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ORIGINAL PAPER

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Estrogenic regulation of HSP90 kD synthesis in rat urinary bladder

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Abstract The role of heat shock protein (HSP90 kD) has been investigated in regard to its association with steroid receptors. HSP90 kD may play a role in steroid receptor stabilization and activation. Oophorectomized Sprague-Dawley rats ($n=25$) were placed into five groups and injected subcutaneously with 30 µg β-estradiol 17-benzoate in sesame oil, with one group injected with carrier oil (control). After estrogen administration, the rats were killed, and their bladders removed for immunostaining, immunoblotting and enzyme-linked immunosorbent assay (ELISA). Immunoblot analysis demonstrated a 90-kD band in bladder homogenates, even in the absence of estrogen. However, the bands were more intense 12 and 24 h after administering estrogen. ELISA showed significant differences in HSP90 kD synthesis as early as 6 h compared to controls ($P<0.05$). After 48 h the estrogen-treated rats and controls were identical. The above results were confirmed by immunostaining for HSP90 kD. HSP90 kD synthesis in the rat urinary bladder is under estrogenic regulation. These findings may be relevant in the etiology and pathobiology of interstitial cystitis and menopausal voiding dysfunctions since the bladder is enriched with estrogenic receptors and is under estrogenic influence.

Keywords Bladder · Estrogen · HSP · IC

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Introduction

Heat shock proteins (HSP) are expressed at the cellular level in response to stressful conditions caused by heat, hypoxia, stretch or injury [9, 13, 14]. These highly conserved proteins are also present in unstressed cells and thought to be involved in maintaining cellular homeostasis by insuring proper protein folding and maturation. The regulatory pathways involved in the expression of various HSP's have been characterized.

One of these proteins, HSP90 kD, has been investigated in regard to its in vivo and in vitro association with steroid receptors [25, 27]. The extent to which HSP90 kD impacts on steroid hormone action in the bladder and its role in the pathobiological mechanisms involved in bladder diseases have recently been investigated. Estrogen is known to increase the sensitivity of bladder and urethral smooth muscle to autonomic drugs and to increase urethral tissue mass in animal models. Estrogen can augment mediator release from mast cells that have been shown in interstitial cystitis (IC) patients to have high-affinity estrogen receptors [1, 4, 15, 30].

Symptoms in patients with IC can also fluctuate in relation to estrogen levels. Interestingly, oophorectomy aggravates IC symptoms in young patients, whereas menopausal women (decreased estrogen) may derive significant benefit and clinical improvement. When estrogen is elevated during ovulation, IC symptoms may worsen; during late pregnancy when estrogen levels are low (and progesterone elevated), patients often have symptomatic relief [6, 24]. Investigating the distribution of this particular stress protein in the urinary bladder may therefore be important for undersatnding bladder diseases where estrogen levels vary (i.e., IC, menopausal voiding dysfunctions, and possible urinary tract infections, UTIs). To examine the relationships between estrogen and HSP90 kD regulation we measured expression of this stress protein in rat bladder tissues from experimental rats exposed to the hormone.

Materials and methods

Animals and chemicals

Oophorectomized Sprague-Dawley female rats weighing approximately 250–300 g were obtained locally (Harlan Sprague-Dawley, San Diego, Calif.). Monoclonal anti-HSP90 kD (clone AC88-IgG1), anti-HSP72 kD (clone C92F3A-5, IgG1), and purified HSP90 kD bovine brain antigen (SPP-780) (StressGen, Vancouver, B.C, Canada); mouse IgG1, kappa (M-1398), anti α-smooth muscle (actin) and B-estradiol 17-benzoate (Sigma, St. Louis, Mo.); Bio-Stain Super ABC immunohistochemical (rat/mouse IgG specific) staining kit (Biomeda, Burlingame, Calif.) were purchased from the indicated vendors and appropriately stored.

Estrogen treatment model

Rats were injected subcutaneously with 30 µg β-estradiol 17-benzoate (estradiol) in sesame oil 1–2 weeks after oophorectomy. Control rats were injected with only the sesame oil. The experimental rats which were given estrogen were assigned to groups for tissue collection at 6, 12, 24 or 48 h after treatment.

Immunochemical staining

The bladder tissue was cut in half by coronal dissection viewed with a biomicroscope. Each piece contained approximately equal portions of the body and neck region. Half of the tissue was snap-frozen in liquid nitrogen and embedded in OCT. The blocks were stored at –18°C. Cryosections (4 µm) were later cut and dried on polylysine-coated slides, then fixed briefly in acetone. The slides were washed with phosphate buffered saline (PBS) and incubated in PBS-10% horse serum. Sections were incubated overnight (15 h) with anti-HSP90 kD (1:100) diluted in blocking buffer. After washing in PBS, sections were incubated with biotinylated anti-mouse IgG antibody. Endogenous peroxidase activity was inhibited by cold methanol-0.5% H_2O_2 treatment. Complexed biotinylated antibody was detected by addition of peroxidase-conjugated streptavidin (Biomeda) and finally incubating in DAB substrate (Sigma). Controls consisted of: (1) omitting the monoclonal antibody, (2) incubating sections with an anti-smooth muscle actin, monoclonal antibody (housekeeping control) or with a non-immune mouse IgG1 ascites fluid control.

Immunoblotting

Western blots were made by taking half of the bladder tissues obtained from the experimental and control rats and homogenizing them in 1.0 ml extract buffer (10 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF, 10 mM NaCl, pH 7.5). Homogenates were centrifuged at 16,000 *g* for 10 min and the supernatants collected. Protein concentrations were determined colorimetrically using the Bradford reagent and values extrapolated from a standard curve using bovine albumin, diluted in extract buffer. The tissue homogenates were adjusted to 1 mg/ml protein and mixed 1:1 with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2-mercaptoethanol. A small aliquot (0.1 ml) was saved for dot blot assay. The SDS-treated samples were heated in a boiling water bath for 2 min and kept on ice until being loaded in duplicate 10% polyacrylamide gels (25 µl/lane) for electrophoresis. Separated proteins were visualized by Coomassie blue staining on one of the gels. Separated proteins on the other gel were transferred to nitrocellulose (Schleicher and Schuell, Keene, N.H.) for immunoblotting. Detection of the HSP90 kD (or HSP72kDa) antigen was accomplished by washing the nitrocellulose in PBS, followed by 60 min incubation in blocking buffer (5% milk-0.05% tween 20-PBS). The nitrocellulose blots were placed in heat-sealable pouches (Scotchpak) with appropriately diluted anti-HSP90 kD, HSP72kDa or control antibody (ascites IgG1). Blots were washed in PBS and incubated with alkaline phosphatase-conjugated anti-mouse immunoglobulin (Ig; Dako). The washed blots were developed with BCIP substrate solution (Sigma) and photographed.

Dot blots (Fig. 1) were made by spotting 10, 1 and 0.1 µg total protein from the non-denatured extracts of the experimental rat bladders (24-h estrogen treated and untreated) onto separate strips of nitrocellulose squares (approximately 3×3 cm), then air drying. A fourth blot was spotted with diluted HSP standards (HSP90 kD and HSP72 kD). The nitrocellulose blots were then incubated overnight in separate heat sealed pouches (Scotchpak) with anti-HSP90 kD (blots 1 and 3) and with monoclonal anti-smooth muscle actin (blot 2) and anti HSP72 kD (blot 4). The blots were washed (3×15 min) the next day in 0.01 M Tris buffered saline (TBS) and then incubated with anti mouse-alkaline phosphatase conjugate (1:500, Dako) for 60 min at room temperature. The blots were washed a final time in TBS, then incubated with the BCIP substrate. Developed blots were photographed.

Competitive enzyme-linked immunosorbent assay

HSP90 kD (StressGen) antigen (0.1 µg/ml) diluted in phosphate buffered saline (PBS) coating buffer was added (200 µl) to a 96-well enzyme-linked immunosorbent assay (ELISA) plate (Corning, N.Y.). After a 2-h incubation at 37°C, the plate was washed 3× with PBS-0.05% tween 20. Blocking solution (PBS-5% horse serum) was added for a 1-h incubation on a shaker. A standard curve was generated by incubating serial dilutions of HSP90 kD antigen (range 0–1000 ng) with an equal volume (50 µl) of monoclonal anti-HSP90 kD (1:100 dilution). Fifty microliter test

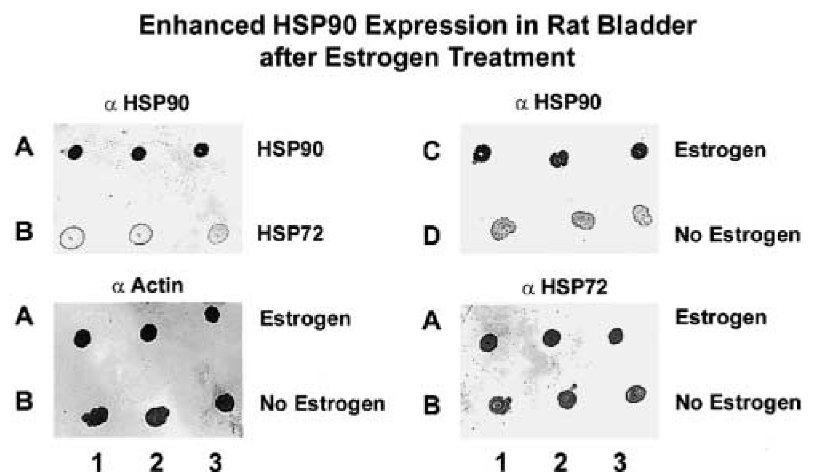

Fig. 1 Dot blot analysis of rat bladder tissue extracts (10, 1 and 0.1 µg protein spotted left to right) with and without estrogen treatment (24 h) and probed for smooth muscle actin (blot 2, *lower left*), HSP72 kD (blot 4, *lower right*), and HSP90 kD (blots 1 and 3, *upper blots*)

samples (rat bladder extracts) adjusted to 100 ng/ml protein, were also incubated with the same monoclonal antibody (1:100). After 6 h incubation at room temperature, 75 μl of the reaction mixtures were transferred to the antigen-coated assay plate. After another 6-h incubation, the plate was rinsed 3× with PBS and goat anti-mouse (biotinylated) antibody was added (100 μl/well, 1:1000 dilution) for 1 h. The assay plate was then washed 3×, followed by the addition of freshly prepared streptavidin-peroxidase conjugate (60 min). Substrate solution (o-phenylenediamine dihydrochloride, buffer, and urea in 20 ml water) was added (100 μl/well) after washing and the resulting color was quantitated by optical density measurements using a microplate reader (Bio-Rad) (540 nm). The amount of HSP90 kD in the test samples could then be extrapolated from the standard curve. Final results were expressed as μg HSP90/mg protein in the extract. The Student's *t*-test was used to compare the HSP90 kD concentration extracted from tissue and corrected for total protein in the extracts. Student's *t*-test was used to determine the statistical significance of the HSP90 kD data.

Results

Immunostaining

HSP90 kD expression was higher in the rat bladder tissues from the estrogen-treated group than in similar tissue sections from the control group rats (Fig. 2a–d). The immunostaining for HSP90 kD was strongest in the mucosa and detrusor (Fig. 2b, c). Cytoplasmic staining for the HSP90 kD antigen appeared to be more intense then any nuclear staining in the mucosal cells (Fig. 2c). However, the precise differentiation of nuclear vs cytoplasmic staining will require further study since immunostaining methods were not adequate to resolve this issue. Connective tissues in the lamina propria and endothelial cells lining blood vessels were negative for HSP90 kD. The detrusor muscle tissue and mucosal region in the rat bladders that were not estrogen treated and incubated with the anti HSP90 kD probe did not stain for HSP90 kD (Fig. 2d) in comparison with the estrogen-treated rat tissues (Fig. 2a, c). Sections that were incubated with irrelevant antibody (isotype control) also did not demonstrate any staining upon incubation with conjugate and subsequent development in substrate (not shown). The absence of staining for red blood cells indicated that endogenous peroxidase was

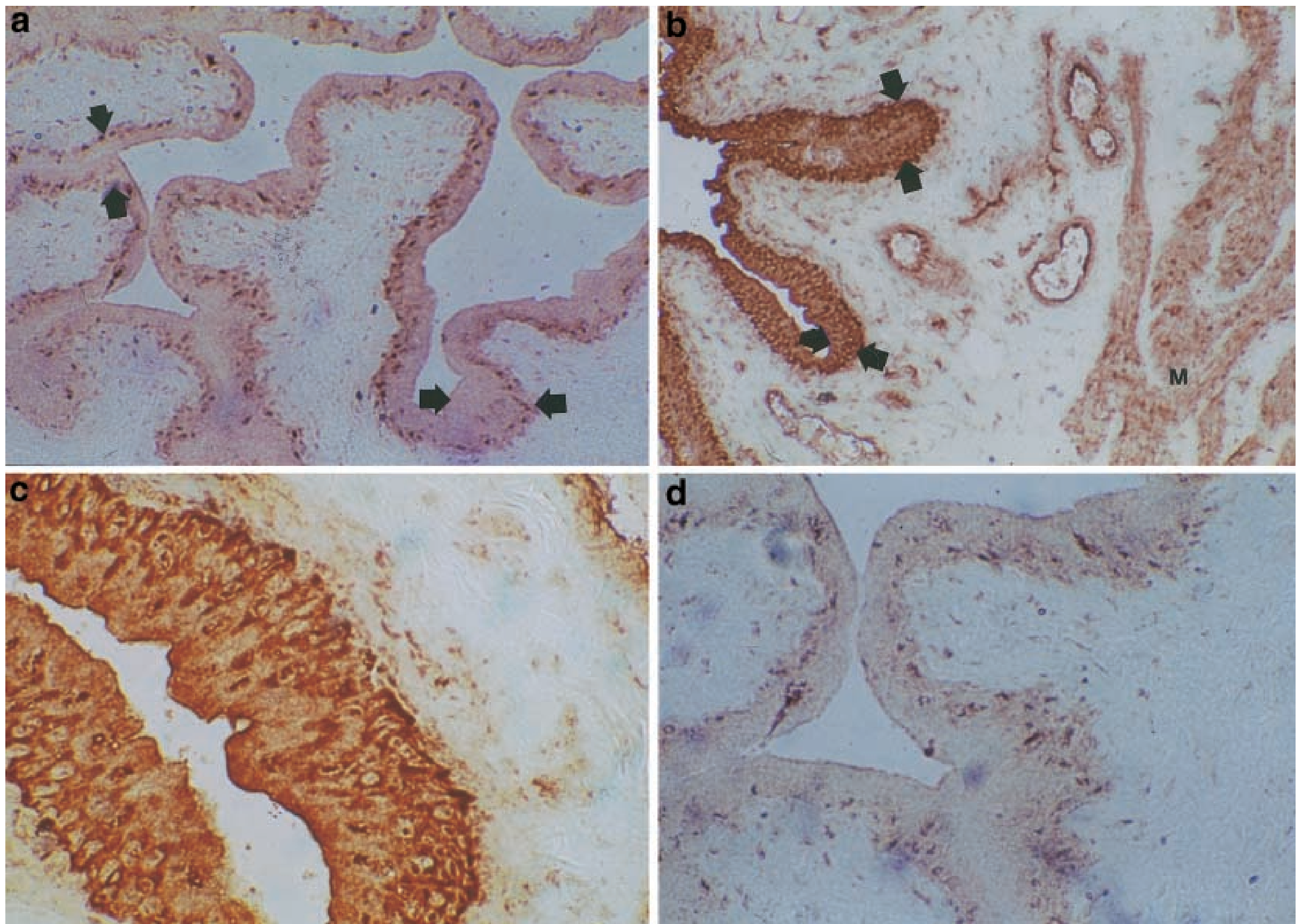

Fig. 2a–d Immunochemical localization of HSP90 kD in rat bladder tissue sections after estrogen treatment. **a** Absence of mucosal staining in experimental rat not estrogen treated (100×). **b** Bladder mucosal tissue 24 h after estrogen treatment, positive staining for HSP90 kD in mucosa (*arrows*) and muscle (*M*) (100×). **c** Higher magnification of mucosal immunostaining of HSP90 kD showing a positive reaction in the urothelial cells basal region 24 h after estrogen treatment (400×). **d** Rat bladder tissue not estrogen-treated and without any discernable staining for HSP90 kD (400×)

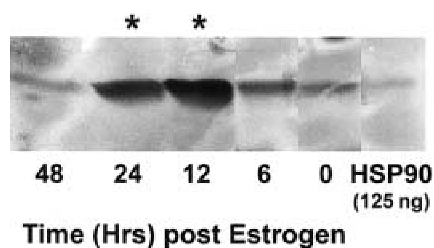

Fig. 3 Immunoblot analysis of extracts from experimental rat bladders demonstrating increased synthesis of HSP90 kD 6, 12, 24 and 48 h after estrogen, peak levels after 12–24 h (*asterisks*). After 48 h HSP90 kD tissue concentration similar to levels detected in rat bladders at start of experiment (0 time, no estrogen). HSP90 kD standard, 125 ng in *Lane 1*. Extract samples normalized for protein concentration (1 mg/ml)

adequately blocked and not contributing to any artifactitious staining.

Western blot analysis

Rat bladder tissue extract samples were also shown by immunoblotting to have strong reactivity for HSP90 kD. Control rat bladder tissue extracts had detectable levels of HSP90 kD indicating constituitive expression. An immunoreactive band (HSP90 kD) was detected in all the tissue extracts. The strongest HSP90 kD signals were observed from rat bladder tissues collected 12 and 24 h after the estrogen treatment (Fig. 3, 4). After 48 h HSP90 kD apparently returns to baseline levels as indicated by equal band staining at these time intervals. Immunoblot of extracted proteins (Fig. 4, HSP90/blot) from estrogen treated (c) and control rat bladder extracts (d) showed a distinct increase of HSP90 kD 12 h after treatment, whereas stress protein HSP72 kD remained at the same level when estrogen treated (a) or not (b) (Fig. 4, HSP72/blot).

Dot blot data substantiates the fact that gene expression and synthesis of HSP90 kD is enhanced by estrogen and this observation is not just a generalized response to estrogens by upregulation of all protein synthesis. This is shown by the dot blot analysis at several dilutions of extract that were probed for actin, HSP72 kD and the HSP90 kD for direct comparison. The HSP90 kD antibody probe specifically reacts with purified HSP90 kD and does not recognize other HSPs (Fig. 4, top left blot). The HSP90 kD reactivity appears strong in the estrogen treated extracts but dramatically less in the untreated rats (top right blot) as we have shown in the immunostaining studies. Furthermore, two other proteins ("housekeeping" protein smooth muscle actin and HSP72 kD) were not differentially expressed in the same tissues that showed upregulation of HSP90 kD after estrogen (Fig. 4, bottom blots). Trace amounts of non-estrogen-treated constitutive expression of HSP72 kD were detected in the tissue extracts and this amount remained the same even after estrogen treatment.

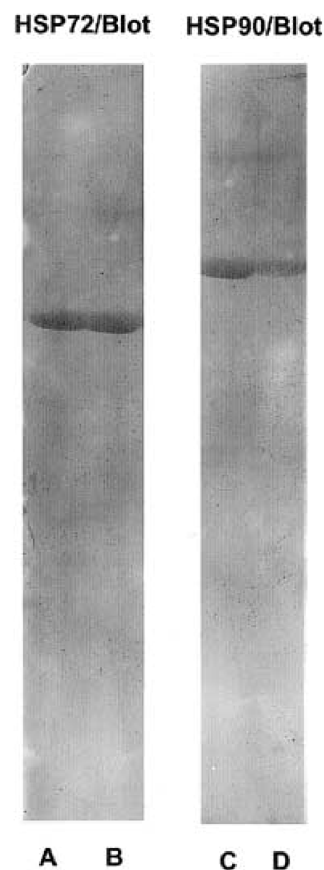

Fig. 4a–d Western blot analyses of bladder extracts probed for HSP90 kD and HSP72 kD. HSP90 kD is upregulated after 12 h estrogen treatment (**c**) compared to control rat bladders (**d**). No change in expression of HSP72 kD was observed estrogen treated (**a**) or control (**b**)

Enzyme-linked immunosorbent assay

The coated HSP90 kD antigen was uniformly distributed over the 96-well plate. Extraction of bladder tissue was also complete since no remaining HSP90 kD could be detected. The mean concentration of HSP90 kD in control rat bladder tissue extracts ($n = 5$) was 0.2 μg/mg protein (extrapolated from a standard curve) using the values obtained after ELISA (Fig. 5). HSP90 kD levels increased 6 h after estrogen injection and reached a peak level after 24 h. The mean concentration of HSP90 kD in the tissue extracts from rat bladders ($n = 5$/group) at 6, 12, and 24 h after estrogen treatment was 0.71, 0.83 and 1.2 μg/mg protein, respectively. These levels were significantly different than the HSP90 kD levels measured in the control rat tissues ($P < 0.01$) (Fig. 5) and indicated that estrogen treatment could increase the amount of HSP90 kD in the bladder wall tissues by almost threefold. HSP90 kD levels in extracts after 48 h compared to control tissue extracts (baseline, 125 ng) were not significantly different ($P > 0.05$) indicating a return to the non-estrogen-treated baseline level for expression of this protein.

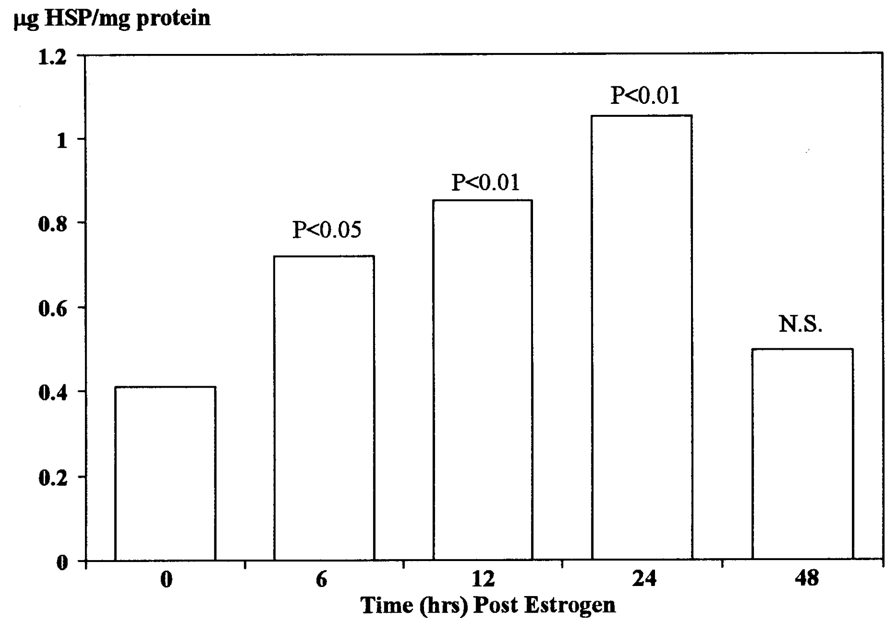

Fig. 5 Relative amounts of HSP90 kD (µg/mg protein) in extracts from experimental and control rat bladders. HSP90 kD concentrations extrapolated from standard curve and corrected for protein weight. Tissues harvested 6, 12, 24 and 48 h after estrogen injection ($n = 5$ rats/group). Increased synthesis 6–24 h after administering estrogen compared to baseline ($P < 0.05$)

Discussion

Immunolocalization of HSP90 kD antigen in bladder tissue sections, immunoblot (Western and dot blot) analysis, and ELISA of bladder tissue homogenates (normalized for protein) consistently demonstrated elevated HSP90 kD levels after administering estrogen to experimental rats. Non-estrogen (and estrogen) -treated controls did not show similar changes in HSP90 kD expression, or expression of other proteins (HSP72 kD, β-actin). These results indicate that HSP90 kD is significantly regulated in the rat bladder by estrogen level. Hormone levels were controlled in these studies by administering a single dose (30 µgm) of estrogen intramuscularly to oophorectomized rats. This dose is considerably higher than the minimum effective daily clinical dose (625 µgm) given by mouth in humans. However, this amount has been administered effectively to regulate HSP90 kD synthesis in rat brain and uterine tissue and in other rat bladder functional studies [15, 17]. HSP90 kD was found to be expressed constitutively even in the oophorectomized rats, indicating that regulation of this stress protein is under additional control mechanisms. HSP90 kD expression dramatically increased in a time-dependent manner after a single estrogen treatment. Similar conclusions have been obtained from other studies examining estrogen effects in the reproductive tissues of the uterus and breast and in studies investigating estrogen effects on various tumor cell lines. A similar time course for estrogen-dependent HSP90 kD expression was reported in breast tumor cell line [29], in the uterus [23], and in the hypothalamus [17]. Cells lacking estrogen receptors do not show any increased gene expression or protein synthesis for HSP90 kD [1]. Many attempts have been made to reveal estrogen effects on bladder tissues in vivo and in vitro [4, 15]. Generally, estrogen increases bladder and urethral mass, sensitivity of smooth muscle to α-adrenergic agonist and field stimulation as well as density of α-adrenergic receptors. These findings, however, have limited clinical implications. The precise physiologic cellular function of HSP90 kD is not yet established. It is synthesized constitutively, like other HSPs, and is considered to have important roles in protein folding, protein-protein interactions and protein kinase activity in normal cells [3, 19, 31]. HSP90 kD bound to nonactive estrogen receptor prevents receptor protein interaction with enhancer elements in DNA. Upon contact with hormone, HSP90 kD dissociates from receptors. This enhances the transcriptional activity. HSP90 kD may paradoxically facilitate transcriptional activity in other types of steroid receptors by direct association with hormone [10, 21, 22].

Immunohistochemical staining showed that oophorectomized rats express low levels of HSP90 kD in the bladder mucosa and muscle. In the estrogen-treated rats, HSP90 kD synthesis is significantly upregulated with more HSP appearing in the mucosal than in the detrusor muscle. In normal rat kidney, Matsubara [16] reported that HSP90 kD is located exclusively in the cytoplasm of epithelial cells. This is in agreement with the urothelial cytoplasmic location observed in this study. Others showed HSP90 kD immunostaining in the cytoplasm and nucleolus in neurons from rat hypothalamus [17]. HSP90 kD can translocate into the nucleus from the cytoplasm in response to external stimuli like heat or hypoxia and was recently shown to occur in cardiac myocytes treated with estrogen [9]. Binding of HSF (heat shock transcription factors) to HSE (heat shock element) forming the heat shock gene promoter upregulates synthesis of many HSPs in response to hypoxia, stretch, heat, or injury [8, 9]. Eukaryotic heat shock genes are regulated via common transactivating pathways induced by stressful stimuli

and may have cell protective functions. HSP70 kD and HSP60 kD can also complex with HSP90 kD. This interaction can determine the state of activation of hormone receptors [28].

A competitive ELISA procedure was utilized to better quantitate the differences observed between the control and estrogen treated groups with immunostaining or immunoblotting. The absolute concentration of HSP90 kD (μg/mg protein) was determined by extrapolation from the standard curve and correcting for total extracted protein. The values were compared and analyzed by statistical analysis (Student's *t*-test).

HSP90 kD in urinary bladder extracts comprises up to 0.2–1.5% of total proteins in agreement with the 1–2% levels reported in other tissues and species. HSP90 kD began to rise 6 h after estrogen treatment, peaked at 24 h and then decreased to baseline values after 2–3 days. This is in agreement with the time course change of HSP90 kD after estrogen injection in the uterus and hypothalamus reported in a previous study [17].

In summary, our data shows that HSP90 kD synthesis in the rat urinary bladder is under the control of estrogen in a time-dependent manner. HSP90 kD may, therefore, be an important factor in estrogen–receptor interactions in the rat and, quite likely, in the human bladder. HSP (and other HSPs) can form associations with steroid receptors and modulate their activity, maintaining receptors in a high affinity hormone binding conformation [5, 33]. In the bladder, it is noteworthy that HSP90 kD induced by stretch (i.e., filling/emptying) can activate eNOS [26]. Estrogens can also indirectly activate HSFs since this study shows that they increase HSP90 kD synthesis, and HSP90 activates these transcription factors [34]. As a consequence, estrogen levels may play a role in cytoprotective mechanisms of the urothelium by upregulating HSPs, which are known to be beneficial under conditions of stress. For this reason, we feel these studies are important in understanding bladder disease and dysfunction, especially in IC where the mucosa is injured. In IC, where estrogens may have an etiological role based on symptomatology and a preponderance of women over men with this disorder, HSP90 kD may have a mucosal protective role similar to that shown in experimental rats in which acid induced colitis was dramatically prevented by increasing the intracellular HSP90 kD levels by hypothermia [18]. Also, in postmenopausal women known to have increased incidence of UTIs, irritative voiding symptoms, and incontinence, it is reasonable to suggest that these problems relate to declining estrogen levels with age and, likely, HSP90 kD expression as well. In fact, these conditions have a good clinical response to estrogen replacement therapy [8, 20, 32].

This study suggests that upregulating HSP90 kD responses in the bladder mucosa (via gene therapy) may be a future goal for treating certain bladder dysfunctions where tissues are under chronic stress (ischemia, inflammation, toxic factors) and not responding to conventional treatments [7].

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