

Changes in Endothelin Receptor Type B and Neuronal Nitric Oxide Synthase in Puromycin Aminonucleoside-Induced Nephrotic Syndrome

Eun Hui Bae, and Soo Wan Kim

Department of Internal Medicine, Chonnam National University Medical School, Gwangju 501-757, Korea

The collecting duct endothelin (ET) system, which involves ET-1 and its two receptors, may play a role in the regulation of renal sodium in association with the nitric oxide synthase (NOS) system. We determined whether sodium retention is associated with changes in the endothelin and NOS systems at different stages (i.e., a sodium retaining stage and a compensatory stage) of nephrotic syndromes. On day 7 after puromycin aminonucleoside (PAN) injection, urinary sodium excretion was decreased, ascites had developed, and there was a positive sodium balance. ET-1 mRNA expression was increased in the inner medulla of the kidney, whereas protein expression of ET receptor type B (ET_BR) was unchanged. The expression of neuronal NOS (nNOS) was decreased in the inner medulla. On day 14, urinary sodium excretion was unchanged compared with controls. The expression of ET_BR increased, while nNOS expression in the inner medulla was comparable to controls. These findings suggest that decreased nNOS plays a role in the development of sodium retention in the nephrotic syndrome. Recovery of nNOS and increased renal ET_BR synthesis may promote sodium excretion in later stages of the nephrotic syndrome (on day 14).

Key Words: Nephrotic syndrome, Endothelin receptor type B, Nitric oxide synthase, Puromycin aminonucleoside

INTRODUCTION

Nephrotic syndrome is a common manifestation of renal disease and is associated with avid sodium retention that leads to the development of edema and ascites [1]. However, urinary sodium excretion may change according during the different stages of the nephrotic syndrome [2] and of liver cirrhosis [3,4]. The time course of puromycin aminonucleoside (PAN)-induced nephrosis consists of urinary sodium retention followed by natriuresis that is comparable to control [2]. PAN-treated rats display a positive sodium balance during the initial sodium retaining period. During the maintenance or compensatory period, urinary sodium excretion increases progressively so that sodium balance becomes negative. These findings suggest that there are dynamic changes in sodium retention at different stages of the nephrotic syndrome.

Endothelin-1 (ET-1), the principal isoform of the ET family, activates 2 subtypes of receptors, the type A ET receptor (ET_AR) and the type B ET receptor (ET_BR). The activation of ET_AR, which is located in vascular smooth muscle cells, increases intracellular Ca²⁺, leading to prolonged vasoconstriction [5]. In contrast, the activation of ET_BR, which was first described on endothelial cells, induces a release

of nitric oxide (NO), thus leading to vasodilation [6]. Recent evidence suggests that the stimulatory effects of ET-1 on water and sodium excretion are mediated through ET_BR. First, it is well known that the renal medulla is rich in ET_BR [7,8], where its activation provokes the release of NO in large amounts [9]. Second, several studies have demonstrated that medullary NO plays a pivotal role in the regulation of renal medullary hemodynamics and excretory functions [10]. Finally, ET-1 rapidly decreases the probability that the rat collecting duct epithelial sodium channel (ENaC) is open through ET_BR, and this effect is dependent on src kinases and the nitric oxide pathway [11]. It is conceivable that the changes in renal ET and in the NO system may contribute to the pathogenesis of sodium retention at different stages of the nephrotic syndrome, i.e., at a sodium retaining stage and a compensatory stage. The underlying mechanism for the time course changes may be causally associated with dynamic changes in ET and in the NO system in the kidney. The purpose of this study was to determine whether PAN induced nephrotic syndrome in rats is associated with altered regulation of ET and the NO system in the kidney in order to elucidate the underlying molecular mechanisms responsible for the increased sodium retention.

Received July 1, 2010, Revised July 29, 2010,
Accepted August 6, 2010

Corresponding to: Soo Wan Kim, Department of Internal Medicine, Chonnam National University Medical School, 8, Hak-dong, Gwangju 501-757, Korea. (Tel) 82-62-220-6271, (Fax) 82-62-225-8578, (E-mail) skimw@chonnam.ac.kr

ABBREVIATIONS: ET, endothelin; NOS, nitric oxide synthase; PAN, puromycin aminonucleoside; ET_BR, type B endothelin receptor; ENaC, epithelial sodium channel; ET_AR, type A endothelin receptor; NOx, nitric oxide metabolites.

METHODS

Animals

Our animal study was approved by the Ethics Committee of Chonnam National University Medical School. Male Sprague-Dawley rats weighing 160 to 180 g were used. PAN-induced nephrotic syndrome was induced in 8 mice by a single intravenous injection of PAN (180 mg/kg body weight; Sigma, St. Louis, MO) via the femoral vein. Control rats (n=8) received vehicle alone (i.e., sterile 0.9% saline). Rats were maintained on a standard rodent diet and allowed free access to drinking water and paired feeding at all times. In the control group, rats were offered the amount of food corresponding to the mean intake of food that the PAN rats consumed during the previous day. During the last 3 days, rats were maintained in metabolic cages to allow urine collections for measurement of Na^+ and creatinine. Water and food intake and body weight were monitored. Rats were killed at two different time points (day 7 and day 14 after PAN or vehicle injection) and tissues used for immunoblotting and real time PCR. Rats were anesthetized with isofluran, and a large laparotomy was made. Blood was collected from the inferior vena cava, and the right kidneys were taken and kept at -70°C until assayed. The right kidney was rapidly removed, and the inner medulla was dissected and processed for immunoblotting as described below. The left kidney was excised and kept at -70°C until being assayed for mRNA expression by real time-PCR. Drugs were purchased from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise.

Isolation of total RNA

Kidney was homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was extracted with chloroform, precipitated with isopropanol, washed with 75% ethanol, and then redissolved in distilled water. The RNA concentration was determined by the absorbance read at 260 nm (Ultraspec 2000; Pharmacia Biotech, Cambridge, UK).

Real-time PCR

cDNA was made by reverse transcribing 5 μg of total RNA using oligo (dT) priming and superscript reverse transcriptase II (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was quantified using a Smart Cycler II System (Cepheid, Sunnyvale, CA) and

SYBR Green was used for detection. Each PCR reaction contained the following final concentrations: 10 μM forward primer, 10 μM reverse primer, 2 \times SYBR Green Premix Ex Taq (TAKARA BIO INC, Seta 3-4-1, Japan), 0.5 μl cDNA and H_2O to bring the final volume to 20 μl . Relative levels of mRNA were examined by real-time PCR using a Rotor-GeneTM 3000 Detector System (Corbette research, Mortlake, New South Wales, Australia) according to the manufacturer's directions. Primers were prepared as in Table 1. Real-time reverse transcription-PCR was done using the following steps: 1) 95°C for 5 min; 2) 95°C for 20 s; 3) 58 to 60°C for 20 s (optimized for each primer pair); 4) 72°C for 30 s; and 5) 85°C for 6 s to detect SYBR Green (nonspecific products melt at temperatures $<85^\circ\text{C}$, and therefore were not detected). Steps 2~5 were repeated for an additional 45 cycles. At the end of the last cycle, the temperature was increased from 60 to 95°C to produce a melting curve. The ratio of each gene to the GAPDH level (relative gene expression number) was calculated by subtracting the threshold cycle number of the target gene from that of GAPDH and raising 2 to the power of this difference [12].

Semiquantitative immunoblotting

The dissected inner medulla was homogenized in ice-cold isolation solution containing 0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, pH 7.2. Homogenates were centrifuged at 1,000 g for 15 min at 4°C to remove whole cells, nuclei and mitochondria. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 9 or 12% polyacrylamide gels. Proteins were transferred by gel electrophoresis (Bio-Rad Mini Protean II, Bio-Rad, Hercules, CA, USA) onto nitrocellulose membranes (Hybond ECL RPN3032D, Amersham Pharmacia Biotech, Little Chalfont, UK). Blots were subsequently blocked with 5% milk in PBST (80 mM Na_2HPO_4 , 20 mM NaH_2PO_4 , 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 hour and incubated overnight at 4°C with primary antibodies, followed by incubation with secondary anti-rabbit (P448, DAKO, Glostrup, Denmark) or anti-mouse (P447, DAKO, Glostrup, Denmark) horseradish peroxidase-conjugated antibodies. Labeling was visualized by an enhanced chemiluminescence system. The primary antibodies to eNOS, nNOS, iNOS (Transduction Laboratories; Lexington, KY, USA), soluble guanylyl cyclase (sGC; Santa Cruz Biotechnology; Santa Cruz, CA, USA) and ET_BR (Alomon Lab, Jerusalem, Israel) were obtained from commercial sources.

Colorimetric assay of nitrite/nitrate

As an index of the synthesis of NO, its stable metabolites (nitrite/nitrate, NO_x) were measured by a colorimetric NO assay kit (Oxford Biochemical; Oxford, MI, USA). Microplates were used to carry out enzyme reactions in vitro. For spectrophotometric assays of nitrite with the Griess reagent, 80 μl MOPS (50 mM) / EDTA (1 mM) buffer and 5 μl samples were added to the microplate wells. Nitrate reductase (0.01 U) and 10 μl NADH (2 mM) were added to the reaction mixture, and the plate was shaken for 20 min at room temperature. Color reagents, sulfanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride were added, and absorbance values were read at 540 nm in a microtitre plate reader (Bio-Rad, Model 3550).

Table 1. Primers used in the polymerase chain reaction

Primers	Sequences
GAPDH	Sense:ATCAAATGGGGTGATGCTGGTGCTG Antisense:CAGTTTCTCCAGGCGGCATGTCAG
ET-1	Sense:CATGGGCTCCTTCTCCATCA Antisense:TCAAGAGGGCAGATCTATCG
ET _A R	Sense:GGTGATAGTAGCCCTCATCT Antisense:GTCTGGGGAACCTCAAACCTG
ET _B R	Sense:CGAGGTGCTTGTGCTATTGC Antisense:GCGAGTACTCCGTGTCCTTG

GAPDH, glyceraldehydes-3-phosphate dehydrogenase; ET-1, endothelin-1; ET_AR, endothelin A receptor; ET_BR, endothelin B receptor.

Statistical analysis

Results are expressed as mean±SEM. The statistical significance of differences between groups was determined by using an unpaired student t-test. *p* values <0.05 were considered significant.

RESULTS

Renal function

Table 2 shows parameters of renal function. On day 7,

PAN-treated rats developed ascites, as indicated by visible pools of fluid in the lateral abdominal gutters. The plasma creatinine level was increased, and its clearance decreased. Total and fractional excretion of sodium decreased, and a positive sodium balance was present. On day 14, PAN-treated rats still developed significant ascites. Creatinine clearance was increased, and its plasma level decreased. Total and fractional excretion of sodium was normalized and sodium balance was unchanged.

Changes in the endothelin system in the kidney

Fig. 1 shows the expression of ET-1, ET_AR and ET_BR in the kidney. On day 7, in rats with PAN-induced nephrotic

Table 2. Changes in renal function

	Day 7		Day 14	
	Control (n=8)	PAN (n=8)	Control (n=8)	PAN (n=8)
Body weight (g)	170±10	214±8*	195±4	171±18*
Pcr (mg/dl)	0.25±0.06	0.41±0.16*	0.31±0.05	0.17±0.05*
Ccr (ml/min)	1.47±0.4	0.84±0.32*	1.25±0.09	1.90±0.74*
U _{Na} V (mEq/day)	2.88±0.30	0.62±0.24*	2.79±0.30	3.57±0.82
FE _{Na} (%)	0.92±0.12	0.48±0.09*	1.12±0.15	1.02±0.35
Na balance (mmol/day)	-1.38±0.29	0.31±0.09*	-1.57±0.35	-2.27±0.79

Values are expressed as mean±SEM. These values were measured on the last day of the experiment. P-Cr, plasma creatinine; Ccr, creatinine clearance; U_{Na}V, rate of urinary sodium excretion; FE_{Na}, fractional excretion of sodium into urine; Na balance, the difference between dietary sodium intake and urinary sodium excretion. **p*<0.05, when compared with the corresponding control group.

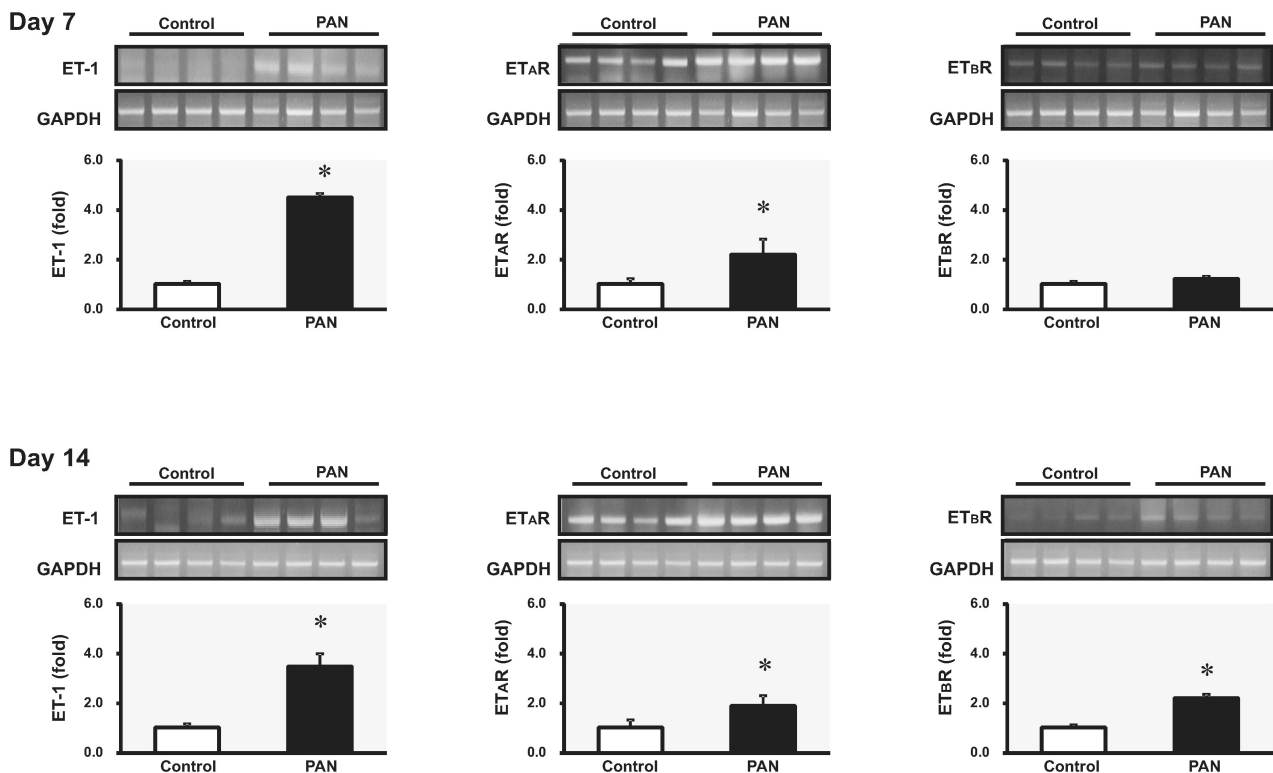


Fig. 1. The expression of ET-1, ET_AR and ET_BR mRNA in kidney. Fluorographs show ethidium bromide stained agarose gels containing reverse transcription-PCR products, and columns show real-time PCR data representing control and PAN-induced nephrotic syndrome groups (mean±SEM, *n*=8 each). **p*<0.05 vs. control.

syndrome, mRNA expression for ET-1 was significantly increased, while ET_BR was unchanged. On day 14, mRNA expression for ET-1 and ET_BR was increased. On days 7 and 14, mRNA expression for ET_AR was increased in PAN-induced nephrotic syndrome. Fig. 2 shows immunoblotting of renal ET_BR. Immunoblotting of ET_BR was unchanged in the inner medulla on day 7 and was increased on day 14.

Changes in the nitric oxide system in the kidney

Fig. 3 and Table 3 show the expression of nNOS, eNOS, iNOS and sGC in the inner medulla of the kidney. On day

7, nNOS protein expression was decreased. On day 14, nNOS protein expression was unchanged compared with controls. On the other hand, expression of eNOS and iNOS proteins was not different between groups at different stages. Fig. 4 shows the urinary nitric oxide metabolite data. On day 7, the amount of urinary NO_x was decreased, while on day 14 it was increased.

DISCUSSION

In the present study, PAN-treated rats showed decreased fractional excretion of sodium and positive sodium balance

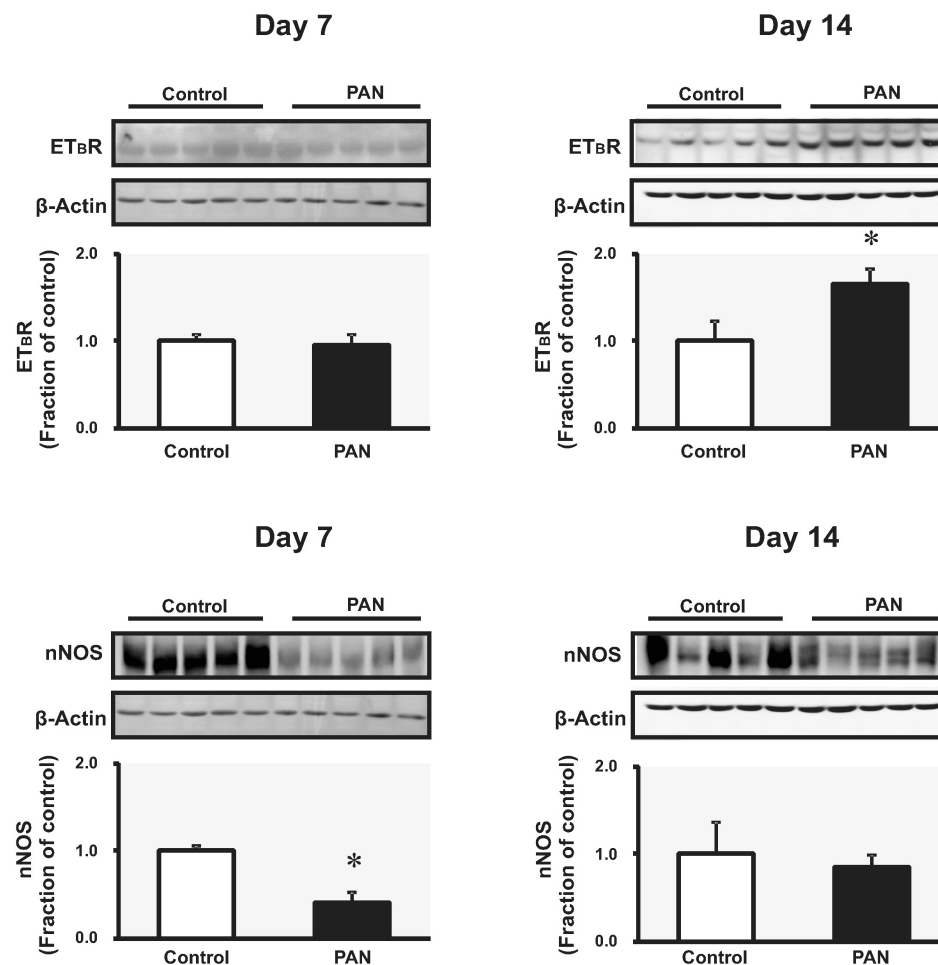


Fig. 2. The expression of ET_BR in kidney. Immunoblotting data of ET_BR in inner medulla was unchanged in PAN-treated rats compared with controls; it was increased on day 14 (mean±SEM, n=8 each). *p<0.05 vs. control.

Fig. 3. The expression of neuronal NOS in kidney. On day 7, nNOS protein expression was decreased. On day 14, nNOS protein expression was increased compared with controls (mean±SEM, n=8 each). *p<0.05 vs. control.

Table 3. Protein expression of nitric oxide synthase and soluble guanylyl cyclase in the kidney

	Day 7		Day 14	
	Control (n=5)	PAN (n=5)	Control (n=5)	PAN (n=5)
eNOS	1.00±0.08	1.17±0.08	1.00±0.20	1.26±0.34
nNOS	1.00±0.05	0.41±0.12*	1.00±0.05	0.81±0.13
iNOS	1.00±0.08	0.98±0.10	1.00±0.07	1.13±0.08
sGC	1.00±0.20	1.20±0.21	1.00±0.14	0.96±0.12

Values are expressed as mean±SEM. eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; sGC, soluble guanylyl cyclase. *p<0.05, when compared with the corresponding control group.

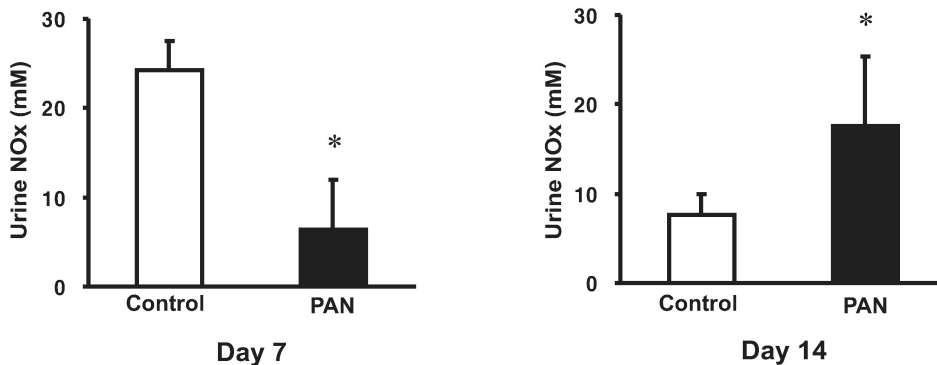


Fig. 4. Amount of urinary nitric oxide metabolite (NOx). Data are the mean \pm SEM of 8 rats for each group. * $p < 0.05$ vs. control.

on day 7. The sodium retention and ascites formation may be causally related with increased tubular reabsorption of sodium. There are dynamic changes in sodium retention at different stages (i.e., a sodium retaining stage and a compensatory stage) of the nephrotic syndrome. On day 14 there was no evidence of a positive sodium balance despite the marked ascites.

ET-1 and ET_AR mRNA expression was increased at both stages, while mRNA and protein expression for ET_BR showed dynamic changes at different stages. In addition, nNOS expression was decreased during the sodium retaining phase (day 7) and it was restored to control value on day 14. In the kidney, both NO and ET have been shown to inhibit tubular sodium reabsorption [13] and there are a number of possible interactions between the renal ET and NO systems [14]. In the thick ascending limb, there is evidence that ET_BR serves to inhibit chloride reabsorption through an NO-dependent pathway [15]. Additionally, rats treated chronically with an ET_BR antagonist have elevated arterial pressures compared with control animals, and this increase in blood pressure was much greater in rats given a high salt diet compared with a low salt diet [16]. A NOS inhibitor, *N*-nitro-L-arginine methyl ester (L-NAME), and a specific ET_BR antagonist, A-192621, both acted independently to abolish the diuretic and natriuretic effects of ET-1 in the kidneys of anesthetized rats [17]. Although these experiments demonstrate an NO mediated diuretic and natriuretic action of ET-1 in the kidney, it is not possible to identify which cell type is the major source of NO, and which isoform of NOS is responsible for these effects. All three isoforms - endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) - are present in the kidney. Therefore, it is important to distinguish whether ET_BRs are linked to the three type of NOS. As there are no highly specific inhibitors of these isoforms, pharmacological approaches are unlikely to be helpful. In this study, eNOS and iNOS expression did not show changes at different stages. This suggested the possibility of a link between nNOS linked and ET-1. The mechanisms by which collecting duct ET receptors reduce Na transport have been partially resolved. ET-1 rapidly decreased rat collecting duct ENaC open channel probability (within 5 min) through ET_BR and this effect was dependent on src kinases and MAPK 1/2 (mitogen-activated protein kinase 1/2) signaling, while being independent of phospholipase C or protein kinase C [11]. NO also was involved in the natriuretic and hypotensive effects of ET-1, which, through ET_BR activation, stimulates nNOS-dependent NO production by cultured rat inner medullary collect-

ing duct cells [18]. Infusion of an ET_BR agonist, sarafotoxin S6c, into the rat renal medulla caused a diuresis and natriuresis that was suppressed by the inhibition of nNOS or protein kinase G expression [19]. Therefore, in this study, decreased expression of nNOS may contribute to decreased sodium excretion on day 7 in PAN-induced nephrotic syndrome, while a restored level of nNOS on day 14 may induce natriuresis. Additionally, urinary nitric oxide metabolite was decreased on day 7 and increased on day 14, which support the idea that nNOS is associated with sodium retention and natriuresis at different stages in PAN-induced nephritic syndrome.

In contrast to ET_BR, the physiologic significance of renal medullary ET_AR is poorly understood despite the fact that ET_AR is expressed in the collecting duct [20]. In vitro studies have found no evidence for collecting duct ET_AR modulation of sodium transport [21]. Although in vitro studies do not support such a role for ET_AR, very recent studies suggest that ET_AR may, in fact, be natriuretic. Infusion of ET-1 into the renal medulla of ET_BR-deficient rats surprisingly caused a natriuresis that was blocked by the administration of an ET_AR antagonist [22]. Therefore, it is assumed that increased ET_AR may contribute to natriuresis on days 7 and 14 in puromycin-induced nephrosis. However, a role for ET_AR in puromycin-induced nephrosis remains to be identified.

In conclusion, decreased nNOS in PAN-induced nephritic syndrome may contribute to sodium retention on day 7. The subsequent recovery of nNOS expression associated with increased ET_BR may play a role in promoting sodium excretion at a later stage (on day 14).

ACKNOWLEDGEMENTS

This study was supported by a grant (CRI10013-1) from Chonnam National University Hospital Research Institute of Clinical Medicine, and by a grant from the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A084869).

REFERENCES

1. Vande Walle JG, Donckerwolcke RA. Pathogenesis of edema formation in the nephrotic syndrome. *Pediatr Nephrol.* 2001;16: 283-293.
2. Deschenes G, Doucet A. Collecting duct Na,K-ATPase activity

- correlates with urinary sodium excretion in rat nephrotic syndrome. *J Am Soc Nephrol*. 2000;11:604-615.
3. Kim SW, Schou UK, Peters CD, de Seigneux S, Kwon TH, Knepper MA, Jonassen TE, Frokiaer J, Nielsen S. Increased apical targeting of renal epithelial sodium channel subunits and decreased expression of type 2 11 β -hydroxysteroid dehydrogenase in rats with CCl₄-induced decompensated liver cirrhosis. *J Am Soc Nephrol*. 2005;16:3196-3210.
 4. Kim SW, Wang W, Sassen MC, Choi KC, Han JS, Knepper MA, Jonassen TE, Frokiaer J, Nielsen S. Biphasic changes of epithelial sodium channel abundance and trafficking in common bile duct ligation-induced liver cirrhosis. *Kidney Int*. 2006;69:89-98.
 5. Marsen TA, Schramek H, Dunn MJ. Renal actions of endothelin: Linking cellular signaling pathways to kidney disease. *Kidney Int*. 1994;45:336-344.
 6. Tsukahara H, Ende H, Magazine HI, Bahou WF, Goligorsky MS. Molecular and functional characterization of the non-isopeptide-selective ETB receptor in endothelial cells. Receptor coupling to nitric oxide synthase. *J Biol Chem*. 1994;269:21778-21785.
 7. Kohan DE. Endothelins in the normal and diseased kidney. *Am J Kidney Dis*. 1997;29:2-26.
 8. Abassi Z, Gurbanov K, Rubinstein I, Better OS, Hoffman A, Winaver J. Regulation of intrarenal blood flow in experimental heart failure: role of endothelin and nitric oxide. *Am J Physiol*. 1998;274:F766-F774.
 9. Zou AP, Cowley AW Jr. Nitric oxide in renal cortex and medulla. An in vivo microdialysis study. *Hypertension*. 1997;29:194-198.
 10. Romero JC, Lahera V, Salom MG, Biondi ML. Role of the endothelium-dependent relaxing factor nitric oxide on renal function. *J Am Soc Nephrol*. 1992;2:1371-1387.
 11. Bugaj V, Pochynyuk O, Mironova E, Vandewalle A, Medina JL, Stockand JD. Regulation of the epithelial Na⁺ channel by endothelin-1 in rat collecting duct. *Am J Physiol Renal Physiol*. 2008;295:F1063-F10670.
 12. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25:402-408.
 13. Stoos BA, Carretero OA, Farhy RD, Scidi G, Garvin JL. Endothelium-derived relaxing factor inhibits transport and increases cGMP content in cultured mouse cortical collecting duct cells. *J Clin Invest*. 1992;89:761-765.
 14. Plato CF, Garvin JL. Nitric oxide, endothelin and nephron transport: potential interactions. *Clin Exp Pharmacol Physiol*. 1999;26:262-268.
 15. Plato CF, Pollock DM, Garvin JL. Endothelin inhibits thick ascending limb chloride flux via ET(B) receptor-mediated NO release. *Am J Physiol Renal Physiol*. 2000;279:F326-F333.
 16. Pollock DM, Pollock JS. Evidence for endothelin involvement in the response to high salt. *Am J Physiol Renal Physiol*. 2001;281:F144-F150.
 17. Hoffman A, Abassi ZA, Brodsky S, Ramadan R, Winaver J. Mechanisms of big endothelin-1-induced diuresis and natriuresis: role of ET(B) receptors. *Hypertension*. 2000;35:732-739.
 18. Stricklett PK, Hughes AK, Kohan DE. Endothelin-1 stimulates NO production and inhibits cAMP accumulation in rat inner medullary collecting duct through independent pathways. *Am J Physiol Renal Physiol*. 2006;290:F1315-F1319.
 19. Nakano D, Pollock JS, Pollock DM. Renal medullary ETB receptors produce diuresis and natriuresis via NOS1. *Am J Physiol Renal Physiol*. 2008;294:F1205-F1211.
 20. Kohan DE. The renal medullary endothelin system in control of sodium and water excretion and systemic blood pressure. *Curr Opin Nephrol Hypertens*. 2006;15:34-40.
 21. Ge Y, Bagnall A, Stricklett PK, Strait K, Webb DJ, Kotelevtsev Y, Kohan DE. Collecting duct-specific knockout of the endothelin B receptor causes hypertension and sodium retention. *Am J Physiol Renal Physiol*. 2006;291:F1274-F1280.
 22. Nakano D, Pollock DM. Contribution of endothelin receptors in endothelin 1-dependent natriuresis in female rats. *Hypertension*. 2009;53:324-330.