

# Alterations in Caveolin Expression and Ultrastructure After Bladder Smooth Muscle Hypertrophy

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**Purpose:** Partial bladder outlet obstruction in male rabbits causes detrusor smooth muscle hypertrophy and voiding dysfunction similar to that observed in men with benign prostate hyperplasia. Using this model, we analyzed the protein expression and ultrastructure of caveolae and the intermediate size filament in detrusor smooth muscle following partial bladder outlet obstruction induced hypertrophy.

**Materials and Methods:** Detrusor smooth muscle sections from bladder body were processed for immunofluorescence and electron microscopy. Western analysis was performed to determine the expression of caveolin isoform-1, 2 and 3, and intermediate size filament proteins.

**Results:** Detrusor smooth muscle cells from both normal and hypertrophied bladders contain orderly arrays of thick and thin myofilaments, interspersed with dense bodies. In addition, there was an increase in intermediate size filaments in the hypertrophic detrusor smooth muscle cells. The dense plaques in the inner membrane of hypertrophied detrusor smooth muscle were longer than those of the control. Detrusor smooth muscle from hypertrophied bladder revealed a decreased number of caveolae and a lack of their orderly distribution at the plasma membrane. Western blotting showed decreased expression of caveolin-1, 2 and 3 in hypertrophied detrusor smooth muscle.

**Conclusions:** Caveolae serve as platforms for proteins and receptors that have a role in signal transduction. The decreased number of caveolae and caveolin protein expression in hypertrophied detrusor smooth muscle might contribute to alterations in signal transduction pathways that regulate the downstream effects of agonist induced contraction, including calcium sensitization, observed in obstructed bladder. In addition, the increased number of intermediate size filaments in the hypertrophied detrusor smooth muscle is likely to alter the cytoskeletal structure and affect the cellular transmission of passive and/or active force.

**Key Words:** urinary bladder; muscle, smooth; caveolae, caveolins; myosins; intermediate filaments

FOLLOWING bladder outlet obstruction, DSM undergoes compensatory hypertrophy in an attempt to empty the bladder against the obstruction. Benign prostatic hyperplasia is a common disorder in aging men, causing PBOO and DSM hypertrophy. Despite the DSM

hypertrophy, dysfunction persists in some bladders, showing increased voiding frequency, decreased voided volume and increased post-void residual urine. The rabbit model of PBOO also reveals DSM remodeling and bladder dysfunction. Detrusor remodeling includes al-

## Abbreviations and Acronyms

DSM = detrusor smooth muscle

EM = electron microscopy

IF = intermediate size filament

PAGE = polyacrylamide gel electrophoresis

PBOO = partial bladder outlet obstruction

PBS = phosphate buffered saline

PVDF = polyvinylidene

SDS = sodium dodecyl sulfate

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tered expression of various regulatory and contractile proteins. Expression of myosin isoforms,<sup>1</sup> thin filament associated protein caldesmon,<sup>2</sup> and RhoA-activated kinase, an enzyme involved in the calcium sensitization pathway,<sup>3</sup> are altered in detrusor remodeling.

Previous studies of the ultrastructure of smooth muscle showed that smooth muscle contractile proteins (myosin and actin) form filaments as in striated muscle, but the myosin-containing thick filaments and the actin-containing thin filaments are not organized into sarcomeric structural units.<sup>4,5</sup> Other distinctive structural elements in smooth muscle are dense bodies, which are analogous to Z-bands in striated muscle, distributed throughout the cell and dense plaques at the plasma membrane, serving as attachment sites for actin filaments.

In addition to cytoplasmic filaments, consisting of contractile proteins that form the contractile apparatus, smooth muscle contains IFs composed of desmin and vimentin.<sup>6</sup> In addition to a role in maintaining cell shape, IFs are thought to be actively involved in the transmission of contractile force.<sup>6</sup> Previous studies of obstruction induced changes showed an increase in IF proteins.<sup>7</sup>

EM studies of smooth muscle cell membrane showed caveolae, flask-shaped invaginations of the plasma membrane between 50 and 100 nm in diameter.<sup>8</sup> Caveolins, the main membrane proteins of caveolae, are required for the formation of caveolae.<sup>9</sup> The caveolin family proteins consist of 3 isoforms, caveolin-1 and caveolin-2, which are present in most cell types, but are enriched in adipocytes, endothelial cells and myocytes,<sup>9</sup> and caveolin-3, which is muscle specific.<sup>10,11</sup> Previous studies from our laboratory showed that PBOO induced DSM hypertrophy is associated with altered signal transduction (eg via RhoA/RhoA-activated kinase-mediated calcium sensitization).<sup>3</sup> We thought it would be of interest to determine whether altered signal transduction is associated with changes in caveolae and caveolin expression in hypertrophied DSM.

We report that the hypertrophied DSM contains well-organized myosin and actin filaments, as observed in normal DSM, but the hypertrophied DSM cells revealed an abundance of IFs compared to normal DSM. Furthermore, EM and immunofluorescence analyses revealed alteration in the structure of caveolae and the expression of caveolin isoforms in hypertrophied DSM.

## MATERIALS AND METHODS

### Rabbit Bladder Surgical Obstruction

PBOO was surgically induced by partial ligation of the urethra in adult (2.5 to 3 kg) male New Zealand White rabbits, as previously described,<sup>3</sup> by a procedure approved

by the Children's Hospital of Philadelphia and University of Pennsylvania institutional animal care and use committees. Preliminary studies showed no difference between normal (unoperated) and sham operated rabbits. Thus, normal rabbits served as controls. Mean  $\pm$  SD weight of the obstructed rabbit bladder was  $10.05 \pm 0.42$  gm compared to  $2.17 \pm 0.17$  gm in control rabbits. Bladder function was measured by keeping the animals in metabolic cages 24 hours before sacrifice.<sup>1</sup> Severely dysfunctional bladders from obstructed rabbits showed increased voiding frequency ( $43 \pm 13$  vs  $4 \pm 3$  for control) and decreased voided volume ( $2.5 \pm 1.0$  vs  $26.0 \pm 16.0$  cc for normal). The bladder dysfunction did not always correlate with bladder mass in the obstructed group. Thus, measuring bladder function is crucial to determine the pathophysiology of the obstructed bladder. In this study, DSM tissue from control and severely dysfunctional obstructed rabbits (4 from each group) were removed free of serosal and urothelial layers, and used for immunofluorescence, EM and Western analyses.

### Electron Microscopy

For EM examination, DSM tissues taken from the bladder body were cut into small ( $1 \times 1$  mm) pieces, fixed in 3.5% glutaraldehyde solution in 0.1 M Na-cacodylate buffer (pH 7.4) and subjected to a standard EM procedure, as previously described.<sup>12</sup> Ultrathin sections were stained in uranyl acetate and in a mixture of lead salts, and observed using a 410 transmission EM (Philips Medical Systems, Andover, Massachusetts). Images were scanned and analyzed.<sup>13</sup>

### Western Analysis of Proteins

Protein extracts in SDS sample buffer from control and obstructed bladders were separated by SDS-PAGE and transferred to PVDF membranes.<sup>1</sup> Membranes were probed with antibodies, including caveolin-1, 2, and 3 (each dilution 1:1,000, BD Transduction Laboratories™). Proteins were detected with horseradish peroxidase-conjugated anti-mouse secondary antibody (dilution 1:5,000, Amersham Biosciences, Little Chalfont, United Kingdom) and visualized with enhanced chemiluminescence.

### Immunofluorescence and Confocal Microscopy

DSM tissue was fixed in formalin (10% phosphate buffered) and embedded in paraffin. Sections ( $5 \mu$ ) were deparaffinized, treated with descending grades of ethanol and washed several times in PBS (pH 7.4). Sections were blocked with 5% bovine serum albumin in PBS for 1 hour at 25C to minimize nonspecific binding. Sections were treated with caveolin-1 (mouse monoclonal) antibody overnight at 4C using 1:100 dilution of the antibody in 1% bovine serum albumin solution made in PBS. After several rinses, sections were treated with secondary antibody (anti-mouse IgG) conjugated to Cy3 (Sigma®), rinsed several times with PBS and mounted with UltraCruz™ mounting medium. Negative controls were made by treating the section as described, except the primary antibody was preabsorbed with caveolin-1 peptide (Abcam®). Sections were examined elsewhere using a TCS SP2 confocal microscope (Leica, Solms, Germany).

### Quantitation of Protein Expression

The amount of protein in the tissue extract was analyzed by Western blotting using specific antibodies and the protein bands were quantified by scanning densitometry using Quantity One®, version 4.6. The intensity of protein bands on Western blots was adjusted to be within the linear range of the optical density by loading the correct amount of protein extract on the gel. As an internal standard,  $\alpha$ -smooth muscle actin was used for protein loading and for protein quantization.

### Statistical Analysis

Based on sample size analysis, 4 animals in each group were found to be sufficient to show a difference between control and obstructed animals at a power greater than 0.9 for Western blot analysis. Sample size calculation was based on preliminary data suggesting a difference between caveolin expression between control and obstructed rabbits with  $\alpha = 0.05$  and power = 0.9. The bands on the Western blots were quantified and statistically analyzed using the Student *t* test (4). Differences were considered significant at  $p < 0.05$ . JMP® 7 was used.

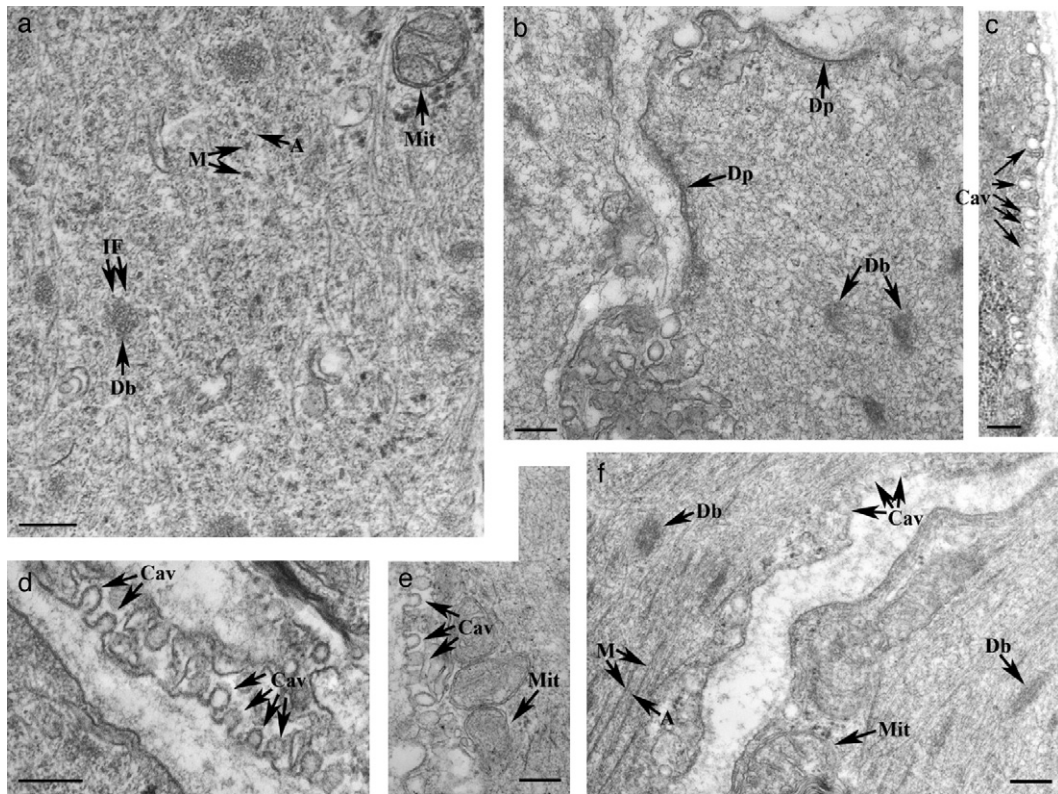
## RESULTS

### Bladder Smooth Muscle Ultrastructure

High magnification EM images of bladder smooth muscle from control rabbit showed myosin-contain-

ing thick (15 to 20 nm) and actin-containing thin (6 to 7 nm) filaments distributed throughout the cell. **Figure 1**, *a* and *f* show cross and longitudinal sections. There were several dense bodies throughout the cells connected to IFs (10 nm) and actin filaments (**fig. 1**, *b*). Dense plaques were well-defined at the inner cell surface (**fig. 1**, *b*). **Figure 2** shows high magnification EM images of hypertrophied DSM from a 2-week obstructed bladder. IFs were more numerous in the hypertrophied DSM cells from obstructed bladders than from normal bladders (**fig. 2**, *f* and *g*). IFs meet dense bodies (**fig. 2**, *g*), and may be seen to start from the dense bodies, but it is not clear whether they go through the dense bodies. In some areas, connection of dense bodies to actin filaments and IFs may be visualized. Myosin filaments, surrounded by actin filaments, were abundant in both longitudinal and cross-sectional views (**fig. 2**, *a*, *b* and *e*).

Caveolae were distributed at the plasma membrane, often forming a row of caveolae in normal DSM cells (**fig. 1**, *c* to *e*). Caveolae were not well formed and were sparse, appearing as rows of 1 to 5, in the DSM from obstructed bladder (**figs. 1**, *d* and *2*, *d*).



**Figure 1.** Cross-sectional (*a* to *e*) and longitudinal (*f*) views of high magnification EM images of bladder smooth muscle from control rabbit. Dense bodies (*Db*) are connected to IFs and actin-containing thin filaments (*A*). Caveolae (*Cav*) often form rows (*c* to *e*). Some myosin filaments have smaller diameter because of lower magnification (*b*). *M*, myosin-containing thick filaments. *Mit*, mitochondria. *Dp*, dense plaques. Scale bars represent 0.2  $\mu\text{m}$ .

**Figure 2.** Longitudinal (*a* and *b*) and cross (*c* to *g*) section high magnification EM views of hypertrophied bladder smooth muscle from 2-week PBOO rabbit. IFs filaments are more numerous (*c* and *f*) than in control tissue (*fig. 1, b*). Dense plaques (*Dp*) are long at plasma membrane (*c*) and caveolae (*Cav*) are less frequent (*d*). Few dense bodies (*Db*) are connected to actin-containing thin filaments (*A*) and IFs (*g*). *M*, myosin-containing thick filaments. *Mit*, mitochondria. *cf*, collagen fibrils. Scale bars represent 0.2  $\mu\text{m}$ .

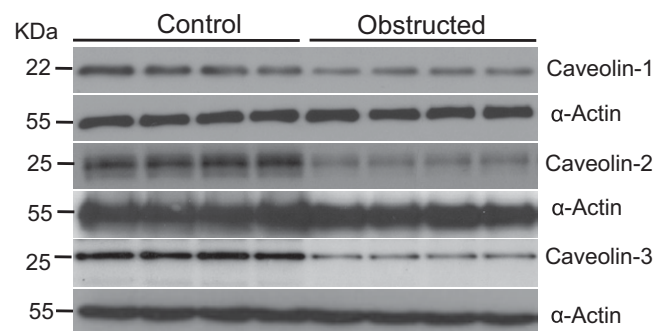
### Altered Caveolin Protein Expression in PBOO Induced DSM Hypertrophy

To determine whether the low number of caveolae present in the hypertrophied smooth muscle was associated with a low amount of caveolin, we performed Western blot analysis for the presence of 3 caveolin isoforms, caveolin-1, 2 and 3. The antibodies to caveolin-1, 2 and 3 recognize proteins with a molecular size of 22, 25 and 25 kDa, respectively. The expression of all 3 caveolin proteins in the DSM from obstructed bladders was decreased compared with control values (*fig. 3*). As a loading control,  $\alpha$ -smooth muscle actin was used. The quantification of the caveolin isoforms from all the experiments was normalized to  $\alpha$ -actin (*fig. 4*). All caveolin isoforms were significantly decreased, although the decrease in caveolin-3 was more prominent than that of other isoforms.

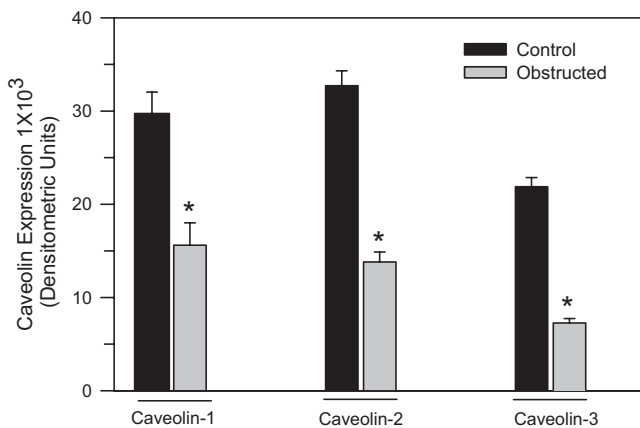
### Caveolin-1 Immunofluorescence and Confocal Microscopy in Control and Obstructed Rabbit Bladder DSM

Caveolin-1 is the major component of caveolae and it has been shown that it is required for the formation of caveolae. The bladder smooth muscle from transgenic mice lacking caveolin-1 expression showed a decreased number of caveolae.<sup>11</sup> Because caveolin-1 seems to be the major caveolin protein required for

the formation of caveolae, we performed immunofluorescence microscopy to determine the localization of caveolin-1 in the DSM tissues. Caveolin-1 was distributed at the plasma membrane of the muscle bundles in control and hypertrophied bladders (*fig.*



**Figure 3.** Altered caveolin protein expression during rabbit bladder smooth muscle hypertrophy induced by PBOO. Equal amount of protein samples (50  $\mu\text{g}$ ) from control and 2-week obstructed rabbit bladder smooth muscle were separated on 12% SDS-PAGE and transferred to PVDF membrane. Membranes were probed with caveolin-1, 2 and 3 antibodies. Blots were stripped and reprobbed with  $\alpha$ -smooth muscle actin antibody as loading control. Expression of all 3 caveolin proteins decreased in obstructed tissue compared to that in control tissue.



**Figure 4.** Caveolin protein isoforms were quantified in DSM using scanning densitometric analysis in protein extract from control and obstructed rabbit bladder smooth muscle tissue samples. Caveolin protein expression was normalized to  $\alpha$ -actin, which served as loading control. Control and 2-week obstructed samples were run on same gels. Protein bands from blots were quantified separately and presented in densitometric units [optical density (intensity  $\times$  mm<sup>2</sup>)]. Optical density represents values used to calculate SD and error bars. Expression of each caveolin isoform decreased following PBOO. Asterisk indicates significantly different in obstructed detrusor vs controls ( $p < 0.05$ ).

5, *c* and *h*). Confocal microscopy revealed large numbers of orderly arranged caveolae on the surface membranes of the normal smooth muscle (fig. 5, *a*), whereas the intensity of the caveolin-1 antibody binding was less and the caveolae were sparse in the

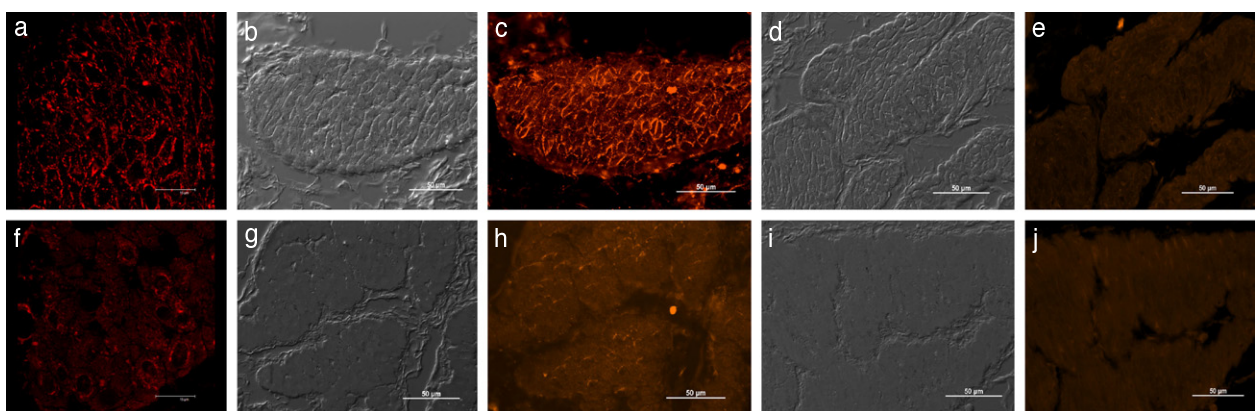
DSM from obstructed bladder (fig. 5, *f*). When the antibody was pre-absorbed with immunizing caveolin-1 peptide, the caveolin-1 staining was abolished in DSM sections from control and 2-week obstructed bladders (fig. 5, *e* and *j*). These findings correlate with the results of EM and Western blot analysis. Confocal microscopic images showed a punctated distribution of caveolin-1 at the plasma membrane in both types of tissue, which correlated to the caveolae seen on EM (fig. 1, *c* to *e*).

#### Altered IF Protein Expression in PBOO Induced DSM Hypertrophy

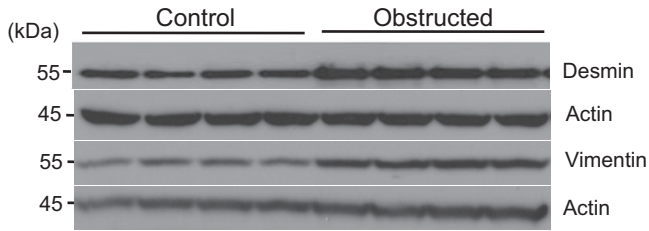
To determine whether the increased number of IFs present in the hypertrophied smooth muscle was associated with an increased amount of IF proteins, we performed Western blot analysis for desmin and vimentin. The antibodies to desmin and vimentin recognize proteins with a molecular size of 55 kDa. The expression of desmin and vimentin in the DSM from obstructed bladders was increased compared with that in controls (fig. 6). The quantification of desmin and vimentin was normalized to  $\alpha$ -smooth muscle actin, which served as a loading control (fig. 7).

## DISCUSSION

The ultrastructural changes in the smooth muscle of the bladder wall during outlet obstruction in rats were described previously.<sup>5</sup> In the current study we describe ultrastructural changes in the bladder wall



**Figure 5.** In situ localization of caveolin-1 in smooth muscle from control and obstructed bladders. Sections (5  $\mu$ ) were made of formalin fixed, paraffin embedded muscle tissues from control (*a* to *e*) and obstructed (*f* to *j*) bladders, and processed for immunofluorescence microscopy using antibody against caveolin-1. Differential interference contrast (*b*, *d*, *g* and *i*) was used to show muscle bundles in fields reacted with antibody (*c*, *e*, *h* and *j*). Note immunofluorescence micrograph (*c*, *e*, *h* and *j*) and confocal microscope (*a* and *f*) images. Immunofluorescence (*c* and *h*) revealed caveolin localization on muscle bundles (*b* and *g*). High resolution confocal images of normal smooth muscle (*a*) show punctated plasma membrane localization of caveolin-1, correlating with caveolae distribution at plasma membrane. Note decreased staining pattern in obstructed DSM (*f* and *h*). Negative control prepared using antibody pre-absorbed with peptide used to raise antibody from normal (*e*) and obstructed (*j*) preparations did not stain significantly vs counterparts. Confocal microscopy negative controls (*a* and *f*) were performed similar to others (*a* to *d* and *e* to *h*). Scale bars represent 15 (*a* and *f*) and 50 (*b* to *e* and *g* to *j*)  $\mu$ m. Reduced from  $\times 40$  (*c*, *e*, *h* and *i*) and  $\times 100$  (*a* and *f*).



**Figure 6.** Altered desmin and vimentin expression during rabbit bladder smooth muscle hypertrophy induced by PBOO. Equal amount of protein samples (50  $\mu$ g) from control and 2-week obstructed rabbit bladder smooth muscle were separated on 10% SDS-PAGE and transferred to PVDF membrane. Membranes were probed with desmin and vimentin antibodies. Blots were stripped and reprobed with actin antibody as loading control. Expression of desmin and vimentin proteins increased in obstructed tissue compared to that in control tissue.

smooth muscle following obstruction induced DSM hypertrophy with special emphasis on the cytocontractile and cytoskeletal filaments, and the expression of caveolin isoforms and the formation of caveolae. We noted a decrease in caveolae and caveolin isoform expression in obstruction induced detrusor hypertrophy. Our finding of an increase in IF proteins and IFs in hypertrophied DSM is in agreement with a previous report of rat bladders subjected to obstruction.<sup>7</sup> Caveolae and caveolins are implicated in many cellular processes, including signal transduction.<sup>14</sup>

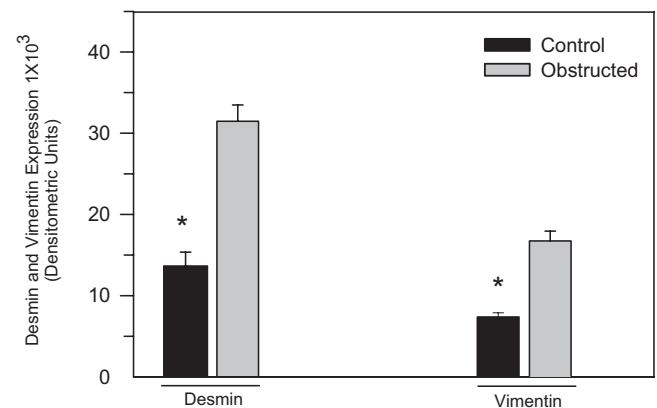
In the hypertrophic guinea pig intestinal smooth muscle, the spatial density of caveolae was marginally smaller than in controls.<sup>15</sup> On the other hand, in the hypertrophic guinea pig ileum, rows of caveolae were similar in shape and size compared to controls.<sup>16</sup> Previous studies of the rat bladder also found no difference between hypertrophic and normal rat bladders in the caveolar structure and distribution.<sup>5</sup> Data in the current study demonstrate a decrease in caveolae and caveolin protein isoforms (figs. 2 to 4). It is not clear whether the lack of changes in the caveolae following smooth muscle hypertrophy in the previous report was due to species or gender difference. In this regard, it is important to note that the bladders used in the current study were severely dysfunctional based on bladder function (increased voiding frequency, decreased volume per void and increased urinary retention). It is possible that the obstruction induced bladder dysfunction as well as the caveolar changes may be corrected by compensatory changes if the obstructed animals were kept for a longer duration than the 2 weeks used in this study. In subsequent experiments, we kept obstructed rabbits up to 5 weeks after obstruction. The bladder function in some animals improved slightly and the protein composition

in these animals has not been analyzed (unpublished data).

The possibility that the observed decrease in the number of caveolae is due to an increase in the cell surface volume due to cellular hypertrophy cannot be ruled out without accurately measuring the cell surface increase and counting the number of caveolae on the membrane. If the decrease in the number of caveolae were due to increased cell surface, the decrease in the caveolin protein in hypertrophied smooth muscle observed in this study must have come from caveolin that is not associated with caveolar structure, since caveolin protein estimation is standardized to extractable protein and  $\alpha$ -actin, which served as an internal loading control. To our knowledge there are no data to date to show that the caveolin unassociated with caveolar vesicular structure is altered by DSM hypertrophy.

Depletion of caveolae and an increased number of dense bodies were also observed in aging human DSM from dysfunctional bladders.<sup>17</sup> The effect of disruption of caveolae on the contractile response depends on the agonists, with no effect on carbachol and KCl induced contractions, whereas contractile responses to angiotensin II and serotonin were attenuated, and responses to bradykinin and phenylephrine were augmented.<sup>18</sup>

The other major structural observation in our study is the increase in the frequency of IFs in the DSM from PBOO bladders (fig. 2). Western blotting analysis also showed increases in desmin and vimentin protein levels (figs. 6 and 7). Our observations are in agreement with an earlier study of



**Figure 7.** Desmin and vimentin proteins were quantified in DSM using densitometric scanning analysis in control and obstructed rabbit bladder smooth muscle tissue samples, as described (fig. 4). Desmin and vimentin protein expression was normalized to actin, which served as loading control. Expression is shown in densitometry units from protein bands. Desmin and vimentin expression increased after PBOO. Asterisk indicates significantly different in 4 obstructed detrusors vs 4 controls (Student's *t* test  $p < 0.05$ ).

hypertrophic guinea pig intestinal smooth muscle by Gabella showing a consistent increase in the number of IFs.<sup>16</sup> The function of the IF system in smooth muscle is not well understood. In hypertrophic smooth muscle of obstructed rat bladder, desmin was identified as a major component of the IFs, and the concentration of desmin was increased in this tissue.<sup>7</sup> Although desmin filaments are not required for force generation and maintenance, the maximum velocity for force generation by Des<sup>-/-</sup> detrusor is slightly lower than that of Des<sup>+/+</sup> both for normal and obstructed bladders.<sup>19</sup> However, studies in mice lacking desmin showed that desmin filaments are not required for force generation and maintenance of force by the DSM, but they appear to have a role in cellular transmission of both active and passive force.<sup>19</sup>

## CONCLUSIONS

Caveolae and caveolin protein levels are decreased in bladder muscle from rabbits with PBOO. All 3

caveolin isoforms are expressed in rabbit DSM, and the obstruction induced DSM hypertrophy and bladder dysfunction are associated with a decrease in caveolin protein level and caveolae formation. These changes might underlie the altered signal transduction events and agonist induced calcium sensitization associated with obstruction induced contractile dysfunction in the bladder. The increased expression of IF proteins and filaments is likely to alter the cytoskeletal structure and transmission of force.

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