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Estrogens do not protect, but androgens exacerbate, collagen accumulation in the female mouse kidney after ureteric obstruction

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ABSTRACT

Aims: Controversy surrounds the gender basis of progression in chronic kidney disease. Unfortunately, most experimental studies addressing this question do not distinguish between direct effects of estrogen and indirect activation of estrogen receptors through conversion of testosterone to 17β-estradiol by aromatase. We examined the pathogenesis of renal fibrosis in female aromatase knockout (ArKO) mice, which lack circulating and stored estrogens, while having normal levels of testosterone.

Main methods: ArKO mice and their wild-type (ArWT) counterparts were subjected to unilateral ureteric obstruction (UUO), with kidney tissue collected at day(D) 0, 3 and 9 post-UUO. Effects of 5α-dihydrotestosterone (DHT) administration on each genotype were also studied. Tissue was assessed biochemically and histochemically for fibrosis. Western blot analysis was used to measure α-smooth muscle actin (α-SMA) expression and TGF-β1 signalling. Matrix metalloproteinase-2 (MMP-2) activity was measured by zymography.

Key findings: UUO increased collagen content over time ($p < 0.05$ (D3) and $p < 0.01$ (D9) vs day 0), with no difference between genotypes in qualitative (collagen IV staining) and quantitative (hydroxyproline concentration) analyses. Systemic administration of non-aromatizable DHT increased collagen content after 3 days of UUO in both genotypes. This was not paralleled by any change in α-SMA (myofibroblast burden) or TGF-β1 signalling but was commensurate with DHT reducing MMP2 activity in both genotypes ($p < 0.05$ vs genotype controls).

Significance: Physiological concentrations of estrogens do not protect the injured kidney from fibrosis progression. Androgens rather than estrogens are the relevant factor involved in regulating disease-related renal scarring in this model.

1. Introduction

Abnormalities in collagen accumulation are a pathological feature of trauma. Chronic kidney disease (CKD) is associated with ongoing generation of pro-fibrotic cytokines, and collagen synthesis by myofibroblasts, the end result being the accumulation of excess collagen, and progressive organ failure [1]. Scarring (so-called fibrosis) is therefore a final common pathway in all ongoing kidney disease.

Despite the universal significance of fibrosis, mounting evidence highlights a population-based heterogeneity. Consistent with this, many have shown a gender bias in the progression of CKD [2], with epidemiological studies suggesting that the prevalence of renal disease and rate of progression is lower in females than males [3–6]. However, this association is not universally accepted in the kidney [7], and it remains more controversial than elsewhere.

In the absence of strong clinical evidence, several experimental studies have examined the role of sex hormones—estrogens and androgens. Although estrogens and their various bioactive metabolites reduce renal collagen synthesis in vitro [8] and prevent both glomerular matrix accumulation and interstitial fibrosis in vivo [9], several investigations imply that it is the detrimental effects of androgens such as testosterone (reviewed in [10]) rather than the protective effects of estrogens that are relevant. Conversely, in some circumstances, testosterone may even be renoprotective [11]. There are nevertheless a number of factors that must be taken into consideration. Many studies [8,9,12,13] have not compared the effects of estrogens/their metabolites to those of testosterone. The latter can also mediate its actions through estrogen

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receptors after being converted to 17β-estradiol by aromatase and exert protective effects in the kidney [14,15] and elsewhere. Second, many studies have used pharmacological doses [15,16], which may not be physiologically relevant. Finally, models of estrogen deficiency, such as ovariectomy, retain the ability to produce extra-gonadal estrogens and therefore do not account for the locally produced estrogens [17].

To overcome these limitations, we have used aromatase knockout (ArKO) mice to determine if sex hormones play a significant role in injury-induced renal fibrosis. We have previously demonstrated that aged (12–14 month old) male ArKO mice, which lack estrogens but have 5–10 times the testosterone levels of age-matched aromatase wild-type (ArWT) mice, have increased kidney collagen concentrations (an absolute measure of fibrosis) [18]. Results suggested that testosterone, rather than estrogens, is the relevant factor regulating renal fibrosis progression. In this study, we extend this work by using female ArKO mice to directly examine the role of estrogens in renal fibrosis. Female ArKO mice are completely free of circulating and tissue-sourced estrogens and have testosterone levels that are comparable to wild-type female mice [19], thus providing a model in which testosterone is not a confounding variable. To determine the effects of androgens on renal fibrosis, we examined the effects of UUO on ArWT and ArKO mice treated with 5α-dihydrotestosterone, an androgen which cannot be converted to estrogens and is the most potent form of androgens.

2. Materials and methods

2.1. Animals

Female aromatase wild-type (ArWT) and knockout (ArKO) C57B6J littermate mice were generated from heterozygous parents and were individually genotyped and housed as described before [20,21]. After weaning (at 6-weeks of age), all mice were provided with water and soy-free rodent lab chow (SF06-053, Specialty Feeds, Western Australia, Australia) ad libitum to deprive them of consuming phytoestrogens, which are present in standard rodent lab chow. Our previous characterisation of this phenotype has shown that female ArKO mice have no circulating estrogens, and normal levels of testosterone [19]. The experiments detailed below were approved by the Florey Institute's Animal Ethics Committee, which adheres to the Australian Code of Practice for the care and use of laboratory animals for scientific purposes.

2.2. Unilateral ureteric obstruction (UUO)

While under general inhalational anaesthesia (Methoxyflurane, Abbott Laboratories, Sydney, NSW, Australia), the left ureter of female ArWT and ArKO mice was ligated with 5.0 surgical silk, while the contralateral ureter was left intact [22]. The incision was sutured, and mice were allowed to recover with temgesic administration (Burprenorphine; Reckitt Benckiser, West Ryde, NSW, Australia).

2.3. 5α-Dihydrotestosterone (DHT) supplementation

In a separate experiment, under methoxyflurane anesthesia, subgroups of ArWT and ArKO mice received subcutaneous implants of continuous-release pellets (Innovative Research of America, Toledo, OH, USA) containing 7.5 mg DHT (90 days release; daily dose ~83 μg) seven days prior to UUO. In accordance with a previous study, this subcutaneous dose of DHT results in serum levels of approximately 100–300 pg/ml [23]. A parallel control group for each genotype consisted of UUO without treatment.

2.4. Tissue collection

ArWT and ArKO mice were killed at 3 and 9 days post-UUO (representative of early and chronic renal tubulointerstitial fibrosis, respectively). In order to measure progression, tissue was taken from unoperated littermates (day 0). The kidneys of all mice were rapidly excised and cut into transverse sections (each containing cortex and medulla) for hydroxyproline and histochemical analysis. Unfixed tissue portions were snap frozen in liquid nitrogen and stored at −80 °C. An adjacent portion was immersion fixed in methyl carnoys, processed and embedded in paraffin wax. To ensure standardization, and to enable inter-group comparisons, each assay used the same anatomical portion of kidney tissue from each animal.

2.5. Histochemistry

Paraffin-embedded kidney tissue sections were dewaxed and dehydrated through graded alcohols. Endogenous peroxidase activity was blocked by treatment with methanol containing 0.3% hydrogen peroxide, washed in PBS and subsequently incubated with normal serum (Vector Laboratories, Burlingame, CA, USA) to block non-specific binding sites. Incubation with a goat anti-human Collagen IV polyclonal antibody (Southern Biotech, Birmingham, AL, USA) was used to identify collagen IV. After washing, primary antibody binding was localised using biotinylated anti-goat IgG (Vector). Labelling was amplified with the ABC Elite kit (Vector), and peroxidase activity visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St-Louis, MO, USA) containing 0.03% hydrogen peroxide, with counterstaining in haematoxylin.

2.6. Collagen concentration

Since collagen is the most abundant protein in the ECM, the measurement of collagen content allows for changes in fibrosis to be identified. The hydroxyproline assay described by us previously [24] is commonly used to measure the collagen content in biological tissue and is based on the frequent occurrence of hydroxyproline in the Gly-X-Y tripeptide repeats in the collagen chain [25].

In brief, frozen portions of kidney tissue were lyophilized for dry weight measurements, before being hydrolyzed in 6 M hydrochloric acid, freeze dried and resuspended in 0.1 M HCl. To determine the content of hydroxyproline in the samples, 10 μl duplicate aliquots of each sample were mixed with 90 μl water, 200 μl isopropanol and 100 μl 7% chloramine-T reagent (Sigma-Aldrich) and then allowed to stand at room temperature for 4 min. Ehrlich's reagent (1.3 ml) was added to each and samples incubated in a 60 °C shaking water bath for 25 min, during which time, increasing hydroxyproline levels in samples induced a colorimetric reaction. The reaction was stopped by cooling samples on ice for 2-3 min, with 3.3 ml of isopropanol added before the absorbance was read by spectrophotometer at 558 nm. Hydroxyproline content of the samples was determined using a standard curve of a hydroxyproline stock (trans-4-hydroxy-L-proline; Sigma-Aldrich), and collagen content was derived by multiplying hydroxyproline content by a factor of 6.94, as hydroxyproline represents ~14.4% of the amino acid composition of collagen in most mammalian species [26]. Collagen content was then expressed as a percentage of the dry weight of the kidney tissue analyzed to yield collagen concentration.

2.7. Western blotting analysis

Western analysis was used to determine changes in total myofibroblast burden (α-smooth muscle actin expression; α-SMA), transforming growth factor-β1 (TGF-β1) expression, and Smad2 phosphorylation, a specific marker of TGF-β1 signal transduction.

Protein samples were isolated from frozen tissue using RIPA (Sigma-Aldrich), with total protein concentration determined by the BCA assay (Thermo Scientific, Rockford, IL, USA). Proteins (5–20 μg/lane) were separated on 10% Mini-PROTEAN TGX stain-free pre-cast gels (Bio-Rad Laboratories, Richmond, CA, USA) and transferred onto PVDF using the Trans-Blot Turbo transfer system (Bio-Rad). Non-specific protein binding sites were blocked with BLOTTO (Thermo Scientific) for 2 h at