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

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 understanding 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 AC8IgG1), 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 (Biomedica, Burlington, 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.

**Immunohistochemical 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 polylsine-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% H2O2 treatment. Complexed biotinylated antibody was detected by addition of peroxidase-conjugated streptavidin (Biomedica) 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 as control fluid.

**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.

**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 3x 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).