

## 17 $\beta$ -estradiol Attenuates Renal Fibrosis in Mice with Obstructive Uropathy

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### = Abstract =

**Purpose :** Men are generally more prone to chronic renal disease and progression to end stage renal disease than women. The purpose of this study is to prove the effect of gender and sex hormone on renal fibrosis in mice with unilateral ureteral obstruction (UUO) and to elucidate the specific underlying mechanisms.

**Methods :** We compared the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) in female and male mice with complete UUO (day 7). After this, we estimated the changes of renal fibrosis in the female mice with oophorectomy and in the female mice with oophorectomy and replacement of 17 $\beta$ -estradiol, respectively.

**Results :** The level of  $\alpha$ -SMA in the female kidney with UUO was significantly lower than that in the male kidney with UUO. oophorectomy and replacement of 17 $\beta$ -estradiol did not change the expression of angiotensin II type 1 (AT1) receptor in the female kidney with UUO, whereas the expression of angiotensin II type 2 (AT2) receptor was significantly more elevated in the intact female (IF) and the oophorectomized female with estrogen (OF+E) than that in the oophorectomized female (OF). The expressions of inducible nitric oxide synthase (iNOS) in the IF and OF+E mice were significantly more elevated than that in the OF mice, which was similar to the expression of AT2 receptor.

**Conclusion :** The female gender is associated with resistance to renal fibrosis in obstructive uropathy and this gender difference may originate from the existence of 17 $\beta$ -estradiol, which has an anti-fibrotic effect via upregulation of the AT2 receptor and iNOS. (*J Korean Soc Pediatr Nephrol* 2011;15:125-137)

**Key Words :** Renal fibrosis, Ureteral obstruction, Gender, 17 $\beta$ -estradiol

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### Introduction

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There are gender differences in the incidence and severity of cardiovascular and renal disease as men are more prone to develop chronic renal disease and to rapidly progress to end stage renal disease than are women [1].

Clinically, men with chronic renal disease of various causes present with a more rapid decline in renal function with time than do women [2]. It has been reported that sex hormones such as testosterone and estrogen have a key role in relation to these gender differences [3–9]. In experimental models of chronic allograft nephropathy, testosterone treatment resulted in increased proteinuria and profound glomerulosclerosis, irrespective of the donor's gender, whereas estradiol reduced glomerulosclerosis and the mononuclear cell infiltration in the allografts of both genders [3]. Testosterone stimulates various key components of the systemic and renal renin–angiotensin–aldosterone system and it produces oxidative stress, which may eventually aggravate the progression of renal disease [4, 5]. We recently demonstrated that testosterone administered to orchietomized male or female mice reverses the protective phenotype and increases the vulnerability to kidney ischemia/reperfusion injury [6]. On the other hand, estrogen attenuates renal injury in various animal models [7–9]. In the aging Dahl salt sensitive rat, estrogen inhibits the proliferation of mesangial cells in vitro, it suppresses the synthesis of collagen type I and type IV and it reduces glomerulosclerosis and tubulointerstitial fibrosis [8, 9]. Catanuto et al. reported that tamoxifen and 17 $\beta$ -estradiol treatment reduce the podocyte transforming growth factor- $\beta$  (TGF- $\beta$ ) mRNA expression, but it enhances the mRNA expression of estrogen receptor subtype  $\beta$  protein [10].

Obstructive uropathy is caused by blockage of the flow of urine and this may damage the

kidney, including hydronephrosis, infiltration of leukocytes and tubular atrophy and dilation, as well as causing increased interstitial fibrosis [11, 12]. Unilateral ureteral obstruction (UUO) is a well-established experimental model of renal injury that reproduces the human effects of obstructive uropathy [13]. There are several pathways that regulate the renal response to UUO: tubular apoptosis, interstitial inflammation and interstitial fibrosis [14]. UUO-mediated interstitial fibrosis is related to various cytokines. Angiotensin II (AngII) is one of major stimulants of renal fibrosis and it produces direct vasoconstriction and it controls the extracellular fluid volume [15]. AngII signal transduction is started by two receptors, that is, the AT1 and AT2 receptors [16]. Most of the effects of AngII are mediated through the AT1 receptor, which is extensively expressed by most cell types, whereas the AT2 receptor is highly expressed in fetal tissue and its expression decreases to a low level in adult animals and humans [17–19]. It is thought that the AT2 receptor counteracts the effects of AngII and so it plays a role in the protection of the kidney [20]. Nitric oxide (NO) in the kidney has many important functions such as the regulation of the renal hemodynamics, maintenance of medullary perfusion, mediation of pressure–natriuresis, blunting of tubuloglomerular feedback, inhibition of tubular sodium reabsorption and modulation of the renal sympathetic neuronal activity [21]. NO is also known to function as one of the antifibrotic factors in UUO [22] and it may be a target for intervention in the fibrotic processes in obstruction, which are due to the interaction of

NO and TGF- $\beta$  induced by AngII [23]. All three isoforms of nitric oxide synthase (NOS), namely, neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) are reported to contribute to NO synthesis in the kidney [24]. While expressions of nNOS and eNOS have been reported in the kidney, there is controversy regarding the expression of iNOS in the kidney [21]. However, there is constant evidence that the iNOS expression in the kidney is remarkably increased by pro-inflammatory stimuli such as ischemia-reperfusion [25, 26] and lipopolysaccharide [27, 28].

The purpose of our study is to prove the effect of gender and sex hormones on renal fibrosis in mice with UUO and to elucidate the specific mechanisms of this. In our complete UUO model, the renal fibrosis in male mice was more prominent than that in the female mice, and oophorectomy in the female mice offsets this gender difference. Moreover, replacement of 17 $\beta$ -estradiol attenuated the renal fibrosis in the oophorectomized female mice, similar to that in the intact female mice, and the expressions of AT2 receptor and iNOS were significantly increased in the intact female mice and the oophorectomized female mice with the replacement of 17 $\beta$ -estradiol. Therefore, our results show that 17 $\beta$ -estradiol may attenuate renal fibrosis in mice with complete UUO via upregulation of the AT2 receptor and iNOS.

## Material and Methods

### 1. Animal preparation

The experiments were performed using age-

matched (8 week old) male and female C57BL/6 mice (weight: 20–25g each). The mice were allowed free access to water and standard mouse chow. In all cases, the studies were approved by the Kyungpook National University Institution Animal Care and Use Committee. Each animal group consisted of at least four mice. UUO was carried out as previously described [29]. The mice were anesthetized with pentobarbital sodium [60 mg/kg body weight (BW); Sigma]. The right kidney was exposed through the site of a right flank incision. The right ureter was completely obstructed near the renal pelvis using a 6-0 silk tie. The sham-operated mice had the same surgical procedure except for the ureter ligation. Harvesting of the kidney was performed 7 days after the UUO surgery.

### 2. Experimental protocol

In a preliminary experiment, we compared the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) of the kidneys of intact male and female mice with UUO. The expression of  $\alpha$ -SMA, which originates from myofibroblasts and this expression is used for the evaluation of fibrosis [30, 31] was determined in the mice kidneys by Western blot analysis using an  $\alpha$ -SMA antibody. In the first experiment, the animals were divided into four experimental groups: intact males (IM), intact females (IF), castrated males (CM), oophorectomized females (OF). The expression of  $\alpha$ -SMA was compared between each group. The operations for castration in the males and oophorectomy in the females were performed 2 weeks before the UUO surgery.

In the second experiment, the female mice were divided into four experimental groups: 1 & 2) IF and OF with vehicle (sesame oil), 3) IF with subcutaneous injection of tamoxifen (IF+T) and 4) OF with replacement of 17 $\beta$ -estradiol (OF+E). The IF+T and OF+E groups were injected with tamoxifen (5 mg/kg; Sigma) and 17 $\beta$ -estradiol benzoate (100  $\mu$ g/kg; estrogen, Sigma), respectively, subcutaneously daily for a total of 3 weeks (from 2 weeks before the UUO operation to harvest). The expression of  $\alpha$ -SMA was compared between each group. In the third experiment, female mice were divided into four experiment groups: 1) sham, 2 & 3) IF and OF with vehicle (sesame oil) and 4) OF+E. The expression of  $\alpha$ -SMA, angiotensin II type 1 (AT1) receptor, angiotensin II type 2 (AT2) receptor, inducible nitric oxide synthase (iNOS) and endothelial NOS (eNOS) were compared between each group. The kidneys were either perfusion-fixed in PLP (4% paraformaldehyde, 75 mM L-lysine, 10 mM sodium periodate; Sigma) for histological studies, or they were snap-frozen in liquid nitrogen for biochemical studies. The kidneys fixed in PLP were washed with PBS three times for 5 minutes each time, embedded in paraffin at room temperature, and then cut into 4- $\mu$ m frozen sections or 2- $\mu$ m paraffin sections using a cryotome (Leica, Bensheim, Germany) or a microtome (Leica, Bensheim, Germany), respectively.

### 3. Measurement of collagen deposition

To evaluate collagen deposition, the paraffin-sections were stained with Masson trichrome according to a standard protocol. Briefly, the

2- $\mu$ m paraffin kidney sections were incubated in Bouin's solution (Sigma) overnight at room temperature (RT), and then they were stained sequentially with Weigert's haematoxylin for 10 minutes, Biebrich scarlet-acid fuchsin for 5 minutes, phosphotungstic-phosphomolybdic acid (Sigma) for 5 min and aniline blue (Sigma) for 10 minutes. The section was incubated in 1% acetic acid (Sigma) for 2 min, dehydrated through a graded series of ethanol solutions to xylene, mounted with Permount (Fisher Scientific, Pittsburgh, PA) and examined under an Axioplan-2 microscope (Carl Zeiss, Munich, Germany). The images were collected using a digital camera (Carl Zeiss). Each experimental animal group consisted of more than four mice. The collagen deposition was analyzed in 10 fields (0.1 mm<sup>2</sup>/field) of the outer medulla using LabWorks 4.5 software (Ultra-Violet Products, Cambridge, UK).

### 4. Western blot analysis

Western blot analyses were performed as described previously [32]. Briefly, equal amounts of renal tissue proteins were separated on 10 % SDS-PAGE gels and then transferred to an Immobilon membrane (Millipore Corp., Bedford, MA). The membranes were blocked and incubated overnight at 4°C with the following antibodies against various proteins:  $\alpha$ -SMA (1:5,000 dilution; Sigma),  $\beta$ -actin (1:5,000 dilution; Sigma), AT1 receptor (1:1,000 dilution; Santa Cruz), AT2 receptor (1:500 dilution; Santa Cruz), iNOS (1:500 dilution; BD Biosciences), eNOS (1:500 dilution; BD Biosciences). After washing, the membranes were incubated

for 1 hour at room temperature with horseradish peroxidase-conjugated horse anti-mouse (Vector Laboratories, Burlingame, CA) or goat anti-rabbit (Vector Laboratories) antibodies diluted 1:5,000. Finally, the membranes were exposed to a Western Lighting Chemiluminescence Reagent (PerkinElmer LAS, Boston, MA). The density of the immunoblot was analyzed using LabWorks 4.5 software (Ultra-Violet Products).

## 5. Statistical analysis

The results were expressed as means  $\pm$  standard errors of the means (SEM). Statistical differences between the groups were calculated using Student's *t*-test. Differences between the groups were considered statistically significant at a *P* value of <0.05. In all cases, the animal groups consisted of 4 mice.

## Results

### 1. The expression of $\alpha$ -SMA in the male kidney with UUO is significantly higher than that in the female kidney

Seven days after UUO, definite dilatation of the renal pelvis and hydronephrosis appeared in the obstructed kidneys, whereas they were not presented in the sham-operated kidney. UUO resulted in marked tubular dilatation and atrophy, and interstitial inflammation in the ipsilateral kidney. The expression of  $\alpha$ -SMA in the kidney with UUO was more prominent than that in the sham-operated kidney without UUO (*P*=0.000) (Fig. 1A). The levels of  $\alpha$ -

SMA in the male kidney with UUO were significantly higher than those in the female kidney with UUO (*P*=0.017).

### 2. Oophorectomy exacerbates renal fibrosis in the female mice with UUO

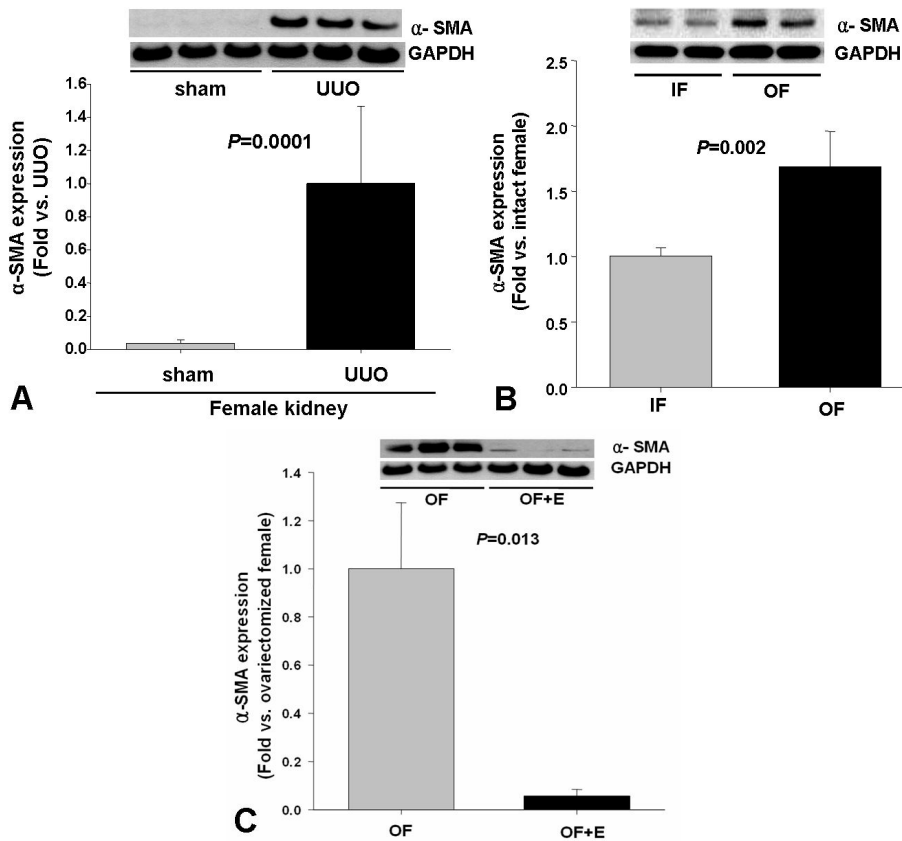
There was no significant difference in expression of  $\alpha$ -SMA due to UUO between the IM and CM groups. However, the level of  $\alpha$ -SMA in the OF with UUO mice was significantly higher than that in the IF mice with UUO (*P*=0.049) (Fig. 1B).

### 3. Replacement of 17 $\beta$ -estradiol attenuates renal fibrosis in the oophorectomized female mice with UUO

Injection of tamoxifen in the IF+T group did not produce a significant change of expression of  $\alpha$ -SMA compared with that of the IF group. However, replacement of 17 $\beta$ -estradiol in the OF+E group significantly attenuated the expression of  $\alpha$ -SMA compared with that of the OF group (Fig. 1C). The deposition of collagen, as detected using Masson trichrome staining also showed a pattern equal to the expression of  $\alpha$ -SMA (Fig. 2A & B).

### 4. 17 $\beta$ -estradiol induces activation of the AT2 receptor and iNOS

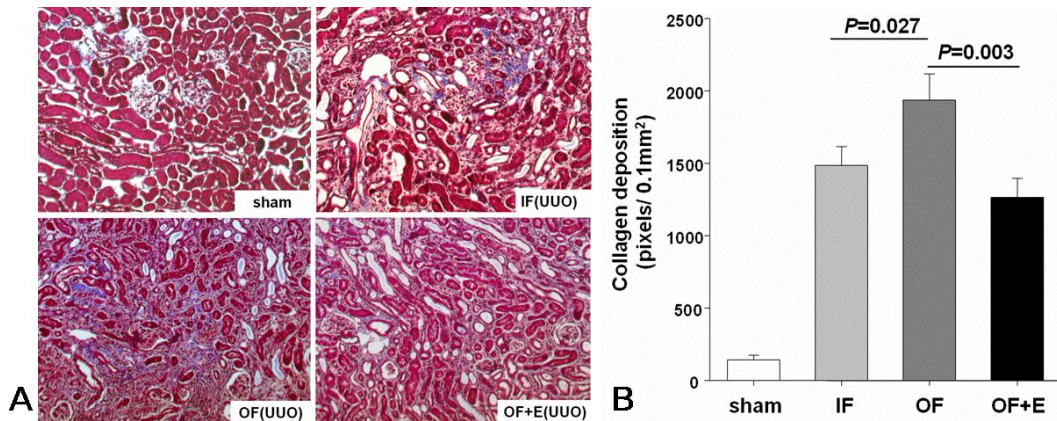
These female animals were divided into four experimental groups: 1) sham, 2) IF, 3) OF, 4) OF+E. The expressions of AT1 and AT2 receptors in the female kidney with UUO were more prominent than that in the sham-operated



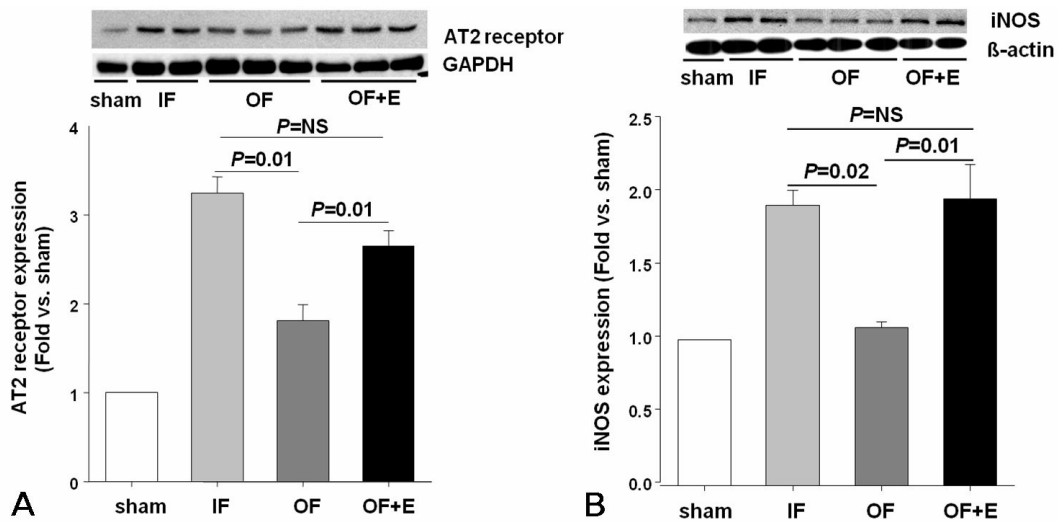
**Fig. 1.** (A) Effect of ureteral obstruction on the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) in C57BL/6 female mice. The expression of  $\alpha$ -SMA in the kidney with UUO was more prominent than that in the sham-operated kidney without UUO ( $P=0.000$ ). The values are expressed as means $\pm$ SEMs ( $n=4$ ). (B) The expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) in the intact (IF) and oophorectomized female (OF) mice. Seven days after UUO, the level of  $\alpha$ -SMA in the OF with UUO was significantly higher than that in the IF with UUO ( $P=0.049$ ). The values are expressed as means $\pm$ SEMs ( $n=4$ ). (C) The expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) in the oophorectomized female (OF) and OF mice with injection of 17 $\beta$ -estradiol (OF+E). Replacement of 17 $\beta$ -estradiol in the OF+E group significantly attenuated the expression of  $\alpha$ -SMA compared with that of the OF group ( $P=0.013$ ). The values are expressed as means $\pm$ SEMs ( $n=4$ ).

female kidney without UUO. oophorectomy and replacement of 17 $\beta$ -estradiol did not change the expression of AT1 receptor in the female kidney with UUO, whereas the expression of AT2 receptor was more significantly elevated in the IF and OF+E kidneys than that in the OF kidney (Fig. 3A). The expression of eNOS in the IF kidney was also more significantly ele-

vated than that in the sham kidney, but there was no significant difference between the OF and OF+E kidney. The expressions of iNOS in the IF and OF+E kidneys were more significantly elevated than that in the OF kidney, which was similar to the expression of the AT2 receptor (Fig. 3B).



**Fig. 2.** (A) Deposition of collagen in the kidneys 7 days after UUO. The female animals were divided into four experimental groups: 1) sham, 2) intact female (IF), 3) oophorectomized female (OF) and 4) OF with replacement of 17 $\beta$ -estradiol (OF+E). (A) Kidney sections (2- $\mu$ m thickness) were stained with Masson trichrome staining. (B) The collagen deposition was analyzed in 10 fields (0.1 mm<sup>2</sup>/field) of the outer medulla using LabWorks 4.5 software (Ultra-Violet Products, Cambridge, UK). The values are expressed as means $\pm$ SEMs (n=4).



**Fig. 3.** (A) The expression of angiotensin II type 2 (AT2) receptor. Seven days after UUO, the expression of AT2 receptor in the female kidney with UUO was more prominent than that in the sham-operated kidney without UUO. Moreover, the expression of AT2 receptor was more significantly elevated in the intact females (IF) and oophorectomized females with replacement of 17 $\beta$ -estradiol (OF+E) than that in the OF. The values are expressed as means $\pm$ SEMs (n=4). (B) The expression of inducible nitric oxide synthase (iNOS). Seven days after UUO, the expressions of iNOS in the intact female (IF) and ovariectomized female with replacement of 17 $\beta$ -estradiol (OF+E) were more significantly elevated than that in the OF, which was similar to the expression of angiotensin II type 2 receptor. The values are expressed as means $\pm$ SEMs (n=4).

### Discussion

Testosterone and estrogen have been re-

garded as two major key factors for the gender difference of renal disease [9, 15, 33-37]. First of all, testosterone is responsible for enhanced

vulnerability of males to renal injury. Testosterone exacerbates renal injury due to ureteral obstruction by the production of tumor necrosis factor- $\alpha$  [34] and orchietomy reduces the post-ischemic oxidative stress and ischemia/reperfusion renal injury in mice [33]. On the other hand, estrogen is responsible for the protective effect of female gender against renal injury. Replacement of estrogen (17 $\beta$ -estradiol) improves renal function and the pathological findings associated with diabetic nephropathy [35], it reduces glomerulosclerosis and tubulointerstitial fibrosis [9] and inhibits apoptosis as well as the expression of TGF- $\beta$  [36, 37]. In an experimental model of chronic allograft nephropathy, estradiol reduced the glomerulosclerosis and mononuclear cell infiltration in allografts of both genders, and this paralleled a decreased mRNA expression of TGF- $\beta$ 1 [3]. McGuire et al. reported that 1) renin-angiotensin activity is positively controlled by androgens and it is antagonized by estrogens 2) endogenous estrogen has an anti-hypertensive effect as well as protective effects against cell and organ damage through increased nitric oxide production [15]. Our preliminary study also showed a definite sex difference in obstructive uropathy, and this was similar to a previous report [34] in which females were less prone to renal fibrosis than males after UUO. According to this result, our study was designed to reveal the specific mechanism of reno-protective effect of the female gender in mice with UUO, especially for the role of 17 $\beta$ -estradiol. oophorectomy aggravated the renal fibrosis in female mice with UUO, whereas replacement of 17 $\beta$ -estradiol attenuated renal fibrosis in

oophorectomized female mice with UUO. This result indicates that in obstructive uropathy, the existence of 17 $\beta$ -estradiol is the major factor of a reno-protective effect of the female gender. Tamoxifen is an orally taken selective estrogen receptor modulator that is used in the treatment of breast cancer and there are no clinical studies on the effects of tamoxifen on renal disease [10]. Although tamoxifen and 17 $\beta$ -estradiol treatment reduces the podocyte TGF- $\beta$  mRNA expression and it increased the expression of estrogen receptor subtype  $\beta$  protein, 17 $\beta$ -estradiol treatment, but not tamoxifen treatment, diminishes extracellular-regulated kinase phosphorylation, and so, 17 $\beta$ -estradiol is more effective than tamoxifen [10]. In our study, injection of tamoxifen in the IF+T group did not produce a significant change of the expression of  $\alpha$ -SMA compared with that of the IF group.

Most of the effects of AngII, including inflammation, vasoconstriction and matrix deposition, are mediated through the AT1 receptor. Although there is controversy, it is thought that the AT2 receptor counteracts the effects of AngII and it plays a role in protection of the kidney [20]. Expression of the AT2 receptor is exceedingly developed in fetal and neonatal kidneys, but the expression level quickly decreases during adolescence [38]. In intact female spontaneous hypertensive rats, the expression of AT2 receptor increases significantly to mediate hypotensive responses via a negative effect on renin synthesis and AngII formation [39, 40]. AT2 receptor knockout mice show an anti-natriuretic shift [41]. However, there are only a small number of experi-



mental studies on the role of the AT2 receptor in the pathogenesis of chronic kidney disease [20]. In the renal ablation model, which was achieved by unilateral nephrectomy and removal of two-thirds of the other kidney, the AT2 receptor knockout mice had a higher mortality rate compared to that of wild type mice [42] and this finding is significantly similar to a report showing progression of renal injury in AT2 receptor knockout mice during UUO [43]. Armando et al. reported that estrogen administration elevates the expression of AT2 receptors predominantly in the capsule and inner medulla and the protective effects of estrogen may be partly mediated by enhanced AT2 receptor stimulation [44]. In our present study, oophorectomy exacerbated renal fibrosis and it reduced the expression of AT2 receptor in the female mice with UUO, whereas replacement of 17 $\beta$ -estradiol attenuated the renal fibrosis and increased the expression of AT2 receptor. Based on this result, we demonstrate that 17 $\beta$ -estradiol may attenuate renal fibrosis in obstructive uropathy through upregulation of the AT2 receptor.

NO plays an important role in the control of the renal hemodynamics and function [45]. In the human UUO model, it was demonstrated that the activity and expression of iNOS in the medulla and eNOS in the cortex are increased [46]. Estrogen stimulates the activity of eNOS, which is secondary to non-transcriptional activation via Akt phosphorylation [47]. Neugarten et al. reported that 1) the eNOS and iNOS levels are significantly higher in the renal medulla of female rats compared with that of the male rats, 2) oophorectomy reduces the renal medullary

eNOS and iNOS levels to that of the intact male rats and 3) estrogen replacement therapy highly increases the medullary eNOS and iNOS levels in the oophorectomized animals [48]. On the other hand, it has been reported that the induction of iNOS can have harmful effects on renal ischemia/reperfusion injury, which is unlike our study [49]. However, iNOS reduces apoptosis and it enhances the thickness of the renal parenchyme in mice with complete UUO, and this is unlike partial UUO, so the degree of ureteral obstruction may influence the effect of iNOS on long-term renal injury [50]. In our experimental model with complete UUO, although expressions of eNOS and iNOS in the IF with UUO group was significantly increased compared with that of the IF with a sham operation, replacement of estrogen induced the activation of iNOS, but not eNOS, and this result suggests that the roles of NOS can be variable according to circumstances, as was seen in the above mentioned reports.

There have been several reports about the relationship between the AT2 receptor and NO. AngII at the AT2 receptor in the kidney stimulates a vasodilator cascade of bradykinin, NO and cyclic GMP, which is activated only during conditions of increased AngII, such as sodium depletion [51]. Palm et al. demonstrated that oxygen availability in the clipped kidney is maintained by the generation of AngII, AT2 receptors and NOS to prevent hypoxia in a kidney challenged with a reduced perfusion pressure [52]. Therefore, NO is a significant physiological mediator of AngII at the AT2 receptor [16,51,52].

In conclusion, there is a gender difference of

renal fibrosis in the murine model with complete UUO and this difference may originate from the existence of 17 $\beta$ -estradiol, which has an anti-fibrotic effect via upregulation of AT2 receptors and iNOS.

### 한 글 요약

#### 폐쇄성 요로병증에서 17 $\beta$ -estradiol에 의한 신섬유화 감소 효과에 대한 연구

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**목적:** 일반적으로 남자는 여자에 비해 만성 신장병의 발병이 많고 말기 신부전으로의 진행이 더 흔한 것으로 알려져 있다. 본 연구는 일측성 요관 폐쇄를 가진 생쥐에서 신섬유화에 대한 성별과 성호르몬의 효과를 규명하기 위해 시행되었다.

**방법:** 일측성 완전 요관 폐쇄 7일째 암컷과 수컷 생쥐의 신장에서  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA)의 발현을 측정 한 후, 암컷 생쥐에서 난소를 제거하거나 제거 후 다시 17 $\beta$ -estradiol을 보충하여 나타나는 신섬유화 정도를 비교 분석하였다.

**결과:** 일측성 요관 폐쇄를 가진 암컷 신장의  $\alpha$ -SMA의 발현이 수컷 신장에 비해 현저히 낮았다. 난소 제거와 17 $\beta$ -estradiol의 보충은 일측성 요관 폐쇄를 가진 암컷 신장의 안지오텐신 II 1형 수용체의 발현에는 의미 있는 영향을 주지 않았지만, 안지오텐신 II 2형 수용체의 발현은 정상 암컷과 난소 제거 후 17 $\beta$ -estradiol을 보충한 암컷에서 현저히 증가되었다. 또한, inducible nitric oxide synthase (iNOS) 역시 유사한 변화를 보였다.

**결론:** 여성은 폐쇄성 요로병증에서 신섬유화에 대한 저항성과 연관이 있으며 이러한 성별의 차이는 17 $\beta$ -estradiol에 의한 안지오텐신 II 2형 수용체와 iNOS의 발현 증가와 연관이 있을 것으로 사료된다.

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