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An early molecular response induced by acute overdistension of the rabbit urinary bladder

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Abstract

In the rabbit, partial urinary bladder outlet obstruction rapidly increases bladder mass and alters several parameters of normal bladder function. Previous experiments on this animal model (Buttyan *et al.* Neurourol. Urodyn. 11: 225–238, 1992) had identified an early molecular response to partial outlet obstruction that involved the profound induction of mRNA encoding a heat-shock gene, hsp-70, as well as induced expression of mRNA for basic fibroblast growth factor and certain protooncogenes. Numerous physiological studies of the hypertrophied rabbit bladder indicate that the primary stimulus for bladder growth may be the initial overdistension that occurs as the bladder fills following partial outlet obstruction. The present study was undertaken to determine if the sequence and characteristics of gene activation during the recovery following a brief period of overdistension of the rabbit bladder are comparable with the gene activity previously described in association with partial outlet obstruction.

Rabbit bladders were overdistended to 20% above capacity for 1 hr and then relieved. Bladders were recovered from control (untreated) rabbits and from rabbits at 1 hr or at 1, 3 or 5 days following relief of overdistension. RNAs extracted from these tissues were examined by Northern blot assays for a number of different mRNA transcripts previously shown to be altered by partial outlet obstruction. By 1 hr following the relief from acute overdistension, there was already a greater than 10-fold increase in the expression of hsp-70 related transcripts as well as a marked increase in the expression of mRNA encoding bFGF and decreased expression of TGF-β_1. Additionally, expression of the early response genes, c-*fos* and c-*jun* were induced at this time. By 24 hrs following overdistension, the expression of all genes returned to near control levels. These results were reproduced for individual specimens from two groups of treated rabbits. In summary, a brief interval of overdistension of the rabbit bladder induces specific alterations in gene activity during the subsequent recovery period that mimics the activity associated with partial outlet obstruction. These results are consistent with the hypothesis that the changes observed following partial outlet obstruction are caused by an initial overdistension of the urinary bladder wall. (Mol Cell Biochem **132:** 39–44, 1994)

Key words: urinary bladder, overdistension, heat shock proteins, growth factors

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Introduction

Rabbits are frequently utilized as an animal model to help better understand the developmental pathology of the dysfunctional human urinary bladder. The rabbit bladder responds to a variety of experimentally-induced stresses with compensatory alterations in micturition frequency and volume; bladder mass, capacity and compliance; and contractile function [1–3]. One of the most familiar effectors of rabbit bladder response is an experimental surgical procedure involving placement of a ligature around the proximal urethra to produce a partial bladder outlet obstruction [1, 4, 5]. Following this procedure, the rabbit bladder mass increases markedly, cells at the inner and outer layer of th bladder undergo DNA synthesis [6] and, with long-term obstruction, bladder function becomes impaired.

Recently, applied analysis of RNAs extracted from a series of partially obstructed rabbit bladders has identified an early molecular response associated with such outlet obstruction-induced bladder hypertrophy [7]. Changes in the expression of a number of different genes could be detected within two hours subsequent to urethral ligation and continued throughout a 48 hr period. The early response was characterized by the acute and intense induction of hsp-70 mRNA, induced expression of certain protooncogenes (c-*myc*, Ha- and N-*ras*) and a growth factor (basic FGF) as well as reduced expression of a negative growth regulator (TGF-β_1). This response is consistent with our concepts of the obstructed bladder as a tissue under stress that is concomitantly experiencing hyperplasia within select cellular compartments [8].

The precise stimulus that initiates this molecular response to outlet obstruction remains to be defined. Upon partial outlet obstruction, the bladder experiences the stress of increased urethral resistance [9]. Physiological studies of the partially obstructed bladder suggest that the primary event which mediates the hypertrophic response is an acute period of overdistension which occurs as the bladder fills in the presence of increased urethral resistance [9, 10]. Therefore, the specific aim of this project was to determine whether the sequence and characteristics of gene activation induced by experimental acute overdistension might be comparable to the changes previously observed following partial outlet obstruction. By correlating the rabbit bladder's molecular response to these two different experimental procedures, we hope to reveal the role of acute and chronic distension in the onset and progression of the hypertrophic response of the obstructed bladder.

Material and methods

Animals and tissues

Mature male New Zealand White rabbits (from Ace Animals, Inc., Boyerstown, NJ) weighing approximately 2.5 kg were used for these experiments. Rabbits were separated into two groups: 1) unoperated controls and 2) animals subjected to overdistension for one hour and euthanized at various periods of time following overdistension.

Each rabbit was sedated with an intramuscular injection (0.7 ml/kg) of ketamine/xylazine mixture (29.2 mg/ml ketamine, 8.3 mg/ml xylazine). Surgical anesthesia was maintained with 0.5 ml pentobarbital (50 mg/ml i.v.). The bladder was catheterized with 8 fr Foley catheter. The urinary bladder was then filled with saline at a rate of 1.5 ml per hour. Intravesical pressure was monitored continually using a Statham pressure transducer and recorded on a Grass model 7 polygraph. An individual rabbit bladder was distended until the cystometrogram output reached the top of the ascending limb, and then the volume was increased by 20% above the maximum value (overdistension). The bladder was maintained at 20% above capacity for one hour. The use of pentobarbital as the anesthetic agent prevented the generation of micturition reflexes to allow for a smooth overdistension of the bladder. At the end of one hour, the saline in the bladder was removed and the rabbit allowed to recover. Individual rabbits were sacrificed (sodium pentobarbitol overdose) at 1 hour, or at 1, 3 or 5 days following the 1 hr period of overdistension. Bladders were recovered and frozen using a clamp precooled in liquid nitrogen. The bladder was stored under liquid nitrogen until analysis.

Extraction and analysis of RNA

Individual bladders were pulverized to a powder under liquid nitrogen. Total RNA was extracted from the frozen powder by homogenization in the RNAzole B reagent (Tel-Test Inc., Friendswood, TX) at a ratio of

100 mg tisue/2 mls reagent. Chloroform was added to the homogenate at 1/10 volume and the mixture was centrifuged at 12,000 × g for 10 min. The aqueous phase was recovered and RNA was precipitated from this phase by the addition of an equal volume of isopropanol. Following centrifugation, the RNA pellet was washed with 70% ethanol, dried and resuspended in TE buffer. Total RNA was quantitated by spectrophotometry at 260 nm. 15 μg aliquots of total RNA from each of five specimen was co-electrophoresed on a 1.2% agarose-formaldehyde gel. After transferring the RNA to a nylon filter (Nytran, Schleicher & Schuell, Inc., Keene, NH), the Northern blot was sequentially hybridized to a series of ^{32}P-labelled probes as previously described [11]. After overnight hybridization at 42° C, the Northern blot was washed at 60° C in a successive series of solutions containing decreasing concentration of SSC, 0.1% SDS and 5 mM EDTA to a final concentration of 0.2 × SSC. The hybridized filter was exposed for overnight to 3 days to Kodak XAR-5 film for autoradiography. Northern blots were reused following removal of radiolabeled probe by a brief rinse in boiling water. At the 6th cycle, the blot was hybridized with 18S rRNA probe to provide a means for estimating RNA loading inequities.

Quantitation of mRNA expression

X-ray autoradiographs were scanned on a Molecular Dynamics Scanning Laser Densitometer. The densitometer readings for 18S rRNA band of each specimen were compared and the correction factor was used to normalize the other densitometry readings.

Probes

Plasmids containing complete and partial cDNA inserts for viral c-*fos* was obtained from Tom Curran, Roche Institute of Molecular Biology; murine c-*myc* from Frederick Alt, Columbia University; bovine *basic fibroblast growth factor (bFGF)* from Judy Abrahams, California Biotechnology, Inc.; 18S rRNA from Dr. Ramreddy Guntaka, University of Missouri at Columbia; murine *heat shock protein-68 (hsp-70)* from Larry Moran, University of Toronto; human *transforming growth factor-*β_1 *(TGF-*β_1*)* from American Type Culture Collection, Rockville, MD; viral c-*jun*, c-*sis* and N-*ras* from Oncor Inc., Gaithersburg, MD. The probes and their use on Northern blots containing RNA from rabbit bladders have been described previously [7, 11]. All cDNA fragments were excised from their plasmid vectors using appropriate restriction enzyme digests and were purified from agarose following gel electrophoresis using the Qiaex DNA extraction kit of Qiagen, Inc. (Chatsworth, CA). Fragments were labeled with ^{32}P-dCTP using the random priming method as previously described [7] and were denatured by boiling prior to hybridization.

Results

Total RNA was extracted from control rabbit bladders and from individual rabbit bladders with recovery periods of 1 hour, 1, 3 and 5 days after one hour acute overdistension. Equal aliquots of RNA from individual specimens were co-electrophoresed in adjacent lanes of a 1.2% agarose-formaldehyde gel. RNA in the gels were blotted onto charge-modified nylon filter paper, and the resulting Northern blots were hybridized to ^{32}P-labeled cDNA probes, as indicated. Changes in bladder gene activity associated with recovery from overdistension were identified by corrected densitometry of the autoradiograph of the hybridized Northern blot. RNAs extracted from two different specimens per experimental group were evaluated to determine the reproducibility of such changes. As summarized in Table 1, the expression of eight different genes were evaluated in this study.

Among the various genes tested, mRNA transcripts for four particular gene products showed a distinct induction subsequent to bladder overdistension. As presented in Fig. 1, the 2.5 kb transcript encoding the mam-

Table 1. Northern blot assays overdistended rabbit bladder RNA

Probe	Control bladder	1 hour recovery	1 day recovery	3 days recovery	5 days recovery
hsp-70	1.00*	10.1	1.20	0.30	0.54
bFGF	1.00*	9.4	1.32	1.56	1.67
c-*fos*	1.00*	7.8	1.12	0.86	0.54
c-*jun*	1.00*	11.3	2.1	2.3	1.9
TGF-β_1	1.00*	0.55	1.37	0.98	1.03
N-*ras*	–	–	–	–	–
c-*myc*	–	–	–	–	–
c-*sis*	–	–	–	–	–

*: control tissue, densitometric value assigned as 1.00; (–): corresponding transcript not detected.

Relative expression of various mRNA transcripts as a function of time after rabbit bladder is released from overdistension. Densitometric values at given times after release from obstruction are compared to values in control (nonobstructed bladder) RNA (assigned an arbitrary value of 1.00).

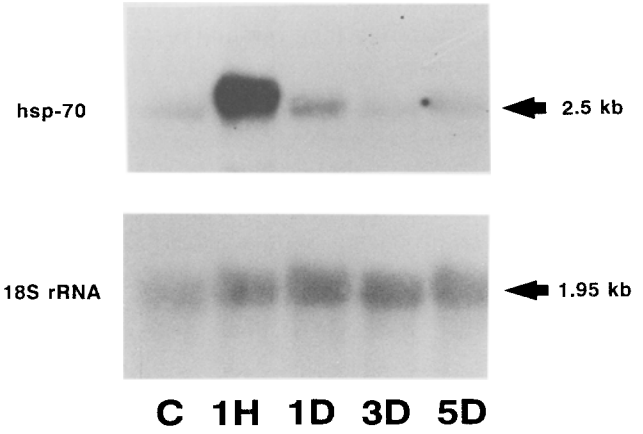

Fig. 1. Induced expression of mRNA encoding hsp-70 after 1 hour rabbit bladder overdistension. Equal aliquots of total RNA (15 μgs) were electrophoresed on a 1.2% denaturing agarose gel and blotted onto a nylon filter. The Northern blot was hybridized to the murine cDNA probe for heat shock protein-70. The top panel is the autoradiograph showing the induced expression of the 2.5 kb hsp-70 transcript. The bottom panel demonstrates the result of hybridization of the same filter with 18S rRNA probe, which provides a means to estimate the RNA loading inequities. Taking densitometry reading for 18S rRNA band of control RNA as standard, the correction factor for each RNA loading are C: 1.00, 1H: 1.80, 1D: 2.28, 3D: 2.27, 5D: 1.91. The corrected densitometry reading indicated more than 10 folds increase of hsp-70 mRNA at 1 hour after the end of overdistension. C: control; 1H: one hour after the end of overdistension; 1D, 3D and 5D: one day, three days and five days after the end of overdistension.

malian heat shock protein, *hsp-70*, demonstrated the most dramatic change. *Hsp-70* mRNA was elevated 10-fold at 1 hr after the end of acute overdistension when compared to its expression in control rabbit bladder RNA. This transcript subsequently returned to control level at 1 day and to even lower than control level at 3 and 5 days. In a similar fashion, the 4.8 kb *bFGF* mRNA became distinctly detectable in bladder RNA only with a 1 hr recovery period from overdistension (Fig. 2). This

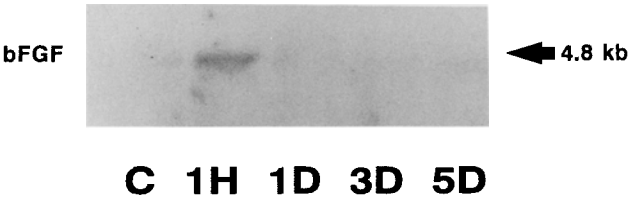

Fig. 2. Induced expression of *bFGF* mRNA after acute overdistension. The Northern blot containing equal aliquots of rabbit bladder RNAs (from Fig. 1) was rehybridized to the bovine cDNA probe for *bFGF*. The autoradiograph demonstrates the detection of 4.8 kb mRNA of bFGF one hour after the end of overdistension. *bFGF* was not detected in control bladder RNA. C: control; 1H: one hour after the end of overdistension; 1D, 3D and 5D: one day, three days and five days after the end of overdistension.

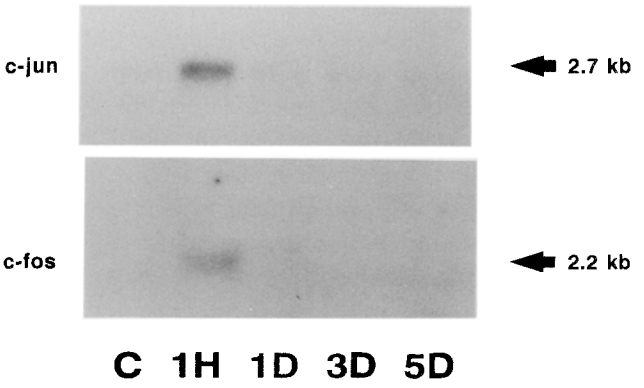

Fig. 3. Induces expression of c-*fos* and c-*jun* mRNA after 1 hour acute overdistension in rabbit bladder RNA. Top panel shows c-*jun* mRNA was distinctly induced in rabbit bladder RNA one hour after overdistension was relieved. Bottom panel shows c-*fos* mRNA was distinctly induced and detected in RNA prepared one hour after the end of acute overdistension (lane 1H). At this level of exposure, the c-*fos* transcript is barely detectable in other lanes. C: control; 1H: one hour after the end of overdistension; 1D, 3D and 5D: one day, three days and five days after the end of overdistension.

mRNA was almost undetectable in control bladder RNA under the conditions of our hybridization and this is consistent with an earlier study of *bFGF* expression in the rabbit bladder [7]. Therefore, it is inappropriate to evaluate the extent of induction experienced by this gene product as well as for the early response protooncogenes, c-*fos* (2.2 kb transcript) and c-*jun* (2.7 kb transcript), which were also highly induced in the 1 hr release bladder specimens (Fig. 3), but were not detectable in control bladder specimens.

TGF-β_1 was the only gene product with decreased expression during the period of recovery from acute overdistension (Fig. 4). At one hour after the end of overdistension, the 2.5 kb mRNA encoding this growth factor was reduced to 55% of control levels, but it subsequently increased back to control levels (1.3-fold at 1 day after acute overdistension), 3 and 5 days after the stress was relieved. It is of note that the enhanced expression of *bFGF*, a known growth enhancer, in conjunction with decreased expression of *TGF*-β, a growth suppressor for many cell types, was previously described [7] in partially obstructed rabbit bladders. This pattern was reiterated in this current study of acute overdistension.

Three gene products that we analyzed for were not changed by acute overdistension of the rabbit bladder, c-*sis*, c-*myc* and N-*ras*. In fact, transcripts encoded by these three genes were never detected, even subsequent to long term X-ray film exposure of Northern blots (not shown). This finding for c-*myc* and N-*ras* was in contrast to the earlier study of partial bladder obstruction in

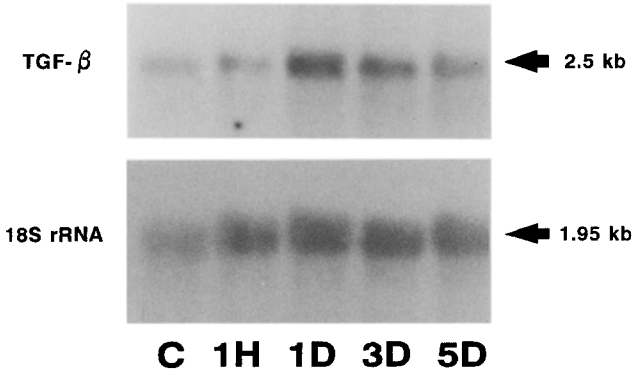

Fig. 4. Decreased expression of *TGF*-β_1 in bladder RNA after acute overdistension. Top panel shows autoradiograph of rabbit bladder RNAs following rehybridization of a Northern blot (from Fig. 3) with a *TGF*-β_1 cDNA probe. *TGF*-β_1 expression declined to 55% at one hour after the end of acute overdistension. Its expression returns to 1.3-fold of control level at one day after overdistension and to control level 3 and 5 days after acute overdistension. Bottom panel is the autoradiograph of the same Northern blot subsequently hybridized with *18S rRNA* cDNA probe, the correction factor for each RNA loading are: C: 1.00, 1H: 1.80, 1D: 2.28, 3D: 2.27, 5D: 1.91. C: control; 1H: one hour after the end of overdistension, 1D, 3D and 5D: one day, three days and five days after the end of overdistension.

which these two particular gene products were distinctly found to be induced [7].

Discussion

Within a day following partial outlet obstruction of the rabbit bladder, there are major decreases in the bladder contractile response to stimulation, extensive radiolabeled thymidine incorporation into nuclear DNA within the urothelium, and increased urethelial permeability [4, 6, 9, 12, 13]. If the obstructive stress is unrelieved, these changes progress to include thymidine labelling within the interstitial tissue compartments and serosal membrane; a marked increase in bladder mass, and continued alterations in both neuronal innervation and contractile responses to autonomic stimulation [6, 9, 12, 13]. Physiologically these changes associated with the earliest period of partial outlet obstruction can be mimicked by a brief period of experimental overdistension of the rabbit bladder [10, 12, 14, 15]. Following this procedure, there are immediate decreases in the contractile response to stimulation (*in vitro*), increases in thymidine incorporation into the urothelium, and an increase in urethral permeability. Since the experimental overdistension experiments are acute, however, both the contractile defects and urethral permeability are returned to normal control levels rapidly when the distension is released. Furthermore, there is no significant increase in bladder mass following one hour overdistension.

As earlier study [7] had examined the changes in gene expression associated with partial outlet obstruction of the rabbit bladder. The results obtained in the current study are consistent with the concept that the initial distension following partial outlet obstruction is sufficient to induce changes in gene activity that subsequently mediate the changes in bladder mass observed. In this study, acute overdistension induced a marked transient stimulation of early response genes, c-*fos* and c-*jun*, the heat-shock gene, *hsp-70* as well as the growth factor, *bFGF*. Also observed was a decrease in the expression of the growth-inhibitor, *TGF*-β_1, within 1 hour after release of the overdistension. These alterations in gene activation are identical to the sequence of gene activation (or inhibition, in the case of *TGF*-β_1) observed within 2 hours following partial outlet obstruction. Since many of these gene products are associated with cellular proliferation and tissue growth, we have proposed that this early response probably mediates the stimulated thymidine uptake and incorporation within the urothelium observed at one day following both acute overdistension and partial outlet obstruction.

Whereas the induced gene activity returns to control levels within 24 hours following acute overdistension, the gene activity alteration associated with partial outlet obstruction is increased at 24 and 48 hours. This is consistent with the continued and expanded thymidine uptake and incorporation into DNA within the entire wall of the bladder observed during the later periods following partial outlet obstruction (but not acute overdistension). In addition, the structural remodelling of the bladder that occurs following partial outlet obstruction (but not following acute overdistension) is preceded by significant and substantial increases in the activation of N-*ras*, c-*myc* oncogene. Thus, these protooncogenes which are activated by partial outlet obstruction, but not activated by acute overdistension, may depend on the continued stress associated with partial outlet obstruction. Therefore these genes (c-*myc* and N-*ras*) may be more directly involved in the bladder obstruction remodeling process.

In conclusion, a brief period of acute overdistension of the rabbit bladder is sufficient to induce specific alterations in bladder gene activity during the recovery period when the overdistension is released. It is of interest that Karim and his colleagues [16] previously reported that simple stretching of bladder cells in culture was sufficient to alter the gene activity of the cells. While this

was attributed to changes in the cytoskeletal/nuclear matrix network by these investigators, it is also important to consider that, *in vivo*, mechanical overdistension of the bladder results in increased permeability of the urothelial boundary, thus making inner layers of the bladder more accessible to urinary growth factors, such as Epidermal Growth Factor [17, 18]. Regardless, our results are clearly consistent with the concept that many of the molecular, biochemical and cellular changes observed in the rabbit bladder following partial outlet obstruction are induced by an initial period of overdistension. Moreover, the continued stress on the bladder associated with chronic outlet obstruction results in the further stimulation of specific oncogenes that may mediate the remodelling of bladder structure observed following partial outlet obstruction, but not following a brief period of overdistension.

Acknowledgements

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