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# Impact of the estrus cycle and reduction in estrogen levels with aromatase inhibition, on renal function and nitric oxide activity in female rats

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ABSTRACT

Estradiol increases mRNA and/or protein expression of the nitric oxide synthase (NOS) isoforms in a variety of tissues including kidney. In this study we determined the relationship between cyclical variations in estradiol levels and renal function and total NO production in the virgin female rat. In addition, we used an aromatase inhibitor (Anastrozole), to inhibit synthesis of estradiol from testosterone. Estradiol levels were higher in proestrus vs. diestrus, and were markedly suppressed by 7 days treatment with aromatase inhibitor. There was no difference in total NO production (from urinary and plasma nitrate + nitrite = $NO_X$) between proestrus and diestrus but aromatase inhibition resulted in *increases* in total NO production. The renal cortical NOS activity and protein abundance also increased in aromatase-inhibited female rats. There were no differences in blood pressure (BP) in any group but the renal vascular resistance (RVR) was low in proestrus, increased in diestrus and did not change further after aromatase inhibition. In summary, the cyclical changes in renal function correlate with estradiol but not NO levels. Pharmacologic castration with aromatase inhibition leads to a marked increase in total and renal NOS. This contrasts to earlier work where surgical castration causes decreased NOS.



## 1. Introduction

In addition to the well recognized protective effects of estradiol and female sex on the cardiovascular system [1,2], the kidney is also protected. Women and female rats are protected from age-dependent kidney damage vs. males [2,3] and premenopausal women and cycling rats with non-diabetic chronic renal disease have a slower rate of progression compared to males [1,4,5]. Estrogen has many cardiovascular/renal actions that could contribute to protection, some of which are mediated by nitric oxide (NO). Estrogens have both acute (nongenomic) effects to stimulate NO release, as well as long-term, genomic actions to increase mRNA and protein expression of NO synthase (NOS) isoforms, and to increase NOS activity in a variety of tissues [1,2]. In the kidney, female rats have higher levels of NOS protein compared to male and ovariectomized rats [6,7].

In this study we investigated the effect of physiological (cyclic) alterations in estradiol levels on systemic and renal hemodynamics and on total NO production in the virgin female rat. As an alternative to ovariectomy we also employed the aromatase inhibitor, Anastrozole (Arimidex), to inhibit synthesis of 17B-estradiol from testosterone. Anastrozole is a potent and highly selective inhibitor of aromatase with no intrinsic hormonal activity [8]. We investigated the renal hemodynamic response to aromatase inhibition as well as the impact on total NO production and renal NOS activity/abundance.

## 2. Experimental

Studies were performed on 21 female Sprague Dawley rats (Harlan Sprague Dawley Inc., Indianapolis, IN) aged 4–5 months as approved by the West Virginia University Animal Care and Use Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were given a low nitrate but complete diet (AIN 76C semi-purified diet, ICN Pharmaceuticals, Costa Mesa, CA) and water ad libitum and maintained in a 12 h light/dark cycle. Stages of the estrous cycle were confirmed by daily vaginal smears. Urine collections (24 h) were made in Nalgene metabolic cages and aliquots were frozen for later analysis.

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Eleven rats were placed on low nitrate diet for 3 days prior to beginning the study and then two 24 h urines were collected, one in proestrus and one in the diestrus stage of the estrous cycle. Next, surgery was performed to implant chronic femoral arterial and venous catheters and a urinary bladder catheter, under general anesthesia and using full sterile technique, as described previously [9]. Two days after surgery, daily vaginal smears were begun to monitor the estrous cycle, while rats were trained to accept the laboratory environment. At least 7 days after surgery and after rats had resumed a normal cycle the first of two "baseline" renal function experiments were performed on each rat; once between 8 AM and 1 PM on the day of proestrus (maximal estradiol levels) and once between 8 AM and 1 PM on day 1 of diestrus (lowest estradiol levels) [10]. After these measurements, each rat was lightly anesthetized and an osmotic mini pump (0.5 μL/h; Alzet; Palo Alto, CA) was implanted under the skin of the back. Minipumps either contained vehicle ($n = 6$) or the aromatase inhibitor Anastrozole ($n = 5$; Zeneca Pharmaceuticals, Macclesfield, UK). Anastrozole was suspended in 25%EtOH:25% polyethylene glycol:50%, 0.9% sodium chloride at a concentration based on the rats' weight at the time of implantation to deliver a dose of 1 mg/kg/day. This dose inhibits ovulation and lowers plasma estradiol without any pharmacological side effects [8,11].

Two additional 24 h urine collections were performed, before each of the renal function experiments conducted in the mornings of days 3 and 7 after minipump implantation. After the last renal function experiment, rats were sacrificed, blood collected and plasma stored frozen for later measurement of estradiol and plasma $NO_X$ and the kidneys were harvested onto dry ice, separated into cortex and medulla, frozen in liquid nitrogen and stored at −80 °C for later analysis.

During renal function experiments, the arterial line was connected to a pressure transducer for measurement of mean arterial pressure (BP) and occasional blood sampling. A continual iv infusion was given of [$^3$H] Inulin (5 μCi/ml; New England Nuclear) and para-aminohippuric acid (PAH; 1 g%) at 5 μl/min/100 g body weight; this low infusion rate allowed rats to remain euvolemic. The bladder catheter was unplugged for urine collection. The clearances of [$^3$H] Inulin and PAH were used to measure glomerular filtration rate (GFR) and renal plasma flow (RPF), respectively, in 2× 20 min clearance periods.

The following analyses were performed: Urine and plasma $NO_3$ and $NO_2$ (=$NO_X$) was measured by the Griess reaction as described by us previously [12]. Plasma concentrations of estradiol were measured by radioimmunoassay by the method of Rozell and Keisler [13]. Plasma estradiol levels were measured in samples from 5 rats treated for 7 days with Anastrozole as well as six samples from control rats, three each in diestrus and proestrus. Samples from the renal function experiments were analyzed for urine volume, and plasma and urine concentrations of [$^3$H]Inulin, PAH and sodium. Blood hematocrit and plasma protein concentration was also measured. These measurements allowed for calculation of GFR, RPF, filtration fraction (FF), RVR and urinary sodium excretion ($U_{Na}V$). Details of these analyses and calculations are given in previous publications [9]. In 10 separate female rats plasma testosterone was measured after 7 days of anastrozole ($n = 6$) and 7 days sham treatment ($n = 4$), using the Count-A-Count total testosterone, Diagnostic Products kit according to the manufacturers instructions.

NOS activity was measured from the conversion of $^3$H-arginine to $^3$H-citrulline in the soluble fraction of renal cortex and medulla, after removal of the endogenous L-arginine and additional of 5 μM L-arginine, all essential cofactors and the arginase inhibitors, 10 mM valine and 10 mM proline. For each sample, two sets of triplicates were run, one at baseline and one in the presence of the non-selective NOS inhibitors, L-NMA (10 mM) and L-NAME (20 mM). Data are expressed as pmoles of [$^3$H]-L-citrulline converted per minute mg protein (pmol Citrulline/min/mg protein) and corrected for background and for the L-NMA and L-NAME inhibitable fraction. Details of this method have been published by us previously [3,14]. The abundance of the NOS3 and NOS1 proteins were measured on homogenates of kidney cortex by Western blot analysis. The NOS3 was detected with mouse monoclonal antibody (Transduction Laboratories, 1:250 dilution, 1 h; secondary antibody goat, anti-mouse IgG-HRP conjugate, Transduction Labs., 1:2000 dilution, 1 h). Separate membranes were probed for detection of NOS1 using rabbit polyclonal antibody (1:5000 dilution, 1 h incubation; secondary antibody, goat, anti-rabbit IgG-HRP, Biorad; 1:3000 dilution, 1 h). Equal protein-loading and transfer was confirmed by Ponceau red staining. The NOS3 and NOS1 abundance was measured by image analysis (Optimas 6.2, Bothell, WA) of the integrated optical density, normalized for total protein using ponceau red. Details of these techniques have been published by us previously [3,14].

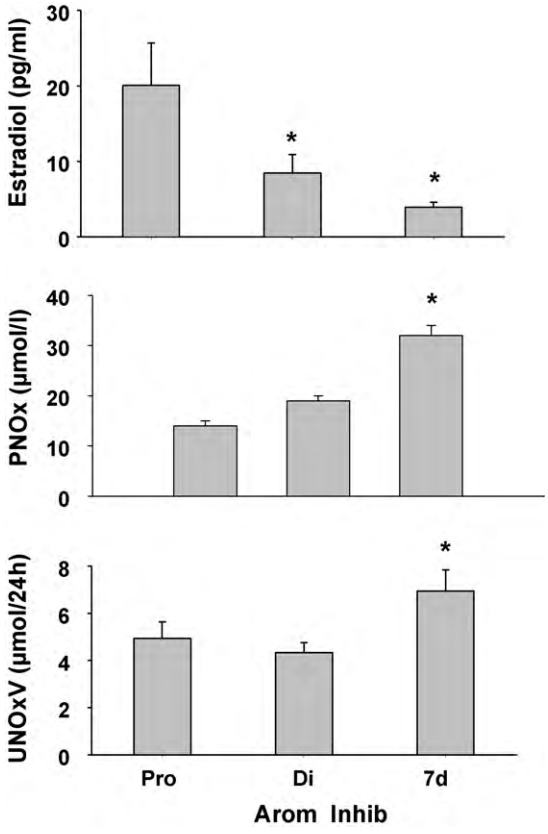


**Fig. 1.** Plasma estradiol concentration, plasma $NO_X$ (nitrite + nitrate) concentration and 24 h urinary $NO_X$ excretion in proestrus (PRO), diestrus (DI) and after 7 days of aromatase inhibition. *$p < 0.05$ difference compared to proestrus, #$p < 0.05$ difference between diestrus and 7 days of aromatase inhibition.

Results are expressed as mean ± SEM. Statistical analysis was by paired and unpaired *t*-test and one-way ANOVA. Values of $p < 0.05$ are considered to be significantly different.

## 3. Results

As previously reported [10], estradiol levels were higher in rats in proestrus than in diestrus and treatment with the aromatase inhibitor, Anastrozole, for 7 days inhibited plasma estradiol further (Fig. 1). In separate rats, 7 days of Anastrozole markedly increased the plasma testosterone compared to sham treated female rats (2728 ± 396 vs. 315 ± 75 pg/ml; $p < 0.001$).