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Sex Hormones Differentially Modulate STAT3-dependent Antioxidant Responses During Oxidative Stress in Renal Proximal Tubule Cells

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Abstract. *Background/Aim: Gender-associated dimorphism in renal oxidative stress may be related to the protective effects of estrogens or the adverse effects of testosterone. Signal transducer and activator of transcription-3 (STAT3)-dependent transcription is vital in renal antioxidant responses, which may be differentially regulated by sex hormones. Materials and Methods: Renal proximal tubule cells were treated with 400 μM H_2O_2 in the presence or absence of 100 nM dihydrotestosterone (DHT), 100 nM 17β-estradiol (E2) or dominant-negative STAT3 (dnSTAT3). Production of reactive oxygen species (ROS), phosphorylation/transcriptional activation of STAT3 and promoter activity of the STAT3-regulated antioxidant gene (MnSOD) were determined. Results: After treatment with H_2O_2, DHT decreased tyrosine phosphorylation/transcriptional activity of STAT3 and promoter activity of MnSOD while E2 increased them. Consequently, DHT augmented while E2 attenuated ROS production. Effects of dnSTAT3 were similar to DHT. Conclusion: Sex hormones may influence renal oxidative stress through differential regulation of STAT3-dependent antioxidant responses.*

Women experience lower incidence of acute renal injury and progression to chronic kidney disease than men (1). This gender-associated dimorphism may be related to protective effects of estrogens (2, 3) and/or adverse effects of testosterone (4, 5). Oxidative stress, a major culprit in renal diseases (6), is due to either excessive formation of reactive oxygen species (ROS) or inefficient activation of the antioxidant system.

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Studies have shown that testosterone promotes oxidative stress in the kidney (7), while estrogens ameliorate it (8). Previously, we showed that testosterone upregulates the promoter of the pro-oxidant p66shc gene thereby increasing mitochondrial ROS production and consequent injury during oxidative stress in renal proximal tubule cells (9). The impact of testosterone and estrogens on the antioxidant system in the kidney, however, remains elusive. Signal transducer and activator of transcription 3 (STAT3) is a transcription factor with adaptive function during oxidative stress (10): in the heart it activates transcription of antioxidant genes such as the manganese superoxide dismutase (MnSOD) (11). Interestingly, STAT3 activation is attenuated by testosterone (12) but augmented by estradiol (13) during ischemia, which maybe due to sex hormone-dependent differential regulation of STAT3.

Accordingly, we hypothesized that testosterone increases oxidative stress in renal proximal tubule cells *via* suppressing activation of STAT3, and hence activation of MnSOD, while estradiol exerts opposite effects.

Materials and Methods

Cell culture. The porcine renal proximal tubule cell line (LLC-PK1) was used as described elsewhere (14). Oxidative stress was established by treating cells with 400 μM H_2O_2 as described elsewhere (15). For dihydrotestosterone (DHT: Sigma-Aldrich, St. Louis, MO, USA) or 17β-estradiol (E2: Sigma-Aldrich) treatment, cells were grown in depleted serum (Life Technologies, Grand Island, NY, USA)-containing medium and serum starved overnight prior to treatment.

Measurement of ROS production. Intracellular generation of ROS was determined using the fluorescent oxidant-sensitive 2',7'-dichlorofluorescein-diacetate dye (DCFDA; Life Technologies) as described elsewhere (15). ROS production was calculated as the increase in fluorescence/30 min/0.5×10^6 cells and expressed as a percentage of untreated cells.

Western blotting. Cell lysates were prepared in a RIPA buffer as described elsewhere (15). 50 μg of lysates were separated on a 4-12% NuPAGE Novex®Bis-Tris gradient mini gel and transferred to

a PVDF membrane by using iBlot (Life Technologies). Blots were hybridized with an anti-phospho-tyrosine (Tyr705)-STAT3 and after stripping with an anti-STAT3 antibody (Cell Signaling, Danvers, MA, USA) and were subsequently visualized by Pierce® ECL Western blotting substrate (Thermo Scientific, Rockford, IL, USA). After exposure to an X-ray film (Midwest Scientific, St. Louis, MO, USA), the films were digitized and analyzed by Un-Scan-It™ Version 6.1 software (Silk Scientific, Orem, UT, USA).

Plasmid transfection. The dominant-negative STAT3 plasmid (dnSTAT3), that harbors a Y705F mutation of the tyrosine residue of *STAT3*, was purchased from Addgene (Cambridge, MA, USA) and transfected into cells by using Lipofectamine 3000 reagent (Life Technologies, Grand Island, NY, USA).

Reporter luciferase assay. To evaluate STAT3-dependent transcription, cells grown in 24-well-plate were transfected with a STAT3-luciferase plasmid (Clontech, Mountain View, CA, USA) together with a Renilla luciferase (Promega, Madison, WI, USA) by using Lipofectamine 3000 reagent (Life Technologies). To determine promoter activity of the MnSOD gene, a MnSOD-promoter luciferase construct (a gift from Dr. Burgering) was used. Firefly (STAT3 or MnSOD) and renilla luciferase activities were determined by using the Dual Luciferase assay kit (Promega) in a Modulus luminometer (Turner Biosystem, Sunnyvale, CA, USA), as recommended by the manufacturer. STAT3-Luc or MnSOD-Luc activity was normalized to the internal Renilla-Luc activity.

Statistical analysis. Continuous variables are expressed as means and standard deviations (S.D.). Statistical differences between the treated and control groups were determined by the 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 suppresses while E2 augments H_2O_2-induced tyrosine phosphorylation and transcriptional activity of STAT3. LLC-PK1 cells were pre-treated or not with either 100 nM DHT or 100 nM E2 overnight prior to treatment with 400 μM H_2O_2. After 30 min, cell lysates were prepared and subjected to SDS-PAGE and western blotting: tyrosine phosphorylation of STAT3 (pTyrSTAT3) and expression of unphosphorylated STAT3 were determined. Figure 1A-B shows that H_2O_2 significantly increased pTyrSTAT3, which was decreased by DHT but augmented by E2. To determine whether the observed changes in pTyrSTAT3 were accompanied by changes in transcriptional activity of STAT3, LLC-PK1 cells were co-transfected with STAT3-Luc and Renilla plasmids. The STAT3-luc plasmid harbors repeats of a consensus STAT3-binding element, and hence, its activation reflects the transcriptional activity of STAT3. Figure 1C shows that H_2O_2 treatment increases transcriptional activity of STAT3, which is abolished by pre-treatment of DHT, but enhanced by E2.

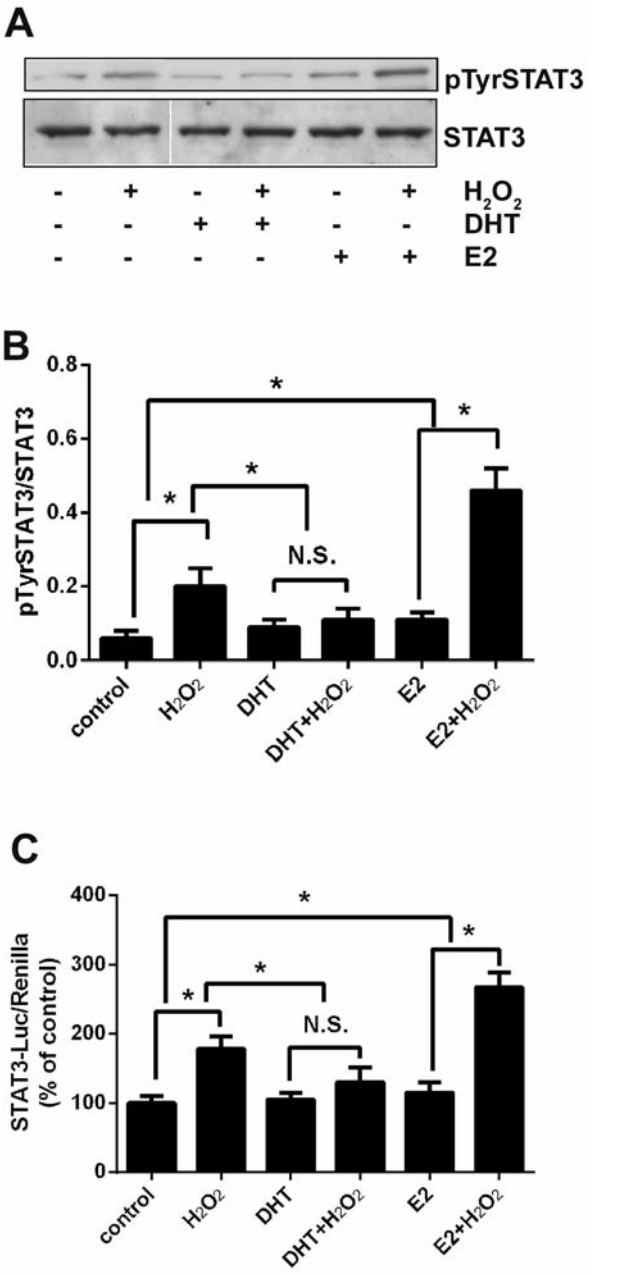

Figure 1. *Dihydrotestosterone (DHT) decreases while estradiol (E2) increases oxidative stress-dependent tyrosine phosphorylation and transcriptional activity of STAT3. (A) Renal proximal tubule (LLC-PK1) cells were treated or not with 100 nM DHT or 100 nM E2 overnight. Tyrosine phosphorylation of STAT3 (pTyrSTAT3) and levels of unphosphorylated STAT3 were determined 30 min after treatment with 400 μM H_2O_2 by western blotting. Results are representative of three independent experiments. (B) Densitometry of the blots is represented in (A). *p<0.05 (C) LLC-PK1 cells were transfected with a STAT3-Luc reporter luciferase plasmid together with a renilla luciferase plasmid. Some cells were pre-treated with either 100 nM DHT or E2 overnight prior to treatment with 400 μM H_2O_2. Luciferase activities were determined 24 h later. Values were calculated as firefly (STAT3)/renilla ratios and expressed as percentage of untreated control. N=3, *p<0.0.5> N.S., Not significant.*

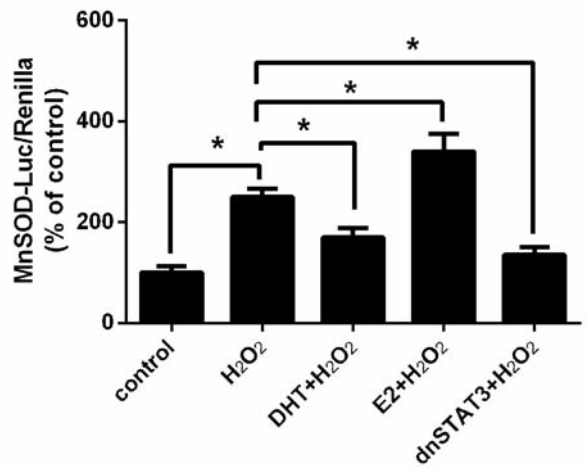

Figure 2. *DHT decreases while E2 increases H_2O_2-dependent activation of the MnSOD promoter. LLC-PK1 cells were transfected with a MnSOD-promoter luciferase plasmid together with renilla luciferase and pre-treated or not with either 100 nM DHT or E2 for 24 h prior to treatment with 400 μM H_2O_2. In some experiments, cells were also co-transfected with a dnSTAT3 plasmid, as indicated. Luciferase activities were determined 24 h later. Values were calculated as firefly (MnSOD)/renilla ratios and expressed as percentage of untreated control. N=3, *p<0.0.5. dnSTAT3, Dominant-negative (Y705F)-STAT3.*

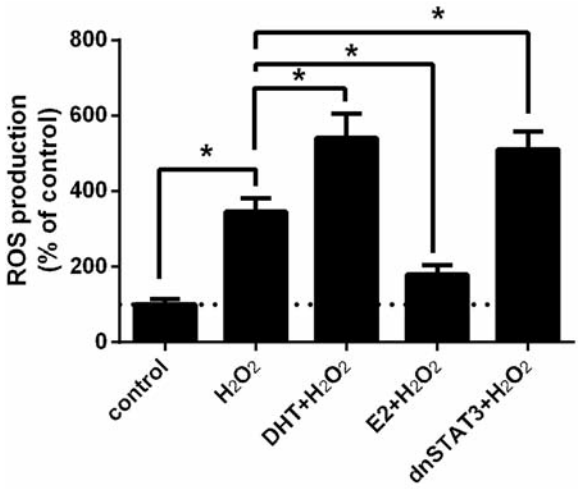

Figure 3. *DHT exacerbates while E2 ameliorates H_2O_2-dependent ROS production. LLC-PK1 cells were pretreated or not with either 100 nM DHT or E2 for 24 h. ROS production was determined after adding 400 μM H2O2. Some cells were transfected with a dnSTAT3n plasmid, as indicated. N=3, *p<0.05.*

DHT treatment suppresses while E2 augments H_2O_2-dependent activation of the MnSOD promoter. Since the antioxidant MnSOD is regulated *via* STAT3, the above results imply that sex hormones may alter H_2O_2-dependent activation of the MnSOD promoter. To determine this scenario, LLC-PK1 cells were transfected with a MnSOD-promoter-luciferase reporter plasmid together with a renilla plasmid and treated with 400 μM H_2O_2 in the presence or absence of DHT, E2 or dnSTAT3. Figure 2 shows that H_2O_2 induces the MnSOD promoter, the extent of which is attenuated by pre-treatment with DHT or transfection with dnSTAT3 while augmented by pretreatment with E2.

DHT increases while E2 decreases H_2O_2-mediated ROS production. To determine whether the above-described effects of sex hormones also reflect on oxidative stress, LLC-PK1 cells were pre-treated with either 100 nM DHT or E2 or transfected with dnSTAT3 prior to treatment with 400 μM H_2O_2, and ROS production was determined. Figure 3 shows that while DHT and dnSTAT3 exacerbate H_2O_2-mediated ROS production E2 ameliorates it.

Discussion

Studies have shown that testosterone promotes oxidative stress in the kidney (7) while estrogens may ameliorate it (8). Oxidative stress is defined as an imbalance between ROS production and elimination of ROS. Previously, we showed that DHT increases ROS production in renal proximal tubule cells through transcriptional activation of the pro-oxidant p66shc gene (9). Herein, we studied whether sex hormones may differentially affect the antioxidant system. MnSOD is important in elimination of intracellular ROS in the kidney (16). MnSOD is transcriptionally activated during oxidative stress, which –among others- involves the activated STAT3 (11). Interestingly, STAT3 activation is modified by sex hormones during oxidative stress: while testosterone decreases it in the ischemic heart (12), estradiol synergistically augments it in ischemic neuronal cells (13). In addition, estradiol is shown to inhibit ROS production in osteoblasts (17) while testosterone increases ROS production in vascular smooth muscle cells. Our studies showed that DHT inhibited oxidative stress-dependent tyrosine phosphorylation of STAT3 (Figure 1A-B) which resulted in a decrease in transcriptional activity of STAT3 including the STAT3-regulated MnSOD (Figure 1C and 2) under oxidative stress. In contrast, E2 treatment augmented oxidative stress-dependent tyrosine phosphorylation of STAT3 (Figure 1A-B) leading to enhanced activity of STAT3 (Figure 1C) and the MnSOD promoter (Figure 2). These results imply that DHT, through suppression of STAT3 activation (Figure 1), inhibits MnSOD expression that may result in increased oxidative stress. In contrast, E2 promotes antioxidant responses *via* up-regulating STAT3-dependent induction of the MnSOD promoter. Indeed, H_2O_2-dependent production of ROS is augmented by DHT and dnSTAT3, but attenuated by E2 (Figure 3).

Our data may explain the observation that men are at higher risk to renal oxidative stress compared to women, and that this difference is due to differential activation of STAT3 and related antioxidant responses by stress hormones.

Acknowledgements

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References

1 Neugarten J: Gender and the Progression of Renal Disease. J Am Soc Nephrol *13*: 2807-2809, 2002.
2 Dixon A and Maric C: 17beta-Estradiol attenuates diabetic kidney disease by regulating extracellular matrix and transforming growth factor-beta protein expression and signaling. Am J Physiol Renal Physiol *293*: F1678-1690, 2007.
3 Muller V, Szabo A, Viklicky O, Gaul I, Portl S, Philipp T and Heemann UW: Sex hormones and gender-related differences: their influence on chronic renal allograft rejection. Kidney Int *55*: 2011-2020, 1999.
4 Park KM, Kim JI, Ahn Y, Bonventre AJ and Bonventre JV: Testosterone is responsible for enhanced susceptibility of males to ischemic renal injury. J Biol Chem *279*: 52282-52292, 2004.
5 Metcalfe PD, Leslie JA, Campbell MT, Meldrum DR, Hile KL and Meldrum KK: Testosterone exacerbates obstructive renal injury by stimulating TNF-alpha production and increasing proapoptotic and profibrotic signaling. Am J Physiol Endocrinol Metab *294*: E435-443, 2008.
6 Nath KA and Norby SM: Reactive oxygen species and acute renal failure. Am J Med *109*: 665-678, 2000.
7 Cvetkovic TP, Stefanovic NZ, Velickovic-Radovanovic RM, Paunovic GJ, Djordjevic VM, Stojanovic DR, Stojanovic IR and Pavlovic DD: Gender differences in oxidative and nitrosative stress parameters in kidney transplant patients on tacrolimus-based immunosuppression. Int Urol Nephrol 2013.
8 Ibrahim IY, Elbassuoni EA, Ragy MM and Habeeb WN: Gender difference in the development of cardiac lesions following acute ischemic-reperfusion renal injury in albino rats. General physiology and biophysics *32*: 421-428, 2013.
9 Reed DK and Arany I: p66shc and Gender-specific Dimorphism in Acute Renal Injury. In Vivo *28*: 205-208, 2014.
10 Lecour S and James RW: When are pro-inflammatory cytokines SAFE in heart failure? Eur Heart J *32*: 680-685, 2011.
11 Negoro S, Kunisada K, Fujio Y, Funamoto M, Darville MI, Eizirik DL, Osugi T, Izumi M, Oshima Y, Nakaoka Y, Hirota H, Kishimoto T and Yamauchi-Takihara K: Activation of signal transducer and activator of transcription 3 protects cardiomyocytes from hypoxia/reoxygenation-induced oxidative stress through the up-regulation of manganese superoxide dismutase. Circulation *104*: 979-981, 2001.
12 Wang M, Wang Y, Abarbanell A, Tan J, Weil B, Herrmann J and Meldrum DR: Both endogenous and exogenous testosterone decrease myocardial STAT3 activation and SOCS3 expression after acute ischemia and reperfusion. Surgery *146*: 138-144, 2009.
13 Sehara Y, Sawicka K, Hwang JY, Latuszek-Barrantes A, Etgen AM and Zukin RS: Survivin Is a transcriptional target of STAT3 critical to estradiol neuroprotection in global ischemia. J Neurosci *33*: 12364-12374, 2013.
14 Arany I, Reed DK, Grifoni SC, Chandrashekar K, Booz GW and Juncos LA: A novel U-STAT3-dependent mechanism mediates the deleterious effects of chronic nicotine exposure on renal injury. Am J Physiol Renal Physiol *302*: F722-729, 2012.
15 Arany I, Faisal A, Clark JS, Vera T, Baliga R and Nagamine Y: p66SHC-mediated mitochondrial dysfunction in renal proximal tubule cells during oxidative injury. Am J Physiol Renal Physiol *298*: F1214-1221, 2010.
16 Macmillan-Crow LA and Cruthirds DL: Invited review: manganese superoxide dismutase in disease. Free Radic Res *34*: 325-336, 2001.
17 Chen JR, Shankar K, Nagarajan S, Badger TM and Ronis MJ: Protective effects of estradiol on ethanol-induced bone loss involve inhibition of reactive oxygen species generation in osteoblasts and downstream activation of the extracellular signal-regulated kinase/signal transducer and activator of transcription 3/receptor activator of nuclear factor-kappaB ligand signaling cascade. J Pharmacol Exp Ther *324*: 50-59, 2008.

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