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**Original article**

# Role of angiotensin II on protein expression of renal nitric oxide synthase in unilateral ureteral obstructive rat

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***Background:*** In the kidney, angiotensin II (ANG II) and nitric oxide (NO) can stimulate each other. Unilateral ureteral obstruction (UUO) activates both substances, where ANG II is stimulated first and NO is augmented later.
***Objective:*** Investigate the role of ANG II on renal nitric oxide synthase (NOS) protein expression in UUO.
***Methods:*** Male Wistar rats were divided into sham and UUO. The UUO rats were treated separately with water, angiotensin converting enzyme inhibitor (ACEI), or angiotensin receptor type 1 blocker (ARB) for one day before UUO and continuously for one or seven days after the operation. The endothelial NOS (eNOS) and inducible NOS (iNOS) protein expressions were examined in histology.
***Results:*** By immunohistochemistry, renal eNOS protein expression in the sham group showed staining in glomerulus and tubular epithelial cells in the cortex and medulla. UUO for one or seven days increased eNOS protein expression. ACEI or ARB reduced the heightened expression caused by UUO in 1-day group. However, in 7-day group, the elevated expression was maintained in the cortex, but was further increased in the medulla after ACEI or ARB administration. Both 1-day and 7-day UUO, with or without angiotensin blockade agents, caused no change in iNOS protein expression. One-day UUO resulted in mild tubular dilatation and cell infiltration. ACEI or ARB could attenuate structural alterations. The 7-day UUO rats demonstrated progressively morphological changes. ACEI was more effective than ARB in reducing tissue destruction.
***Conclusion:*** In UUO, angiotensin blockade could attenuate renal eNOS protein expression in 1-day UUO group but not in 7-day UUO animals. The inhibition of angiotensin system ameliorates nephropathy induced by UUO.

***Keywords:*** Angiotensin II, nitric oxide synthase, unilateral ureteral obstruction

Increasing evidence has suggested an intricate interaction between angiotensin II (ANG II) and nitric oxide (NO) [1]. Acute and prolonged infusion of ANG II could up-regulate nitric oxide synthase (NOS) levels in renal tissues [2]. Administration of NO donor induces a 4.5 fold increase in basal renin secretion rate in isolated perfused kidney [3]. In addition, NO could stimulate renin secretion in conscious animals [4].

Unilateral ureteral obstruction (UUO) increases various mediators including ANG II and NO [5]. Besides vasoconstrictory effects that could deteriorate renal hemodynamics, ANG II plays a central role in the initiation and progression of tubulointerstitial fibrosis in UUO by increasing several growth factors and cytokines [6]. Angiotensin converting enzyme inhibitor (ACEI) and angiotensin receptor blocker (ARB) blunts the expression of such substances in concomitant with amelioration of histological changes [7].

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NOS protein expression and urinary NO excretion were elevated in UUO [8]. NO could abrogate the alteration in renal hemodynamics induced by ANG II [6]. Apart from vasodilatory effect, NO could also attenuate tubulointerstitial fibrosis [9]. L-arginine administration in UUO animals could restore renal function and improve nephropathy [10]. By contrast, treatment with NOS inhibitor results in sustained renal damage caused by ureteral obstruction [11]. During UUO, it is likely that ANG II is stimulated first and then NOS is activated later. However, this contention is still unclear. To date, there are no available data showing the effect of ANG II blockade on renal eNOS and iNOS protein expressions during UUO.

In the present study, we investigate the role of ANG II and angiotensin receptor type 1 ($AT_1$) on renal NOS protein expressions in UUO using ACEI and ARB.

## Material and methods

### *Experimental animals*

The study was approved by the Ethics Committee of Research, Faculty of Medicine, Chulalongkorn University. Male Wistar rats, weighing 220 to 250 grams, were used for this study. The animals were housed with controlled temperature (23-25°C) and 12 hours of controlled light-dark time. The animals were given free access to laboratory chow and water as assigned in the experimental protocols.

### *Experimental procedure*

The experimental protocol was performed as described previously [12]. Briefly, the animals were divided into two groups, sham and UUO. The serum creatinine of each animal should be less than 1 mg/dL. In UUO group, the left ureter was ligated with a silk suture at a point one-third the distance from the renal pelvis. In sham group, the left ureter was only wiped without ligation. The UUO rats were treated separately with water, ACEI (Enalapril 200 mg/L, Biolab, Bangkok, Thailand), or ARB (Losartan 500 mg/L; MSD, Hertfordshire, UK) for one day before UUO and continuously for one or seven days after the operation (n = 8/group). The doses of ACEI and ARB had no hypotensive effects [13].

One day before the experiment, the animals were placed in metabolic cages for twenty-four hour urine collection. On the experimental day, the animals of the respective groups were re-operated under anesthesia. Blood sample was collected from the abdominal aorta, and was centrifuged at 1,000 g for 15 minutes. Serum was stored at -80°C until use for measuring blood chemistry using ISE (ion selection electrode) indirect method (Model CX3, Beckman Instrument INC, Deutschland, Germany). The kidneys were removed and fixed in 10% paraformaldehyde overnight, and embedded in paraffin for immunohistochemical detection of endothelial NOS (eNOS) and inducible NOS (iNOS) protein expressions as well as histological evaluation.

### *Immunohistochemical examination*

The detection of protein localization was performed as described previously [14]. Briefly, the sections were incubated in monoclonal antibody against eNOS or iNOS (BD Transduction, Franklin Lakes, USA) at concentrations of 1:100 and 1:200, respectively. Then, the sections were rinsed, incubated with biotinylated anti-mouse immunoglobulin (H+L) (Vector, Burlingame, USA), and reacted with ABC-streptavidin horseradish peroxidase complex (Vector, USA). The sections were reacted for peroxidative activity in a solution containing 0.025% 3, 3-diaminobenzidine (DAB, Sigma, St Louis, USA), washed, and counterstained with haematoxylin (CV Laboratories, Bangkok, Thailand).

Areas of staining were identified and semi-quantitatively scored by three pathologists in a blinded fashion. The intensity of staining was scored from 0 to 3 as described previously [14].

### *Histopathological examination*

Renal tissue injury was examined by the Periodic Acid-Schiff (PAS) reaction and scored in a semi-quantitative manner from 0 to 5 as described previously [15].

### *Statistical analysis*

The intensity scores of renal NOS protein expressions and renal pathological scores were present in descriptive statistics by measuring the central tendency (mode). The results of blood and urine parameters were expressed as mean ± SD. Statistical differences among groups were assessed by ANOVA (analysis of variance) with post hoc comparison by Tukey's test where appropriate. A p-value of $<0.05$ was considered statistically significant. The statistical calculations were performed using the statistical package SPSS for Window 16.

## Results

### *Renal NOS protein expression*

**Figure 1** shows immunohistochemical images to demonstrate the renal eNOS protein expression in the cortex and medulla from the left (obstructed) kidney of rats in sham, UUO, UUO+ACEI, and UUO+ARB for one or seven days after UUO. The intensity scores of renal eNOS protein expression are shown in **Table 1**.

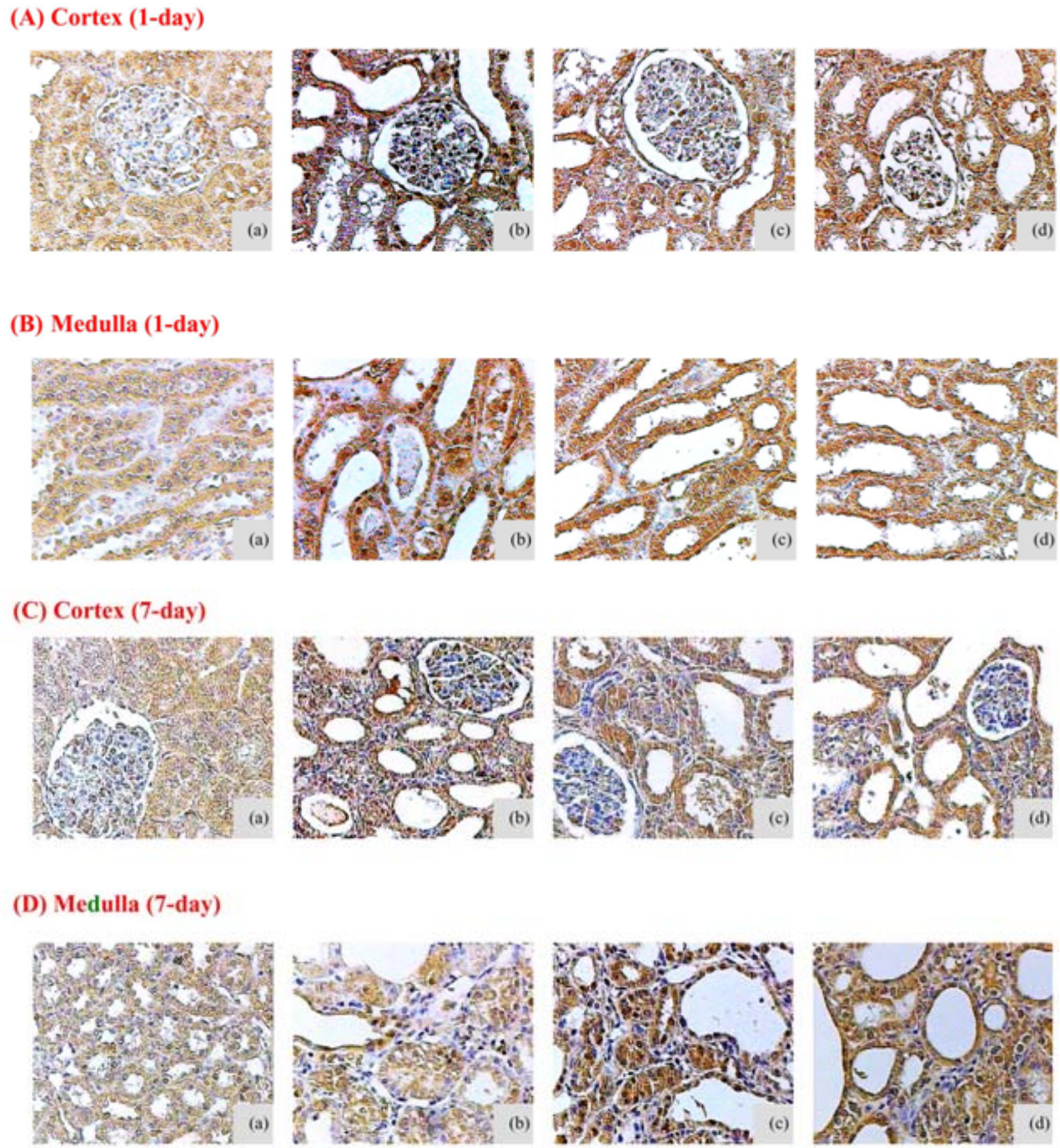


**Figure 1.** Representative immunohistochemical staining of renal eNOS protein expression in the cortex (**A, C**) and medulla (**B, D**) from one-day (**A**, B) and seven-day (**C, D**) UUO groups (**a-d**: original magnification: 200x). **(a)**: sham, **(b)**: UUO, **(c)**: UUO+ACEI, **(d)**: UUO+ARB.

**Table 1.** The intensity scores of renal eNOS protein expression in the cortex and medulla from left (obstructed) and right (non-obstructed) kidney of rats in sham, UUO, UUO+ACEI, and UUO+ARB for one or seven days after UUO (Mode, n = 8/group).

| | | Duration period after UUO | | | |
|---|---|---|---|---|---|
| | | 1 day | | 7 days | |
| | | Cortex | Medulla | Cortex | Medulla |
| **sham** | Lt | 1 | 1 | 1 | 1 |
| | Rt | 1 | 1 | 1 | 1 |
| **UUO** | Lt | 3 | 3 | 2 | 2 |
| | Rt | 1 | 1 | 1 | 1 |
| **UUO+ACEI** | Lt | 2 | 2 | 2 | 3 |
| | Rt | 1 | 1 | 1 | 1 |
| **UUO+ARB** | Lt | 2 | 2 | 2 | 3 |
| | Rt | 1 | 1 | 1 | 1 |

UUO = unilateral ureteral obstruction, ACEI = angiotensin converting enzyme inhibitor, ARB = angiotensin receptor type 1 blocker, Lt = left (obstructed) kidney, Rt = right (non-obstructed) kidney.

In the sham group, the eNOS protein expression was detected in glomeruli and renal tubular epithelium. The staining was present in the cortex and medulla (see A(a) and B(a)). The intensity score was 1 in both areas (**Table 1**). One-day UUO caused an increase in eNOS protein expression in both regions (see A(b) and B(b)) with the intensity scores of 3. This enhancement in eNOS protein expression was still observed in 7-day UUO and the scores were 2 (see C(b) and D(b) and **Table 1**). In the 1-day UUO group, ACEI or ARB could attenuate eNOS protein expression in the cortex (see A(c) and A(d)) and medulla (see B(c) and B(d)). The intensity scores were 2 (**Table 1**). In 7-day UUO rats, ACEI or ARB had no effect on the expression in the cortex area (see C(c) and C(d)). Interestingly, in the medulla, ACEI or ARB augmented eNOS protein expression. The scores were enhanced from 2 to be 3 (see D(c) and D(d) and **Table 1**).

The non-obstructed right kidney showed comparable intensity score of eNOS protein expression with the sham group (**Table 1**).

One-day and 7-day UUO, with or without angiotensin blockade agents, had no influence on iNOS protein expression (data not shown).

***Histopathological study***

In 1-day UUO, the kidney showed moderate tubular dilatation with a few cast formation and mild brush border membrane loss (scores 2-3, **Table 2**). In addition, some interstitial fibrosis and cell infiltration also occurred in the cortex and medulla. Treatments with ACEI or ARB in 1-day UUO rats could reduce the pathological changes (scores 1-2, **Table 2**).

In 7-day UUO, progressive morphological changes were observed (scores 3, **Table 2**). There was more dilatation of Bowman's capsule space with a mild glomerular damage. The glomeruli were compressed while the collecting ducts and distal tubules were much more expanded. Interstitial fibrosis and cell infiltration were prominent. ACEI had more effectiveness in attenuating tissue damage than ARB (scores 1-2; **Table 2**).

The non-obstructed right kidney of UUO rats had normal histological study at both periods after UUO (**Table 2**).

***Blood and urine parameters***

The renal function and serum electrolytes in all UUO groups were not significantly different from the sham group (data not shown). Urine flow rate and fractional excretion of electrolyte were comparable among the studied groups (data not shown).

**Table 2.** The pathological scores of renal cortex and medulla from left (obstructed) and right (non-obstructed) kidney of rats in sham, UUO, UUO+ACEI, and UUO+ARB for one or seven days after UUO (Mode, n = 8/group).

| | | Duration period after UUO | | | |
|---|---|---|---|---|---|
| | | 1 day | | 7 days | |
| | | Cortex | Medulla | Cortex | Medulla |
| **sham** | Lt | 0-1 | 0-1 | 0-1 | 0-1 |
| | Rt | 0-1 | 0-1 | 0-1 | 0-1 |
| **UUO** | Lt | 2-3 | 2-3 | 3 | 3 |
| | Rt | 0-1 | 0-1 | 0-1 | 0-1 |
| **UUO+ACEI** | Lt | 1-2 | 1-2 | 1-2 | 1-2 |
| | Rt | 0-1 | 0-1 | 0-1 | 0-1 |
| **UUO+ARB** | Lt | 1-2 | 1-2 | 2-3 | 2-3 |
| | Rt | 0-1 | 0-1 | 0-1 | 0-1 |

UUO = unilateral ureteral obstruction, ACEI = angiotensin converting enzyme inhibitor, ARB = angiotensin receptor type 1 blocker, Lt = left (obstructed) kidney, Rt = right (non-obstructed) kidney.

## Discussion

The present results showed that eNOS protein expression was normally expressed constitutively in the cytoplasmic area of renal tubular epithelial cells and glomerulus. One day after UUO markedly enhanced renal eNOS protein expression in both cortical and medullary regions. Following seven days after UUO, eNOS protein expression was still higher than the sham group while the magnitude of increase was less than the 1-day UUO group.

UUO increases eNOS protein expression, eNOS activity, and eNOS mRNA level [10, 13]. The increase in ANG II during UUO has been shown to activate $Ca^{2+}$-dependent NOS activity and protein expression [2, 10]. Ureteral obstruction causes increased renal vascular resistance, declined renal blood flow, and reduced oxygen consumption in the renal tissue [16]. During obstruction, the ureteral pressure is progressively elevated, leading to increased tubular wall tension and renal tissue stresses, both of which can cause hypoxia [6]. A previous cell culture study using non-UUO model by Arnet et al. [17] has shown that hypoxia enhanced the amount of eNOS mRNA and protein. In addition, rats subjected to hypoxia (9-10% oxygen exposure) have increments in both eNOS mRNA and protein expression in various tissues including kidney [18]. Thus, the induction of eNOS expression in the UUO model may be partly mediated by a decrement in renal tissue perfusion.

The present study is the first to demonstrate that ACEI and ARB decreased renal eNOS protein expression in 1-day UUO rats. This would underscore an essential role of the heightened ANG II levels in the above mechanisms involving in the stimulation of eNOS in UUO. In 7-day UUO groups, eNOS protein expression was progressively increased although the animals still received either ARB or ACEI. The mechanisms for this alteration are still unclear. Regarding ARB, the inhibition of $AT_1$ receptor would result in stimulation of angiotensin receptor type 2 ($AT_2$) which exerts opposite effects against $AT_1$ receptor [19]. Previous studies showed that $AT_2$ receptor mediates the production of NO in renal interstitial cells in ureteral obstruction and non-obstructive model [20]. It has been demonstrated that the antifibrotic effect of $AT_2$ activation was induced during obstructive nephropathy [21]. The findings in 7-day ARB-treated UUO animals may be caused by the more binding of ANG II to $AT_2$ receptor, leading to sustained activation of nitric oxide pathway.

The increase in eNOS protein expression by ACEI in 7-day UUO was observed in the present study as shown in **Figure. 1(C, D)** and **Table 1**. Besides enhancing bradykinin concentrations, ACEI can activate bradykinin $B_1$ receptor, leading to elevated intracellular calcium and NO release [22]. ANG II can stimulate the synthesis and release of endothelin-1 (ET-1) in endothelial cells [23]. In ureteral obstruction,

the expressions of renal endothelin converting enzyme-1 and endothelin A ($ET_A$)-receptor mRNA in rats are enhanced [24]. ACEI has an inhibitory effect on endothelin and its receptor expression [25]. ACEI could ameliorate the increased expression of ET-1 mRNA in the obstructed kidney but unalters $ET_A$ mRNA expression. Interestingly, ACEI could markedly increase endothelin B ($ET_B$) - receptor mRNA and protein expression in the obstructed kidney [25]. In endothelial cell culture, $ET_B$ receptor agonist stimulates the production of NO and cGMP [26]. Therefore, the increased eNOS protein expression during ACEI treatment in the present study may be mediated, in part, via activation of $ET_B$ receptor.

In the present experiment, both one and seven days after UUO had no effect on iNOS protein expression. These data concur with the result by Knerr et al. [27] who showed comparable iNOS mRNA levels between patients with congenital ureteropelvic junction obstruction and control. Indeed, the results related to iNOS expression in ureteral obstruction have yielded controversial data. Upregulation in iNOS is mainly mediated via ANG II activated cytokine-stimulated NO synthesis, leading to an increase in the magnitude of iNOS mRNA accumulation [28]. On the contrary, transforming growth factor β (TGF-β) induced by ANG II and mechanical stretch could reduce iNOS expression as well as NO production [29].

The present study is the first to compare the effectiveness in reducing UUO-induced renal tissue damage between ACEI and ARB. Of interest, the treatment with ACEI was more effective than ARB as shown in **Table 2**. However, the superiority of ACEI is not related to the NOS levels since both agents had comparable effects on NOS. The underlying mechanisms of this finding are still unestablished. One possible mechanism might be that bradykinin is more available during ACEI treatment. Besides $B_1$ receptor, the heightened bradykinin could also bind to $B_2$ receptor leading to decreased ureteral obstruction-induced renal fibrosis [30].

In conclusion, angiotensin blockade could attenuate renal eNOS protein expression one day after UUO but not seven days after UUO. The inhibition of angiotensin system ameliorates nephropathy induced by UUO. The ANG II-NO interactions in UUO are more subtle and complex. The counterbalancing effects of ANG II and NO could regulate both renal function and renal tissue integrity.

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