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Orchiectomy reduces susceptibility to renal ischemic injury: a role for heat shock proteins

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Abstract

In previous studies we demonstrated that the presence of testosterone, rather than the absence of estrogen, plays a critical role in gender differences in kidney ischemia/reperfusion (I/R) injury. Although molecular chaperones such as heat shock proteins (HSPs) have been implicated as protective agents in the pathophysiology of I/R injury, their roles in gender differences in susceptibility to renal I/R injury remain to be defined. Here we demonstrate that orchiectomy increases the basal and post-ischemic expression of HSP-27 in kidney tubular epithelial cells, but not HSP-72, glucose-regulated protein (GRP)-78 or GRP-94 expression. Orchiectomy prevents the disruption of the actin cytoskeleton and renal functional disorders induced by I/R, when compared with intact male mice or orchiectomized mice treated with dihydrotestosterone, a non-aromatizable isoform of testosterone. Thus, the protection afforded by orchiectomy is associated with increased expression of HSP-27, a heat shock protein important for maintenance of actin cytoskeletal integrity. These findings indicate that testosterone inhibits the heat shock response and may provide a new paradigm for design of therapies for I/R injury.

Keywords: Gender; Kidney; Ischemia; HSP-27; HSP-72; GRP-78; GRP-94; Castration; Testosterone

Molecular chaperones such as heat shock proteins (HSPs) protect cells and tissues from various stresses including ischemia/reperfusion (I/R) [1–4]. I/R in kidney results in characteristic morphological changes including collapse of the actin cytoskeleton, loss of cell–cell contact, and disruption of cell integrity, resulting in loss of epithelial cell function [5–8]. Molecular chaperone proteins are associated with maintenance of cellular integrity, and inhibition of inflammatory, and immune responses, which are involved in I/R injury.

Gender differences result in differential responses to I/R injury in brain, heart, and kidney [9–12]. Recently, we reported that orchiectomy reduced kidney susceptibility to I/R and that the presence of testosterone, rather than an absence of estrogen, plays a critical role in these gender differences [13]. It has been demonstrated that sex hormones regulate HSP expression in various organs [14–16] and that HSP expression has been implicated in gender differences in susceptibility to I/R [15]. We hypothesized that lower susceptibility to I/R seen in orchiectomized mice is associated with HSPs. To test the hypothesis we determined HSP expression in the kidneys isolated from intact, orchiectomized, and dihydroxytestosterone (DHT)-treated orchiectomized mice prior to and 4 h after I/R.

In these studies, we found that orchiectomy increases the expression of HSP-27 in the tubular epithelial cells in kidneys prior to and 4 h after I/R, but has no effect on

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HSP-72, glucose-regulated protein (GRP)-78 or GRP-94. DHT treatment of orchiectomized animals resulted in prevention of this increase in HSP-27 production. Thus, the greater resistance to I/R seen in orchiectomized mice may be explained by the increased expression of HSP-27, which stabilizes the actin cytoskeleton in the proximal tubular epithelial cell, leading to reduced post-ischemic histological damages and functional impairment.

Materials and methods

Animal preparation. Experiments were performed in age-matched (12–14 weeks) BALB/c male mice. In all cases, studies were done according to approved animal experimental procedures by the Animal Care and Use Committee at Kyungpook National University and the Brigham and Women's Hospital. Each animal group consisted of more than four mice. Blood was collected from mice to determine plasma creatinine.

Animals were anesthetized with pentobarbital sodium (60 mg/kg body weight; BW, ip) prior to surgery. Thirty minutes of bilateral renal ischemia was carried out as previously described [3]. In some animals orchiectomy was carried out 15 days before bilateral renal ischemia or sham surgery as previously described [13]. Some groups of animals were administered dihydroxytestosterone (500 μg/kg BW; DHT; Sigma) or vehicle (Sesame oil; Sigma) by subcutaneous injection every day for 14 days prior to ischemia. Kidneys were harvested for Western blot analysis and immunohistochemical studies as previously described [4].

Immunoblot analysis. For the preparation of protein samples from kidney, the organ was homogenized with a Dounce homogenizer and the homogenate was prepared for Western blot analysis as previously described [3]. Immunoblot analyses were performed with anti-HSP-27, -HSP-72, -GRP-78, and -GRP-94 antibodies obtained from Upstate Biotechnology (Lake Placid, NY) and anti-ERK-1 antibody obtained from the Santa Cruz.

Renal functional parameters. Seventy microliters of blood was taken from the retrobulbar vein plexus at the times indicated on the figures. Plasma creatinine concentrations were measured using a Beckman Creatinine Analyzer II.

Immunohistochemistry. Kidneys were perfused via the left ventricle with PBS for 2 min at 37 °C and then 4% PLP (4% paraformaldehyde, 75 mM L-lysine, and 10 mM sodium periodate) fixative. Kidneys were excised, placed in PLP overnight at 4 °C until assay, and washed and stored in PBS containing 0.02% sodium azide at 4 °C. Fixed tissue was embedded in oxytetracycline compound (Sakura FineTek, Torrance, CA) and then cut into 4 μm sections using a cryomicrotome (Cryotome). Sections were mounted on microscope slides and stored at −20 °C until use. To detect HSP-27, the section was dried and washed with PBS. The section was incubated in 2% goat serum for 20 min at room temperature and treated with HSP-27 antibody in 2% goat serum overnight 4 °C. The sections were washed with PBS three times and incubated in the Texas red conjugated anti-rabbit goat IgG (Vecta Laboratories) for HSP-27 staining for 30 min at room temperature. The sections were washed with PBS, mounted with mounting solution (Vectashield, Vector Laboratories), and observed by fluorescence microscope (Nikon). For the phalloidin staining, sections were dried, washed with PBS, and incubated in Cy3-conjugated phalloidin (Sigma) for 15 min.

Statistics. Results are expressed as means ± SEM. Statistical differences among groups were calculated using analysis of variance (ANOVA). Differences between groups were evaluated Student's *t* test. Differences were considered statistically significant at a *p* value of <0.05.

Results

Orchiectomy increases basal and post-ischemic HSP-27 expression in the kidney

Since HSPs have been implicated in protection against I/R injury [1,3,4,17], we determined HSP-27 or HSP-72 expression in intact, orchiectomized, and DHT-treated orchiectomized mice prior to and 4 h after ischemia. The level of HSP-27 expression in orchiectomized males is greater than in intact male mice prior to ischemia (Fig. 1C). DHT administration to orchiectomized mice prevents the increase in HSP-27 expression prior to ischemia (Fig. 1C). Ischemia increases HSP-27 expression in all experimental animal groups at 4 h after ischemia (Figs. 1A and C). The post-ischemic increase of HSP-27 expression is significantly greater in orchiectomized mice than in intact males (Figs. 1B and C). DHT administration to orchiectomized mice attenuates the post-ischemic increase of HSP-27 expression (Fig. 1C).

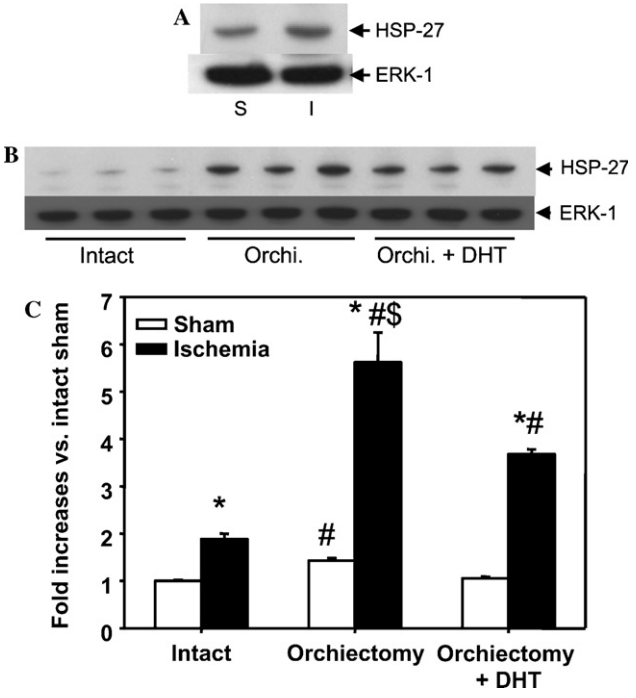

Fig. 1. Effect of hormonal modification on HSP-27 expression in the kidney. Some BALB/c mice were orchiectomized (Orchi.) on day 0. Some mice were administered dihydrotestosterone (DHT, 500 μg/kg BW) or vehicle by subcutaneous injection daily for 14 days. On day 15 mice were subjected to 30 min of bilateral ischemia at 36–38 °C. (A) The expression of HSP-27 in intact males was measured 4 h after either sham-operation (S) or 30 min of bilateral renal ischemia (I) by Western blot analysis using HSP-27 antibody. (B) Post-ischemic expression of HSP-27 in the kidney at 4 h after ischemia. ERK-1 was used as an equal loading marker. (C) The density of Western blot bands was quantified by the NIH Image program. Values presented are expressed as means ± SEM ($n = 4$). $^{*}p < 0.05$ versus their respective control. $^{\#}p < 0.05$ versus intact males before ischemia. $^{\$}p < 0.05$ versus post-ischemic expression in DHT-treated orchiectomized males.

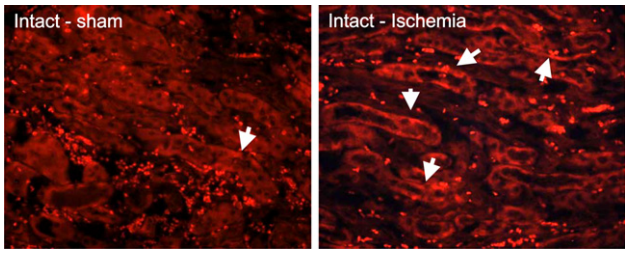

Fig. 2. Localization of HSP-27 in the proximal tubular epithelial cells. Some BALB/c mice were gonadectomized on day 0. Some mice were administered or vehicle (Sesame oil) by subcutaneous injection daily for 14 days. On day 15 mice were subjected to 30 min of bilateral ischemia at 36–38 °C. HSP-27 expression was determined by the immunohistochemical assay. Kidneys were perfusion-fixed with 4% PLP-fixative, cryocut in 4 μm sections, immuno-stained using HSP-27 antibody as a primary antibody, and Texas red was used as a fluorescence marker.

We localized HSP-27 expression by immunohistochemical techniques using HSP-27 antibody. HSP-27 is intensively expressed in the proximal tubular epithelial cells in the outer medulla of the kidney after ischemia (Fig. 2). Consistent with the Western blot analyses proximal tubule HSP-27 expression is greater in the post ischemic kidney when compared to HSP-27 expression in intact sham-operated kidneys (Fig. 2).

Orchiectomy does not change basal and post-ischemic GRP-72, GRP-78 or GRP-94 expression

There are no significant differences in basal HSP-72 expression in either orchiectomized or DHT-treated orchiectomized males when compared with intact males (Fig. 3). Four hours after ischemia HSP-72 expression is not altered in any of the three experimental animal groups (Fig. 3). Since the pathogenesis of ischemia is linked to endoplasmic reticulum (ER) stress and cells exposed to ER stresses produce ER molecular chaperones such as GRP-78 and GRP-94 [18–21], we evaluated GRP-78 and GRP-94 in renal I/R injury in hormonal modified mice. There are no significant differences in the basal levels of either GRP-78 or GRP-94 expression in both orchiectomized and DHT-treated orchiectomized mice when compared with intact males (Fig. 4). Four hours after ischemia the levels of both GRP-78 and GRP-94 expression are not altered in any of the three experimental animal groups (Fig. 4).

Orchiectomy protects the kidney from ischemial reperfusion injury

There are no significant changes of body weight after orchiectomy or DHT treatment of orchiectomized mice. Thirty minutes of bilateral kidney ischemia in intact mice markedly reduces renal function 4 and 24 h after ischemia as measured by plasma creatinine concentration (Fig. 5). Four hours after ischemia the levels of plasma creatinine in both intact and DHT-treated orchiectomized mice increase to approximately four

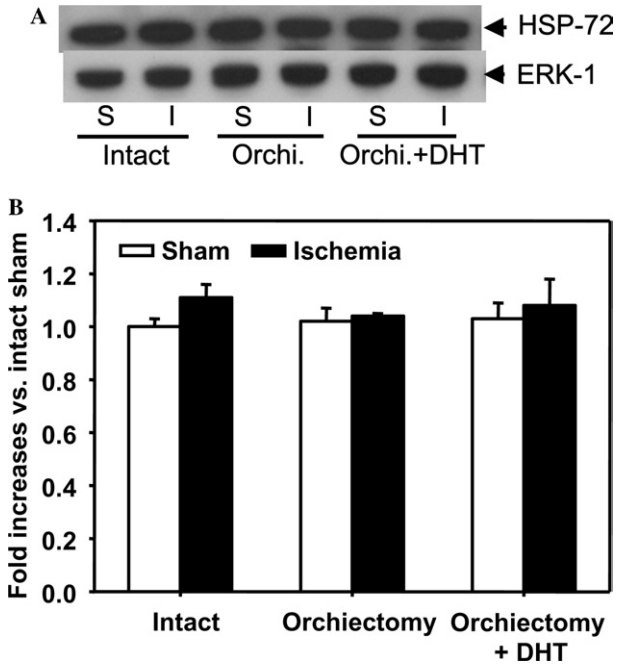

Fig. 3. Effect of hormonal modification on HSP-72 expression in the kidney. Some BALB/c mice were orchiectomized (Orchi.) on day 0. Some mice were administered dihydrotestosterone (DHT, 500 μg/kg BW) or vehicle by subcutaneous injection daily for 14 days. On day 15 mice were subjected to 30 min of bilateral ischemia at 36–38 °C. (A) The expression of HSP-72 in intact males was measured 4 h after either sham-operation (S) or 30 min of bilateral renal ischemia (I) by Western blot analysis using HSP-72 antibody. Post-ischemic expression of HSP-72 in the kidney 4 h after ischemia. ERK-1 was used as an equal loading marker. (B) The density of Western blot bands was quantified by the NIH Image program. Values presented are expressed as means ± SEM ($n = 4$). Intact indicates intact mice.

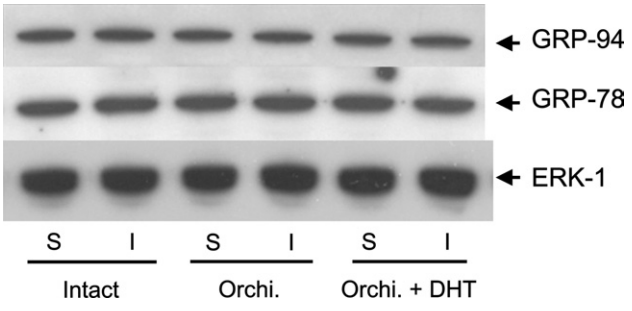

Fig. 4. Effect of hormonal modification on GRP-78 and GRP-94 expression in the kidney. Some BALB/c mice were orchiectomized (Orchi.) on day 0. Some mice were administered dihydrotestosterone (DHT, 500 μg/kg BW) or vehicle by subcutaneous injection daily for 14 days. On day 15 mice were subjected to 30 min of bilateral ischemia at 36–38 °C. The expression of GRP-78 and GRP-94 in intact males was measured 4 h after either sham-operation (S) or 30 min of bilateral renal ischemia (I) by Western blot analysis using GRP-78 and GRP-94 antibodies. The blots represent representative bands of four independent experiments. ERK-1 was used as an equal loading marker.

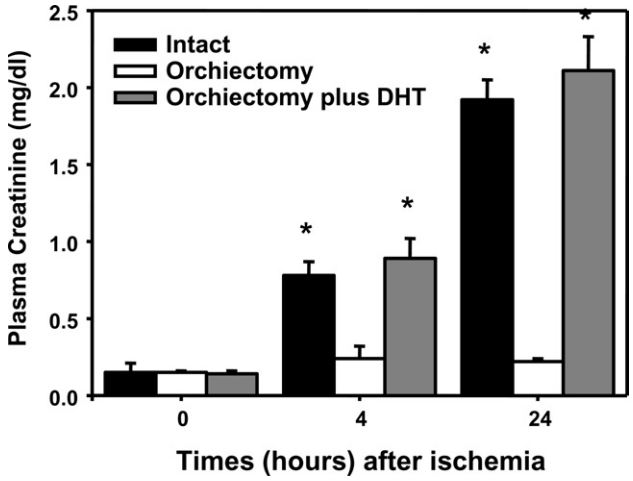

Fig. 5. Effect of hormonal modification in post-ischemic plasma creatinine concentrations. Some BALB/c mice were orchiectomized on day 0. Some mice were administered dihydrotestosterone (DHT, 500 μg/kg BW) or vehicle by subcutaneous injection daily for 14 days. On day 15 mice were subjected to 30 min of bilateral ischemia at 36–38 °C. Blood was collected prior to ischemia (time 0) and at 4 and 24 h after ischemia. Values presented are expressed as means ± SEM ($n = 4$–6). *$p <$ 0.05 versus their respective control.

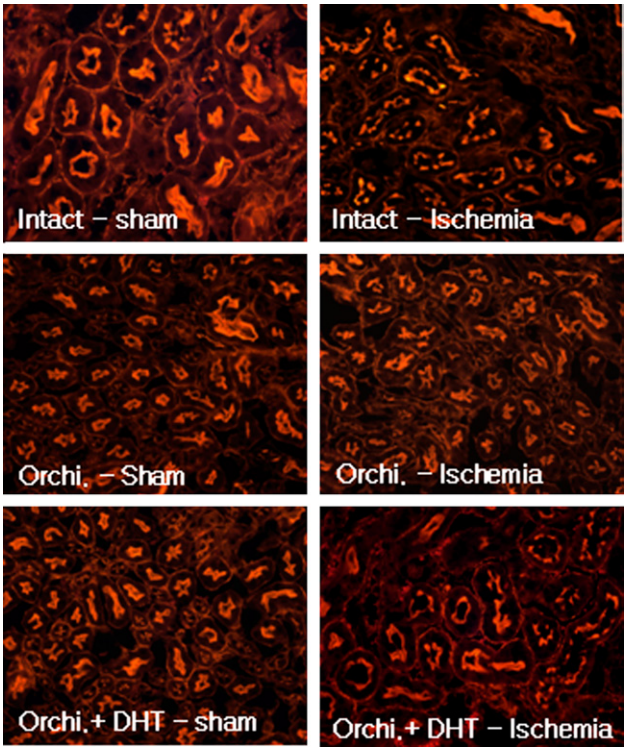

Fig. 6. Post-ischemic disruption of the actin cytoskeleton in the proximal tubular epithelial cells in kidney. Some BALB/c mice were orchiectomized (Orchi.) on day 0. Some mice were administered dihydrotestosterone (DHT, 500 μg/kg BW) or vehicle by subcutaneous injection daily for 14 days. On day 15 mice were subjected to 30 min of bilateral ischemia or sham surgery at 36–38 °C. Kidneys were perfusion-fixed with 4% PLP-fixative, cryocut in 4 μm sections and stained using cy3-conjugated phalloidin.

times of that of basal levels (Fig. 5). By contrast, the same period of ischemia in orchiectomized mice results in no measurable change in the concentration of plasma creatinine 4 and 24 h after ischemia (Fig. 5). Some actin disassembly is observed in the proximal tubule cell in the outer medullary S3 segment in the kidney in intact and DHT-treated orchiectomized mice 4 h after ischemia (Fig. 6). In contrast, there is less actin disruption in orchiectomized mice 4 h after ischemia (Fig. 6).

Discussion

These studies demonstrate that orchiectomy increases the basal and post-ischemic expression of HSP-27 in the proximal tubular epithelial cells in the kidney 4 h after ischemia, but does not result in changes in HSP-72, GRP-94 or GRP-78. These studies further demonstrate that orchiectomy is associated with increased stability of the actin cytoskeleton and functional impairment associated with I/R. DHT administration to the orchiectomized mice increases the kidney susceptibility to I/R injury, with an associated reduction of HSP-27 expression prior to and post ischemia.

Recently we reported that gender differences profoundly affected susceptibility to ischemic acute renal failure in mice and that the presence of testosterone, rather than an absence of estrogen, played a critical role in enhanced male susceptibility to kidney I/R injury. Orchiectomy protected the kidney from I/R, whereas ovariectomy did not alter the resistance of females to I/R injury [13]. In the current studies, we also found that orchiectomy rendered the kidney resistant against I/R injury, consistent with our previous studies [13]. In the current studies, we found that the increases of plasma creatinine in intact and DHT-treated orchiectomized mice were about four times baseline 4 h after ischemia. Although, interestingly, damaged tubular cells were observed in the proximal tubules in intact and DHT-treated orchiectomized mice 4 h after ischemia, histologic evidence of damaged tubules was modest when compared with the increases of plasma creatinine. In previous studies [13] we observed that 30 min of bilateral renal ischemia disrupted most of the proximal tubular cells in the outer medulla 24 h after ischemia [4,22]. These data are consistent with a delay between the histological evidence for severe tubular damage and the functional impairment.

Several studies have demonstrated that estrogen regulates HSP expression in various organs and that HSP expression differs with gender [15].The present studies demonstrate that the ablation of male sexual hormone such as testosterone by orchiectomy results in increased basal and post-ischemic expression of HSP-27 in the proximal tubular cells in the kidney, whereas an administration of DHT into orchiectomized mice prevented

the increase of HSP-27 expression afforded by orchiectomy. We previously reported that kidney resistance to ischemic injury by ischemic preconditioning is associated with HSP-27 expression [3]. In subsequent studies, we also demonstrated that forced expression of HSP-27 in LLC-PK1 cells, an established kidney epithelial cell line cell, by using an adenoviral vector encoding HSP-27, protects the cells from glucose deprivation and reactive oxygen species stress [4]. In the same studies, we found that increased expression of HSP-27 is correlated with kidney resistance afforded by ureteral obstructive preconditioning [4]. Thus, the increased resistance to injury in orchiectomized mice may be explained by the increased expression of HSP-27. The mechanism of protection afforded by HSP-27 may relate in part to stabilization of the actin cytoskeleton [23] in the proximal tubular epithelial cell, leading to reduce the post-ischemic functional impairment and histological damage. Rocchi and colleagues reported that androgen ablation increased HSP-27 expression and the increased HSP-27 confers resistance to androgen withdrawal in hormone-refractory prostate cancer cells. This results from the anti-apoptotic effect of HSP-27 expression [24]. Castration increased the expression of HSP-27 mRNA in the rat ventral prostate [14].

It has been suggested that HSP-72 protects cells/tissues from stress including I/R [17,25,26] and that HSP-72 expression is regulated by sex hormones such as estrogen [16]. In previous studies, we reported that kidney ischemia increased HSP-72 24 h after ischemia; however, in present studies we did not find any significant increases in HSP-72 in the kidney 4 h after ischemia [3,4]. Since the protection was already apparent by 4 h, these data suggest that inducible HSP-72 may not be associated with the renal tolerance conferred by androgen ablation by orchiectomy.

Endoplasmic reticulum stress is common in I/R and ER molecular chaperone proteins, such as GRP-78 and GRP-94, are produced to protect against the stress [18,21]. Recently, our laboratory [19] reported that increased GRP-78 expression is present in cells that have been rendered resistant to damage associated with reactive oxygen species (ROS). Inhibition of GRP-78 gene expression increased the cell susceptibility to ROS. We have also recently found that pretreatment of intact mice with tunicamycin, an inducer of ER molecular chaperones such as GRP-78, protects the kidney from I/R injury (Park et al., unpublished data). In the present study, we did not find any change in GRP-78 and GRP-94 expression 4 h after reperfusion. Thus GRP-78 and GRP-94 may not be associated with the early protection afforded by orchiectomy.

In summary, our data demonstrate that increases of HSP-27 expression may account for the protection against I/R injury afforded by androgen ablation of by orchiectomy. This could result in increased stabilization of the actin cytoskeleton. Our finding may have important implications for understanding the pathophysiology of gender differences in kidney I/R injury and should lead to consideration of androgens as targets for protective strategies to avoid acute renal failure.

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