bladders. The use of cold probes and antibodies allowed for confirmation that these shifts of the oligonucleotide probe did correspond to the transcription factors NFAT and MEF-2. We also showed that calcineurin is activated in the urothelium. This finding is significant because it raises the possibility that this pathway may play an important role in epithelial mesenchymal signaling. Perhaps it contributes via paracrine signaling to the substantial DNA synthesis taking place in the lamina propria following pBOO (18).

The rise in the nuclear translocation of NFAT was also confirmed using a second independent method by carrying out pBOO in a transgenic mouse developed by Wilkins and Molkentin (30) that contained a ubiquitously expressed NFAT-luciferase reporter construct. In this particular transgenic construct, nuclear translocation of NFAT will result in the transcription and translation of luciferase whose activity is readily measured, and which serves as a reporter for activation of the
calcineurin-NFAT pathway. We showed that luciferase activity nearly doubled in mice subjected to pBOO relative to their sham-operated or control littermates and that this activity is sustained as shown in the time course study. Furthermore, this increase was diminished by inhibition of calcineurin with CsA delivered by Alzet pumps at volumes of 0.25 l/h, which would correspond to doses of 10 mg·kg⁻¹·day⁻¹. While this dose is lower than the 15-mg/kg twice daily dosing via an intraperitoneal approach used in the murine cardiac trials, this protocol resulted in a drop in luciferase activity below that seen in the sham controls. This large drop in the presence of persisting obstruction suggests that CsA is effectively inhibiting calcineurin at the treatment dose of 10 mg·kg⁻¹·day⁻¹.

As seen with other experimental models, pBOO resulted in varying degrees of bladder wall hypertrophy, which is reflected in the standard errors we recorded (16, 23). These large standard errors lead to a higher number of mice being needed to show significance in a drug trial such as the one we conducted. In a rabbit model of pBOO, bladder mass declined in the presence of CsA administered by injections twice daily, but did not quite achieve statistical significance (8). In contrast, in this current study, bladder hypertrophy secondary to obstruction was significantly diminished with CsA (P < 0.05). We suspect this is because in this set of experiments, the CsA was delivered via osmotic pump and thus achieved a stable therapeutic level.

The calcineurin-NFAT pathway also impacted on myosin heavy chain isoform mRNA expression. The B isoform that predominates in the normal urinary bladder is the fast isoform and has been associated with a higher ATPase activity as well as a higher velocity of contraction (2, 3). With pBOO, a shift occurs and the slower tonic myosin heavy chain A isoform begins to predominate (10). CsA treatment led to enhanced expression of the SM-B isoform even in the presence of pBOO.

![Fig. 9. Immunohistochemistry showing the NFAT isoform distribution within the bladder wall following pBOO at ×400. Staining for NFATc3 and NFATc4 reveals evidence of staining within the muscle bundles and interstitial cells (M) as well as the urothelium (U). Smooth muscle α-actin was delineated by the use of a FITC-conjugated 2° antibody (green), and the NFAT isoforms were detected by the use of a secondary antibody coupled to Texas Red. Nuclei were stained with DAPI (blue). Color merging revealed expression of NFAT isoforms within the smooth muscle cells as well as within the urothelium. Similar distributions were seen in control and sham control bladders.](http://ajprenal.physiology.org/)

![Fig. 10. Immunohistochemistry showing the NFAT isoform distribution within the urothelium following pBOO at ×400. NFATc3 and NFATc4 (red) were detected within the urothelium just below the intense stain for uroplakin II (green). Nuclei were stained with DAPI (blue). Color merging revealed expression of both NFAT isoforms within the urothelium. Similar distributions were seen in control and sham control bladders.](http://ajprenal.physiology.org/)
It is important to note that in two different models of pBOO, and using three different measures of calcineurin activity, there is strong evidence to suggest that this transition from the smooth muscle MHC B to A isoform is in part mediated by calcineurin, and it is blocked using CsA (8). The exact molecular mechanism(s) whereby NFAT (perhaps acting in conjunction with other transcription factors such as MEF-2) can mediate this MHC isoform shift remains a focus of our current investigation.

This study demonstrated that in vitro whole bladder performance was improved with CsA administration following pBOO. Both the responses to electrical field stimulation as well as direct stimulation with KCl demonstrated that pBOO led to a decline in pressure generation compared with controls or sham-operated controls. In contrast with CsA administration, there was an improvement in the in vitro contractile performance of these bladders as measured using the whole organ model. This improved physiology can be correlated with the shift in the myosin heavy chain mRNA expression back toward the more normal B isoform. We showed in multiple studies, that the most severe outlet obstruction as measured by increases in bladder mass as well as declines in velocity of contraction is associated with increased expression of the slower A isoform (8). Following reversal of the outlet obstruction, bladder mass falls back toward normal, the voiding phenotype improves, and the mRNA expression pattern reverts back to the B isoform (10). The mechanism by which overexpression of the myosin A isoform leads to detrusor malfunction remains unclear. It is clear that following pBOO, rabbit bladder smooth muscle strips are able to maintain the same isometric peak force compared with sham- or nonoperated controls (27).

However, when these fibers were released under isotonic conditions, the strips taken from these obstructed bladders showed a 10-fold drop in velocity of shortening (27). This is critical since in vivo, the bladder smooth muscle must not only be able to generate force, but it must shorten. If the velocity of shortening is markedly diminished, then the maximal power that a bladder can generate is also going to decline substantially as well. In clinical practice, urologists can calculate power by simultaneously measuring the intravesical pressure and the flow rate during micturition. This measurement is not adaptable to the murine model at this time. The in vitro whole organ bladder preparation relies upon a 26-gauge needle through which intravesical pressures can be recorded accurately; however, its small luminal diameter offers too much resistance for the system to be allowed to empty so as to measure maximal power generation. As of this time, the in vitro whole organ bladder only allows for determination of the bladders contractile performance under isotonic conditions.

In this study, we also described the presence in the bladder of mRNA for all four calcineurin-responsive NFAT isoforms using RT-PCR. We did not probe for the NFATc2 isoform that has been characterized as being independent of calcineurin regulation and is responsive to osmotic changes (20) and focused on those isoforms that have been described in striated or smooth muscle systems. NFAT isoforms C1 through C4 are all present in normal bladder wall, but NFATc2 has the lowest level of expression. NFATc2 mRNA expression could only be detected by significantly lowering the concentration of 18S primers in the reaction that implies that it is present in very small amounts. These data correlate nicely with the work of Layne et al. (19), who using single smooth muscle cell preparations were able to show expression of the NFATc1, NFATc3, and NFATc4 isoforms. While Layne et al. showed an absence of NFATc2, it is important to point out an important distinguishing feature between our studies; they relied on a study of isolated smooth muscle cells, whereas we relied on RNA sampling from the whole bladder. As with the Layne paper, we also showed a predominance of the expression of NFATc3, and NFATc4 isoforms, and this finding guided us in designing our chromatin immunoprecipitation experiments. In contrast with the work of Layne, we also demonstrated that with pBOO, there is a drop in the expression of these isoforms at the mRNA level that we feel reflects the increase in total RNA synthesis that is seen with pBOO.

Based on our study of the mRNA isoform distribution and based on prior work in the literature, we opted to carry out a CHIP on CHIP analysis to identify which promoters the NFATc3 and NFATc4 isoforms are binding to. We opted to focus our work on these targets over MEF-2 because this transcription factor is also under the control of the histone deacetylases and the CamIIKinase pathways. Our analysis revealed 269 promoter sites that were binding NFATc3 and 43 promoter sites binding NFATc4. We then chose to focus our attention on two genes whose promoters were bound by these isoforms. The promoter element for Ctgf was bound by NFATc4 with the highest statistical significance based on the MAT analysis algorithm. We were also curious to see whether any potassium channels were targets and noted that NFATc3 was highly associated with binding at the promoter site for the voltage-gated potassium channel α-subunit (KCNS2). In both of these instances, we confirmed the role of the calcineurin-NFAT pathway in their regulation by demonstrating a rise in their expression following pBOO, and a decline in their expression when treated with CsA.

The significance of the calcineurin-NFAT pathway triggering Ctgf expression is that this cytokine is felt to trigger the expression of extracellular matrix elements that are a prominent feature of the bladder’s response to pBOO. As these elements are deposited within the bladder wall, the viscoelastic properties change with a loss of compliance that places the upper urinary tract at risk of structural damage. The ability to manipulate this pathway and minimize bladder wall fibrosis is of translational relevance. While the administration of CsA led to a drop in Ctgf mRNA expression, it did not completely eliminate it. This can be explained by the findings of Chaqour et al. (6) who demonstrated that mechanical stretch leads to a rise in Ctgf and that the Ctgf promoter contains a binding site for the NF-κB transcription factor.

Based on the work of Layne et al. (19) who studied the smooth muscle phenotype of the NFATc3 knockout mouse, we expected to find that the BK α-subunit would contain a binding site for NFATc3. In fact, our CHIP on CHIP analysis showed that the BK channel promoter was not bound by either NFATc3 or NFATc4. This finding was confirmed by our observation that this particular subunit was unresponsive to pBOO and CsA. This discrepancy between our findings can be explained on the basis of our differing models; Layne et al. were studying the NFATc3 knockout mouse and using isolated smooth muscle cell preparations, whereas we were studying the whole bladder following surgically induced pBOO in a wild-type mouse. While Layne et al. clearly demonstrated an increase in...
BK channel expression and ion channel activity as measured by current inhibition with iberiotoxin, they acknowledged that these findings may result from secondary compensatory changes as a result of the genetic deletion and might not reflect direct binding of NFATC3 to the BK promoter.

This paper also differs from the work of Layne et al. in that we studied the distribution of these NFAT isoforms in the intact whole bladder wall. Our immunohistochemical findings demonstrate that NFATC3 and NFATC4 are expressed in the smooth muscle and interstitial cells as well as the urothelial compartments of the bladder wall. This finding correlates nicely with our gel shift assay that demonstrated that there was a marked nuclear translocation of NFAT within the urothelial fraction. This activation of the calcineurin-NFAT pathway within the urothelium is of clinical significance because patients with neurogenic bladders secondary to spina bifida or spinal cord injury must perform intermittent catheterization, which would allow for drug delivery while avoiding systemic CsA toxicity.

For any patient with anatomic obstruction, surgical relief of the obstruction will always be warranted as the initial management. However, up to 30% of patients are left with residual symptoms and hence improved medical management strategies are needed. This treatment has even more potential applicability for patients with neurogenic bladders secondary to spina bifida or spinal cord injury where progressive fibrosis develops that is especially prominent within the lamina propria (11). From a basic science perspective, it provides the rationale for our using a tetracycline-responsive promoter approach to selectively drive calcineurin expression that is spatially localized to the urothelium or to the smooth muscle so as to establish a proof of concept that is independent of the need for surgical manipulation.

These data suggest that treatment with CsA can partially reverse some of the molecular consequences of pBOO when administered at the time the obstruction is created. While encouraging, these preliminary studies do not reveal the mechanism by which calcineurin inhibition leads to these changes. Is this CsA effect primarily driven by its effect on smooth muscle, or within the urothelium? This model system is an ideal one in which to begin to answer these questions before such interventions can be considered ready for a clinical trial in the treatment of clinical bladder hypertrophy seen in patients with pBOO, spina bifida, or spinal cord injury.

In conclusion, we demonstrated that the calcineurin-NFAT pathway is activated following pBOO, within the smooth muscle as well as the urothelium. We also showed that CsA administration can result in a bladder phenotype that demonstrates less hypertrophy, improved myosin heavy chain isoform expression, and improved in vitro whole bladder performance.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


