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p66shc and Gender-specific Dimorphism in Acute Renal Injury

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Abstract. *Background/Aim: Acute renal injury (AKI) is more prevalent in males than in females perhaps due to adverse effects of testosterone. The oxidant sensor p66shc is regulated by testosterone, hence may be responsible for the aforementioned gender disparity. Materials and Methods: Wild-type or p66shc-knockdown renal proximal tubule cells were treated with 400 μM H_2O_2 in the presence or absence of 100 nM dihydrotestosterone (DHT); the production of reactive oxygen species and cell injury were determined. The impact of DHT on p66shc expression and promoter activity as well as gender-dependent expression of p66shc in the mouse kidney was also determined. Results: DHT increased H_2O_2-dependent oxidative stress and injury via p66shc and expression of p66shc via promoter activation. Renal expression of p66shc was higher in male compared to female kidneys. Conclusion: Higher sensitivity of the male kidney to AKI may be due to the testosterone-dependent increase in p66shc expression.*

Ischemia-reperfusion-induced acute kidney injury (IR-AKI) is a severe illness with a high mortality/morbidity rate among patients at ICUs (1). Interestingly, incidence of IR-AKI is significantly higher in males than in females (2). Some studies suggested that this gender-related dimorphism is associated with androgen hormones such as testosterone (3) that promotes oxidative stress in the kidney (4-6). However, the mechanism by which testosterone augments oxidative milieu remains elusive.

In earlier studies of ours we suggested that the adaptor protein p66shc (7) may play a pivotal role in renal injury during IR-AKI (8) by increasing mitochondrial production of ROS and consequent mitochondrial de-polarization (9). We also showed that a number of renal toxicants, including oxidative stress, elicit their renal toxicity by up-regulating the promoter of *p66shc* (10-12). Interestingly, androgens such as dihydrotestosterone (DHT) have been also shown to induce the promoter of *p66shc* in cancer cells (13, 14); hence, p66shc may contribute to gender-specific disparity in the ischemic kidney.

Accordingly, we hypothesized that enhanced sensitivity of the male kidney to IR-AKI is due to, at least partly, androgen-dependent activation of p66shc.

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Materials and Methods

Animal experiments. Kidneys from 8-10-week-old male and female C57Bl/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA).

Cell culture. The immortalized mouse renal proximal tubule cell line (TKPTS) was used as described elsewhere (8). Oxidative stress was established by treatment of cells with 400 μM H_2O_2, as described elsewhere (9). For dihydrotestosterone (DHT) (Sigma-Aldrich, St. Louis, MO, USA) treatment cells were grown in depleted medium (Life Technologies, Grand Island, NY, USA) and serum-starved overnight. The *p66shc*-knockdown variant of TKPTS was developed and maintained as described elsewhere (8).

Assessment of cell injury. The extent of cell injury was determined by the fluorescent CytoTox-One Homogenous Membrane Integrity assay kit (Promega, Madison, WI, USA), as described elsewhere (15).

Determination of ROS production. Intracellular generation of ROS was determined by fluorescent oxidant-sensitive 2',7'-dichlorofluorescein-diacetate dye (DCFDA) (Life Technologies, Grand Island, NY, USA) in a 96-well-plate, as described elsewhere (16). ROS production was calculated as the increase in fluorescence/ 30 min/ 0.5×10^6 cells and expressed as a percentage of that of corresponding untreated cells.

Western blotting, immunoprecipitation. Kidney or cell lysates were obtained in a RIPA buffer as described elsewhere (15). For immunoprecipitation, 400-500 μg total cell lysates were incubated with a p66shc antibody (NanoTools, Germany) overnight at 4˚C by using the "Catch and Release v2.0 reversible immunoprecipitation system" (Millipore, Charlottesville, VA, USA). Immunoprecipitated proteins or 20-50 μg of kidney/cell lysates were separated on a 4-12% NuPAGE Novex®Bis-Tris gradient mini gel (Invitrogen, Grand Island, NY, USA) and transferred to a PVDF membrane by iBlot

(Invitrogen, Grand Island, NY, USA). Blots were hybridized with a p66shc antibody, visualized by Pierce® ECL Western blotting substrate (Thermo Scientific, Rockford, IL, USA), and exposed to X-ray film (Midwest Scientific, St. Louis, MO, USA). Films were digitized and analyzed by Un-Scan-It™ Version 6.1 software (Silk Scientific, Orem, UT, USA).

Reporter luciferase assay. To evaluate promoter activity of p66shc-reporter luciferase activities, cells grown in 24-well plates were transfected with the p66shc-Luc plasmid containing the -1096 to +44 base-pair region (relative to the ATG codon) of the human *p66shc* gene promoter (a gift from Dr. Irani, Cardiovascular Institute, University of Pittsburgh, PA, USA) (17) together with a Renilla luciferase (Promega, Madison, WI, USA) by using Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY, USA). 24 h after treatment(s) firefly and renilla luciferase activities were determined by using the Dual Luciferase assay kit (Promega, Madison, WI, USA) in a Modulus luminometer (Turner Biosystem, Sunnyvale, CA, USA), as recommended by the manufacturer. p66shc-Luc activity was normalized to the internal Renilla-Luc activity.

Statistical analysis. Continuous variables were expressed as means and standard deviations (S.D.). Statistical differences between the treated and control groups were determined by Student's *t*-test. Differences between means were considered significant if $p<0.05$. All analyses were performed using the SigmaStat 3.5 (Systat, San Jose, CA, USA) software package.

Results

DHT augments H_2O_2-induced ROS production and cell injury that depends on p66shc. To determine whether DHT exacerbates detrimental effects of oxidative stress, TKPTS cells were treated with 400 μM H_2O_2 in the presence or absence of 100 nM DHT (see Materials and Methods). Production of ROS and cell injury (by means of LDH release) were determined as described above. As Figure 1A shows, DHT significantly augments H_2O_2-dependent ROS production and LDH release. Next, we determined whether adverse effects of DHT were associated with p66shc. Earlier, we created a TKPTS cell line in which expression of p66shc was knocked-down (k.d.) (8). Hence, we used that p66shc k.d. line and compared responses to the wild-type (w.t.) p66shc cell line after treatment with DHT+ H_2O_2. As is seen in Figure 1B, knocking-down of p66shc significantly attenuated DHT+ H_2O_2-dependent induction of ROS and LDH release. These results suggested that adverse effects of DHT on ROS production and cell injury are mediated through p66shc.

DHT treatment increases expression of p66shc via induction of its promoter. To determine whether DHT treatment affects expression of p66shc as described in cancer cells (14), TKPTS cells were treated with 100 nM DHT and cell lysates were prepared at various time points. Protein levels of p66shc were determined by western blotting. Figure 2A and

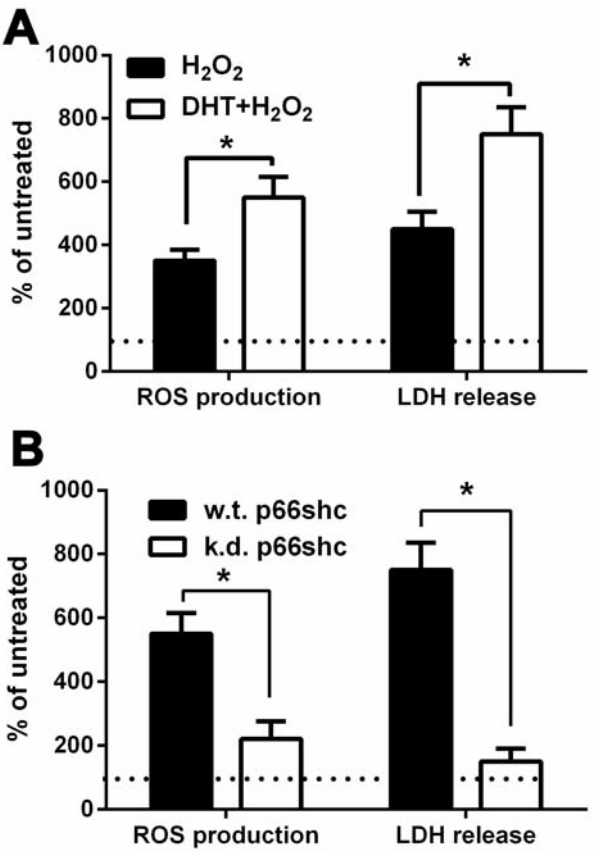

Figure 1. *Dihydrotestosterone (DHT) augments oxidative stress (H_2O_2)-dependent production of reactive oxygen species (ROS) and cell injury (LDH release) via p66shc. (A) Mouse renal proximal tubule (TKPTS) cells were treated with 400 μM H_2O_2 in the presence or absence of 100 nM DHT. ROS production and LDH release were determined as described in Materials and Methods. Results were expressed as % of untreated values, n=3, *p<0.05. Dotted line represents 100% (untreated values). (B) TKPTS cells with p66shc wild-type (w.t.) or knockdown (k.d.) phenotype were treated with 400 μM H_2O_2 in the presence of 100 nM DHT. ROS production and LDH release were determined as described in Materials and Methods. Results were expressed as % of untreated values, n=3, *p<0.05. The dotted line represents 100% (untreated values).*

B demonstrate that 100 nM DHT significantly increases p66shc levels as early as 6 h. Next, we wanted to know whether DHT affects p66shc at the level of transcription or not. Accordingly, TKPTS cells were transfected with a *p66shc*-promoter-luciferase reporter (17) together with a renilla luciferase and treated with various concentrations of DHT for 24 h. Luciferase activities were determined and the activity of the *p66shc* promoter was normalized to renilla activity. As is seen in Figure 2C, DHT increased activity of the *p66shc* reporter, especially at higher concentrations. These data suggested that DHT affects *p66shc* at the promoter level.

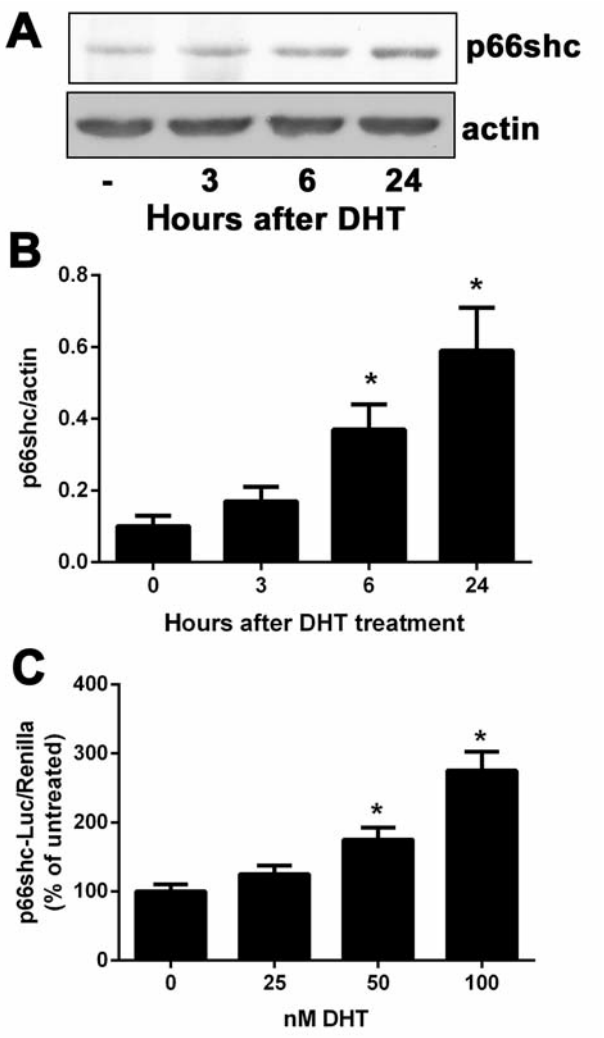

Figure 2. *DHT increases expression of p66shc via up-regulation of its promoter. (A) TKPTS cells were treated with 100 nM DHT for the time points indicated. p66shc expression together with actin was determined from cell lysates by western blotting, as described in Materials and Methods. Results shown are representatives of three independent experiments. (B) Densitometry of western blot results from (A). Expression of p66shc was normalized to actin expression. n=3, *p<0.05 compared to untreated (0-h treatment).*

Renal expression of p66shc is higher in male than in female mice. Results of experiments described above suggested that higher testosterone levels may predict for higher levels of p66shc in the kidney. To test this hypothesis, we compared renal expression of p66shc in kidneys from aged-matched male and female mice. Since p66shc protein levels are relatively low in the kidney, we employed immunoprecipitation to enhance detectability of the p66shc protein and normalized its level to actin expression in the same amount of lysate. Figure 3A-B demonstrates that kidneys from male mice express higher amounts of p66shc than kidneys from female mice. These

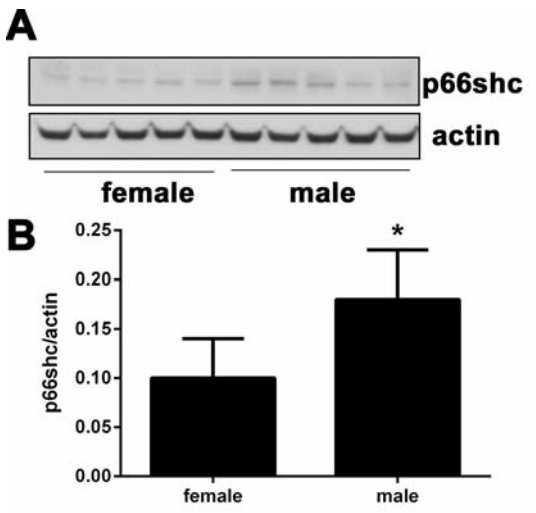

Figure 3. *p66shc expression is higher in kidneys from aged-matched male compared to female mice. (A) Kidneys from 8-10-week-old female or male mice were lysed and analyzed with western blotting as described in Materials and Methods. (B) Densitometry of results from (A). n=5, *p<0.05 compared to females.*

results may further substantiate the connection between testosterone levels and p66shc expression and perhaps sensitivity to IR-AKI.

Discussion

Several studies have demonstrated that androgens promote oxidative stress in the kidney (4-6), that may be the result of increased mitochondrial ROS production, as observed in prostate cancer cells (14). Excess production of mitochondrial ROS contributes to injury during IR-AKI (18) that -as we proposed earlier- could be due to activation of p66shc (9). p66shc -*via* binding to cytochrome *c*- diverts electrons from the respiratory chain resulting in excess formation of hydrogen peroxide (H_2O_2) in the mitochondria and consequent cell injury (19). Our present data support this scenario: DHT exacerbates oxidative stress (H_2O_2)-induced production of ROS and cell injury in a p66shc-dependent manner (Figure 1A-B).

DHT increases expression of p66shc in cultured renal proximal tubule cells (Figure 2A-B) *via* activation of the *p66shc* gene promoter (Figure 2C) similar to observations in prostate cancer cells (14). Importantly, among aged-matched mice, renal expression of p66shc was higher in males than in females (Figure 3A-B). Earlier we demonstrated that H_2O_2-dependent injury incrementally increased in proportion to increasing amounts of p66shc in renal proximal tubule cells, implying that the extent of oxidative injury depends on the levels of p66shc (10). Therefore, we can postulate that the higher sensitivity of the male kidney to IR-AKI may be

due to, at least partly, the testosterone-dependent increase in renal expression of p66shc.

It is elusive, however, how DHT may enhance activity of the *p66shc* promoter. Studies including our own, have suggested that the *p66shc* promoter is up-regulated by p53 (17, 20) and hypo-methylation (10, 21, 22). Some data from the literature show that DHT significantly increases expression of p53 (23) and decreases activity of DNA methyltransferases (24). Hence, a stimulatory effect of DHT on the *p66shc* promoter *via* p53 or hypo-methylation in the kidney is highly plausible and warrants further investigation.

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