Nephrogenic diabetes insipidus in mice lacking all nitric oxide synthase isoforms

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Nitric oxide (NO) is produced in almost all tissues and organs, exerting a variety of biological actions under physiological and pathological conditions. NO is synthesized by three different isoforms of NO synthase (NOS), including neuronal, inducible, and endothelial NOSs. Because there are substantial compensatory interactions among the NOS isoforms, the ultimate roles of endogenous NO in our body still remain to be fully elucidated. Here, we have successfully developed mice in which all three NOS genes are completely deleted by crossbreeding singly NOS$^{-/-}$ mice. NOS expression and activities were totally absent in the triply NOS$^{-/-}$ mice before and after treatment with lipopolysaccharide. Although the triply NOS$^{-/-}$ mice were viable and appeared normal, their survival and fertility rates were markedly reduced as compared with the wild-type mice. Furthermore, these mice exhibited marked hypotonic polyuria, polydipsia, and renal unresponsiveness to an antidiuretic hormone, vasopressin, all of which are characteristics consistent with nephrogenic diabetes insipidus. In the kidney of the triply NOS$^{-/-}$ mice, vasopressin-induced cAMP production and membranous aquaporin-2 water channel expression were reduced associated with tubuloglomerular lesion formation. These results provide evidence that the NOS system plays a critical role in maintaining homeostasis, especially in the kidney.

Nitric oxide (NO), an important intracellular messenger molecule, plays important roles in maintaining homeostasis (1–6). NO is formed from its precursor L-arginine by a family of NO synthases (NOSs) with stoichiometric production of L-citrulline. NO is a gaseous free radical, freely passes through plasma membranes, and elicits multiple actions without receptor coupling either directly or by means of the soluble guanylate cyclase/cGMP-mediated pathway. Three distinct NOS isoforms exist that are encoded by three distinct genes, including neuronal (nNOS; also known as NOS1), inducible (iNOS; also known as NOS2), and endothelial NOS (eNOS; also known as NOS3) (1–6). nNOS and eNOS are constitutively expressed mainly in the nervous system and the vascular endothelium, respectively, synthesizing a small amount of NO in a calcium-dependent manner under basal conditions and upon stimulation. By contrast, iNOS is induced when stimulated by microbial endotoxins or certain proinflammatory cytokines, producing a greater amount of NO in a calcium-independent manner (1–6).

Genetically engineered animals are a powerful experimental tool to study the function of target genes *in vivo*. Animals bearing targeted deletions of singly and doubly NOS isoforms have thus been developed (7–16). nNOS-deficient (nNOS$^{-/-}$) mice exhibit hypertrophy of the pyloric sphincter (7), increased aggressive behavior (17), and protection from cerebral ischemia (18). eNOS$^{-/-}$ mice are hypertensive (12), vulnerable to cerebral ischemia (19), and show accelerated vascular lesion formation (20). The principle phenotype of iNOS$^{-/-}$ mice is an increased susceptibility to infection with pathogens and resistance to hypotensive response when exposed to bacterial lipopolysaccharide (LPS) (10). In doubly n/eNOS$^{-/-}$ mice, but not in singly nNOS$^{-/-}$ or eNOS$^{-/-}$ mice, hippocampal long-term potentiation is blunted (15). Thus, the roles of individual NOS have been extensively studied. However, because there are substantial compensatory interactions among the NOS isoforms (15), the ultimate roles of NO in our body are still poorly understood.

Here, we report the development of mice in which all three NOS isoforms are completely disrupted. The triply n/i/eNOS$^{-/-}$ mice had markedly reduced survival and fertility rates. Furthermore, they showed hypotonic polyuria, polydipsia, and reduced antidiuretic response to exogenous vasopressin, accompanied by impaired renal cAMP production, defective membrane expression of aquaporin-2 water channel, and tubuloglomerular lesions. These phenotypes closely resemble the human disorder, nephrogenic diabetes insipidus, demonstrating a previously uncharacterized aspect of the important roles of endogenous NO in maintaining our body homeostasis, especially in the kidney.

Materials and Methods

Animal Preparation. Singly nNOS$^{-/-}$ (7), iNOS$^{-/-}$ (9), and eNOS$^{-/-}$ mice (12) were mated with each other to produce doubly n/iNOS$^{-/-}$, n/eNOS$^{-/-}$, and i/eNOS$^{-/-}$ mice. The doubly NOS$^{-/-}$ mice were then intercrossed, and triply n/i/eNOS$^{-/-}$ mice were generated. Eight- to 12-week-old male mice were used in most experiments, and 1-week-old male mice were used in experiments for cAMP, aquaporin-2, cGMP, and prostacyclin measurements.

Western Blot Analysis. The brain (cerebellum), heart, and kidney were isolated and homogenized at 4°C. Membrane fraction of the kidney was obtained by ultracentrifugation at 200,000 × *g* for 1 h (21). Western blot analysis was performed as described in ref. 22.

NOS Activity and Nitrite Plus Nitrate (NOx) Concentration. The brain, the heart, and the kidney were prepared freshly. NOS activity was quantified by monitoring the biochemical conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline using a NOS assay kit (Cayman Chemical, Ann Arbor, MI) (23). NOx concentrations in the plasma and urine were analyzed by the Griess method, as reported in ref. 24.

Osmolality, Biochemistry, and Vasopressin Level. Osmolality in the plasma and urine was measured by an osmotic pressure analyzer (Kyoto Daiichi Pure Chemicals, Kyoto, Japan). Plasma and urinary biochemical data were assessed by a Dri-Chem autoanalyzer (Fuji).

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Abbreviations: NOS, nitric oxide synthase; nNOS, neuronal NOS; iNOS, inducible NOS; eNOS, endothelial NOS; LPS, lipopolysaccharide; NOx, nitrite plus nitrate.

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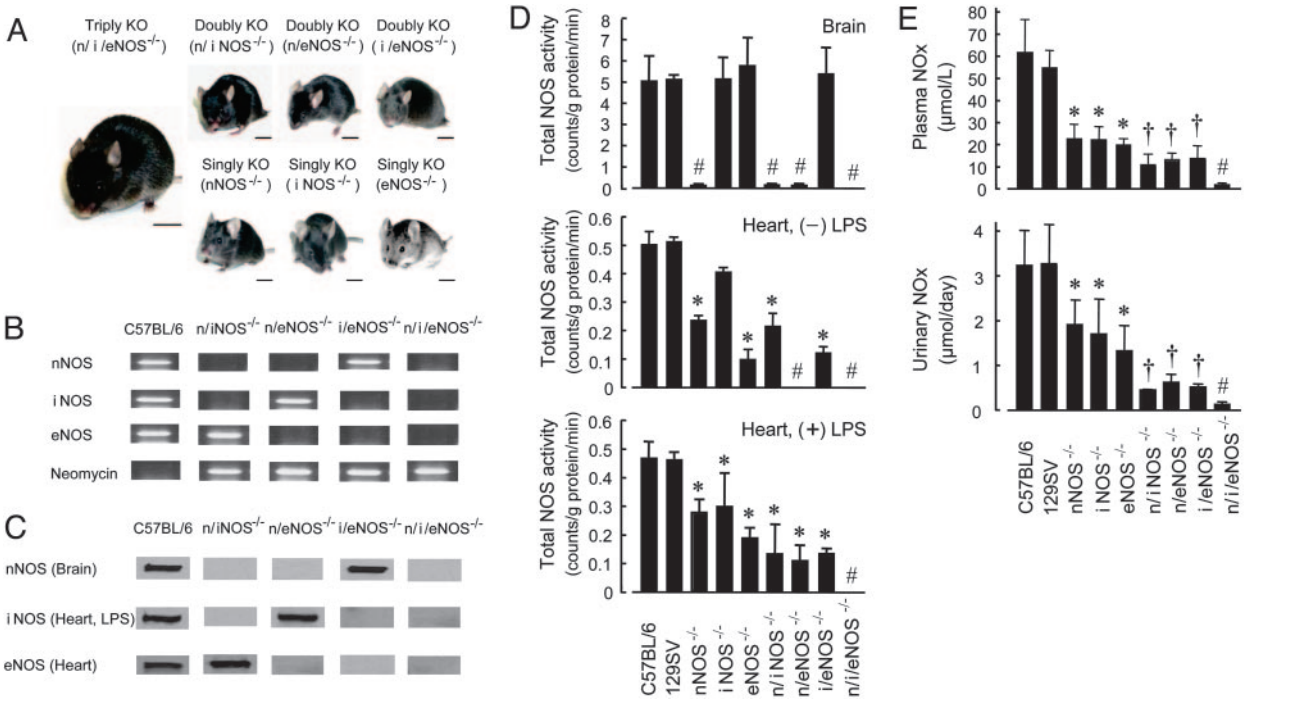

Fig. 1. NOS expression, NOS activity, and NOx level in triply NOS$^{-/-}$ mice. (*A*) Appearance of NOS$^{-/-}$ mice (KO, knockout). (Scale bars, 1 cm.) (*B*) PCR analysis for nNOS, iNOS, eNOS, and neomycin genes in genomic DNA. (*C*) Western blot analysis for nNOS, iNOS, and eNOS in the brain, the LPS-treated heart (20 mg/kg i.p.; 12 h after treatment), and the normal heart, respectively. (*D*) Total NOS activity in the brain, the heart, and the LPS-treated heart ($n = 6$ each). *, $P < 0.05$; #, $P < 0.001$; compared with wild-type C57BL/6 mice. (*E*) Plasma NOx concentrations and urinary NOx excretion ($n = 6$ each). *, $P < 0.05$; †, $P < 0.01$; #, $P < 0.001$; compared with C57BL/6 mice.

Vasopressin concentrations in the urine were determined by a vasopressin RIA kit (Mitsubishi Kagaku, Tokyo).

Renal Sensitivity to Exogenous Vasopressin. Water (1 ml) was administered to the mice orally, and either saline (100 μl) or synthetic vasopressin (0.002 unit per 100 μl of saline, Sankyo) was injected i.p (25). Urinary volume was then checked every hour.

Histological Analysis. The 3-μm-thick sections were stained with hematoxylin–eosin, periodic acid-Schiff, or phosphotungstic acid-hematoxylin for general staining and staining for glomerular abnormalities or thrombi, respectively (26).

Immunostaining. The kidney sections were incubated with rabbit polyclonal antibodies (BD Biosciences) at a dilution of 1:500 for nNOS, 1:250 for eNOS, 1:100 for iNOS, and 1:1,000 for aquaporin-2 for 1 h at room temperature. An avidin biotin immunoperoxidase system was used to detect the antigen (22).

cAMP Level in Renal Collecting Duct. Segments of renal collecting ducts were freshly prepared by using the microdissection procedures reported in ref. 27. The segments were preincubated in oxygenated Krebs–Ringer solution (in mM: 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 0.0026 calcium EDTA, and 11.1 glucose) at 37°C for 30 min and incubated with 0.1 mM 3-isobutyl-1-methylxanthine at 37°C for 30 min. During the last 20 min of the 30-min incubation with 3-isobutyl-1-methylxanthine, certain segments were stimulated with 1 μM vasopressin. Tissue levels of cAMP and protein were measured by a cAMP enzyme immunoassay kit with the acetylation procedure (Cayman, Ann Arbor, MI) and by the Lowry method, respectively.

Renal cGMP and Prostacyclin Levels. The kidney slices were preincubated in oxygenated Krebs–Ringer solution at 37°C for 30 min and incubated in the presence of 0.1 mM 3-isobutyl-1-methylxanthine at 37°C for 30 min. Tissue cGMP levels were assessed by an ^{125}I-cGMP RIA kit (Amersham Pharmacia) (22). Renal prostacyclin levels were evaluated by measuring prostacyclin metabolite 6-keto-prostaglandin $F_{1\alpha}$ with an enzyme immunoassay kit (Amersham Pharmacia) as described in ref. 28.

Statistical Analysis. Results are expressed as mean ± SEM. Survival curves were analyzed by the Kaplan–Meier method. Differences in all other parameters were evaluated by ANOVA.

Results

Development of Triply Mutant Mice. We first produced three kinds of doubly NOS$^{-/-}$ mice (n/iNOS$^{-/-}$, n/eNOS$^{-/-}$, and i/eNOS$^{-/-}$ mice) by crossing singly NOS$^{-/-}$ mice. The doubly NOS$^{-/-}$ mice were then interbred over four to five generations, and triply NOS$^{-/-}$ mice (n/i/eNOS$^{-/-}$ mice) were finally developed. The triply NOS$^{-/-}$ mice were indistinguishable from wild-type animals in phenotype (Fig. 1*A*). These mice developed normally and were able to mate with normal increase in body weight (Table 1).

NOS Expression, NOS Activity, and Plasma NOx Level. We first confirmed the disruption of all three NOS genes in the triply NOS$^{-/-}$ mice by PCR analysis of genomic DNA (Fig. 1*B*). In the wild-type mice, nNOS, iNOS, and eNOS were abundant in the normal brain, the heart 12 h after treatment with LPS (20 mg/kg, IP), and the normal heart, respectively (Fig. 1*C*). Western blot analysis revealed no expression of any NOS protein in the brain or the heart of triply NOS$^{-/-}$ mice either before or 12 h after LPS treatment (Fig. 1*C*). Citrulline assay also demonstrated the absence of any NOS activity in the brain or the heart of the triply NOS$^{-/-}$ mice either before or 12 h after LPS treatment (Fig. 1*D*).

To evaluate systemic NO production, we measured plasma NOx concentrations and urinary NOx excretion. Plasma and urinary

Table 1. Body weight and biochemical data in the urine and plasma

Mice	Body weight, g		Urine	Plasma			
	8 weeks of age	12 weeks of age	Na^+, meq/liter	Urea nitrogen, mg/dl	Creatinine, mg/dl	Ca^{2+}, mg/dl	K^+, meq/liter
C57BL/6	18.7 ± 0.2	23.3 ± 0.3	109 ± 15	21.5 ± 0.6	0.36 ± 0.02	9.1 ± 0.1	8.7 ± 0.3
129SV	20.9 ± 0.8	24.9 ± 0.1	134 ± 17	24.8 ± 2.1	0.34 ± 0.02	8.8 ± 0.2	8.4 ± 0.4
$nNOS^{-/-}$	19.8 ± 0.6	23.4 ± 0.4	80 ± 9	26.4 ± 1.4	0.34 ± 0.02	8.8 ± 0.2	7.8 ± 0.5
$iNOS^{-/-}$	19.8 ± 0.5	26.3 ± 0.8	91 ± 10	28.2 ± 2.4	0.38 ± 0.04	9.0 ± 0.1	8.0 ± 0.2
$eNOS^{-/-}$	20.3 ± 0.5	24.4 ± 0.4	92 ± 19	29.6 ± 2.2	0.40 ± 0.03	9.1 ± 0.2	8.0 ± 0.4
$n/iNOS^{-/-}$	18.8 ± 0.4	23.1 ± 0.8	59 ± 3*	31.1 ± 2.4*	0.38 ± 0.02	9.3 ± 0.1	8.1 ± 0.5
$n/eNOS^{-/-}$	19.7 ± 0.7	23.9 ± 0.9	52 ± 6*	30.7 ± 0.4*	0.40 ± 0.04	9.4 ± 0.2	8.5 ± 0.2
$i/eNOS^{-/-}$	20.9 ± 0.5	25.0 ± 0.6	51 ± 9*	34.5 ± 2.4*	0.42 ± 0.02	9.2 ± 0.1	9.0 ± 0.4
$n/i/eNOS^{-/-}$	18.8 ± 0.6	23.8 ± 0.2	42 ± 6†	41.0 ± 3.8†	0.46 ± 0.07	9.5 ± 0.1	8.1 ± 0.3

Data are expressed as mean ± SEM. The number of mice studied was 8–16 for body weight and 6 for biochemical data. *, $P < 0.05$; †, $P < 0.01$; compared with wild-type C57BL/6 mice.

NOx levels were significantly lower in accordance with the number of disrupted NOS genes (Fig. 1*E*). In the triply $NOS^{-/-}$ mice, both values were extremely low, with only 2.4% and 3.6% of normal plasma and urinary NOx levels noted, respectively (Fig. 1*E*).

Survival and Fertility. We then examined the survival and fertility of the triply $NOS^{-/-}$ mice. During the 10 months of follow-up, all of the wild-type mice lived, whereas only three of 13 triply $NOS^{-/-}$ mice (23%) survived (Fig. 2*A*). Survival rate was significantly worse in accordance with the number of disrupted NOS genes (Fig. 2*A*). The number of offspring produced from breeding pairs was also significantly smaller in the same manner (Fig. 2*B*).

Hemodynamics. To characterize the phenotypical changes in the triply $NOS^{-/-}$ mice, we measured hemodynamic variables under conscious conditions by the tail-cuff method. Systolic blood pressure was significantly higher in the triply $NOS^{-/-}$ mice than in the wild-type mice. The degree of hypertension in those mice was equivalent to that in the eNOS gene-deficient singly and doubly $NOS^{-/-}$ mice ($eNOS^{-/-}$, $n/eNOS^{-/-}$, and $i/eNOS^{-/-}$ mice) (Fig. 2*C*). Heart rate was significantly lower in the triply $NOS^{-/-}$ mice than in the wild-type mice, again equivalent to that in the eNOS gene-deficient singly and doubly $NOS^{-/-}$ mice (Fig. 2*D*).

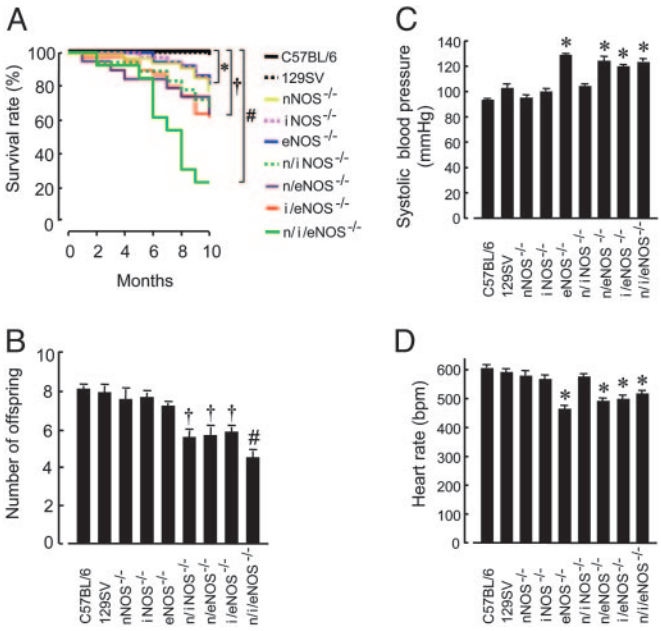

Fig. 2. Survival, fertility, and hemodynamics in triply $NOS^{-/-}$ mice. (*A*) Survival rate 10 months after birth ($n = 13$–40). *, $P < 0.05$ between each singly $NOS^{-/-}$ and C57BL/6 mice; †, $P < 0.01$ between each doubly $NOS^{-/-}$ and C57BL/6 mice; #, $P < 0.001$ between triply $NOS^{-/-}$ and C57BL/6 mice. (*B*) Number of offspring produced from breeding pairs ($n = 10$–26). †, $P < 0.01$; #, $P < 0.001$; compared with C57BL/6 mice. (*C*) Systolic blood pressure ($n = 9$–16). *, $P < 0.05$ vs. C57BL/6 mice. (*D*) Heart rate ($n = 9$–16). *, $P < 0.05$ vs. C57BL/6 mice.

Body Water Balance and Blood Chemistry. Because our initial observation suggested the presence of polyuria in the triply $NOS^{-/-}$ mice, we next investigated body water balance. Urinary volume (Fig. 3*A*), water intake (Fig. 3*B*), and plasma osmolality (Fig. 3*C*) were all significantly increased in accordance with the number of disrupted NOS genes, whereas urinary osmolality was significantly decreased in the same manner (Fig. 3*D*). The triply $NOS^{-/-}$ mice exhibited marked hypotonic polyuria (Fig. 3 *A* and *D*), polydipsia (Fig. 3*B*), and dehydration (Fig. 3*C*) as compared with the wild-type mice. Urinary Na^+ concentrations were significantly lower in accordance with the number of disrupted NOS genes (Table 1), and urinary glucose was undetected in any type of mice. Plasma concentrations of urea nitrogen were significantly higher, whereas those of creatinine tended to be higher, in accordance with the number of disrupted NOS genes (Table 1). By contrast, plasma concentrations of Ca^{2+} and K^+ were comparable among the 9 groups (Table 1).

Vasopressin Release and Renal Sensitivity to Vasopressin. Because the presence of diabetes insipidus was noted in the triply $NOS^{-/-}$ mice, we next examined whether diabetes insipidus is of central or nephrogenic origin. In triply $NOS^{-/-}$ mice, central vasopressin release (assessed by urinary vasopressin excretion) was unchanged (Fig. 3*E*), whereas renal responsiveness to the antidiuretic hormone was markedly reduced (Fig. 3*F*).

Histology. Renal tubular apoptosis and regeneration (increased cell number) (Fig. 4*A*), glomerulosclerosis (Fig. 4*B*), and glomerular thrombus formation (Fig. 4*C*) were noted in the triply $NOS^{-/-}$ mice but not in the wild-type mice (Fig. 4). Quantitative analysis revealed that those abnormalities were exacerbated in accordance with the number of disrupted NOS genes ($n = 6$ each; data not shown). Renal tubular lesions were observed predominantly in distal and collecting tubules than in proximal tubules. We examined whether similar apoptotic change or thrombus formation could also be observed in organs other than the kidney in the triply $NOS^{-/-}$ mice. However, no remarkable lesions were noted in other organs, including the brain, lung, liver, pancreas, and spleen ($n = 5$ each). In contrast, enlargement of the stomach with pyloric sphincter hypertrophy was found in three of five triply $NOS^{-/-}$ mice.

To elucidate the cause of death in the triply $NOS^{-/-}$ mice, we examined the pathology of 10 triply $NOS^{-/-}$ mice that died during the follow-up period. We were unable to examine two mice because of postmortem changes. In the remaining eight mice, one mouse had myocardial infarction, and three mice showed severe renal tubuloglomerular lesions, apparently because of nephrogenic dia-

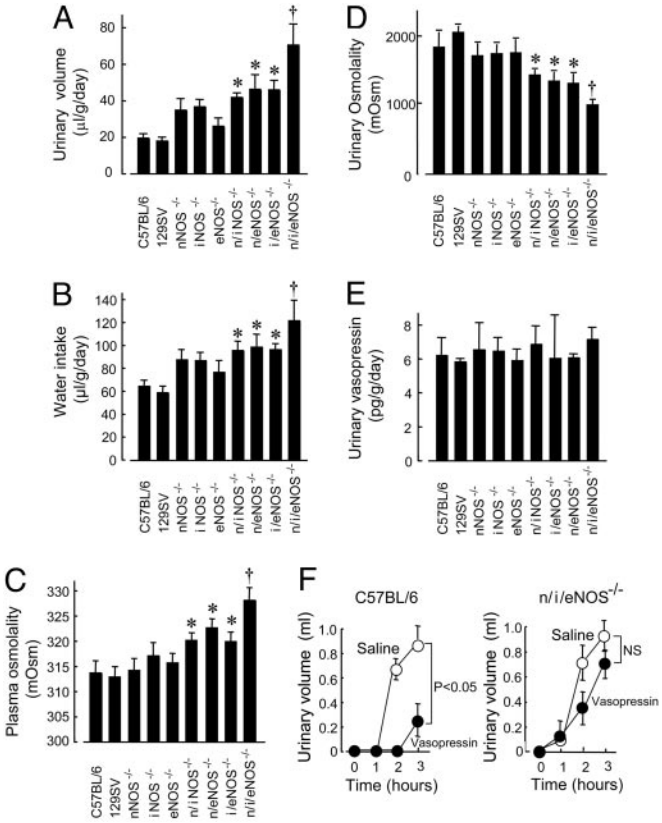

Fig. 3. Body water balance, osmolality, and vasopressin kinetics in triply $NOS^{-/-}$ mice. (*A*) Urinary volume ($n = 6$–13). *, $P < 0.05$; †, $P < 0.01$; compared with C57BL/6 mice. (*B*) Water intake ($n = 6$–13). *, $P < 0.05$; †, $P < 0.01$; compared with C57BL/6 mice. (*C*) Plasma osmolality ($n = 6$–13). *, $P < 0.05$; †, $P < 0.01$; compared with C57BL/6 mice. (*D*) Urinary osmolality ($n = 6$–13). *, $P < 0.05$; †, $P < 0.01$; compared with C57BL/6 mice. (*E*) Urinary vasopressin excretion ($n = 6$ each). (*F*) Changes of urinary volume in response to exogenously administered vasopressin ($n = 6$ each). Open circles, saline i.p.; closed circles, vasopressin i.p. (0.002 unit).

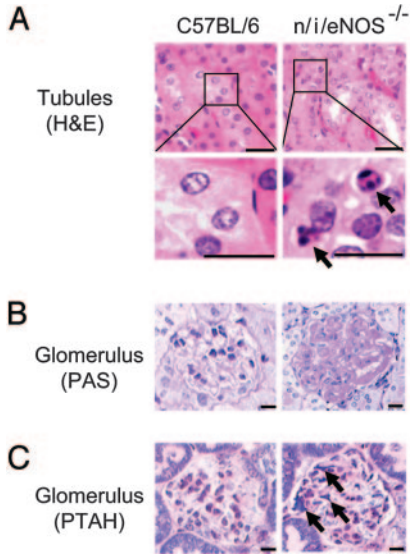

Fig. 4. Histopathology in the kidney of triply $NOS^{-/-}$ mice. (*A*) Renal tubular apoptosis (arrows) and regeneration (increased cell number) in triply $NOS^{-/-}$ mice. H&E, hematoxylin and eosin staining. (Scale bar, 10 μm.) (*B*) Renal glomerulosclerosis (hyalinization, pink color) in triply $NOS^{-/-}$ mice. PAS, periodic acid-Schiff staining. (Scale bar, 10 μm.) (*C*) Renal glomerular thrombi (arrows, dark blue) in triply $NOS^{-/-}$ mice. PTAH, phosphotungstic acid-hematoxylin staining. (Scale bar, 10 μm.)

betes insipidus. The other two mice had pulmonary and liver congestion and acute renal tubular necrosis, compatible with acute circulatory failure (e.g., sudden cardiac death). The remaining two mice had no remarkable histological findings. In all of the dead triply $NOS^{-/-}$ mice, wall thickening, perivascular fibrosis, and adventitial mast cell infiltration of the coronary arteries were noted, suggesting an involvement of coronary vasospasm-induced myocardial ischemia.

Renal NOS Expression and Activity. Western blot analysis showed expression of all three NOS proteins in the kidney of the wild-type mice ($n = 5$ each). Immunostaining revealed that nNOS and eNOS were localized mainly in renal tubules, collecting ducts, and glomeruli, whereas iNOS was found predominantly in renal tubules and collecting ducts. Total renal NOS activity was decreased in accordance with the number of disrupted NOS genes ($n = 5$ each; data not shown). NOS expression and activity were totally absent in the kidney of those mice.

cAMP Level and Aquaporin Expression in Renal Collecting Duct. To elucidate the molecular mechanisms for nephrogenic diabetes insipidus in the triply $NOS^{-/-}$ mice, we examined cAMP levels and aquaporin-2 water channel expression in renal collecting duct. To examine whether functional abnormalities, independent of structural abnormalities, are also involved in the pathogenesis of nephrogenic diabetes insipidus, we used 1-week-old triply $NOS^{-/-}$ mice in which no renal lesions were yet developed. Basal cAMP levels in collecting ducts were comparable between the wild-type and the triply $NOS^{-/-}$ mice (Fig. 5*A*). By contrast, vasopressin-induced cAMP production in collecting ducts was significantly lower in the triply $NOS^{-/-}$ than in the wild-type mice (Fig. 5*A*). Western blot analysis revealed that expression levels of aquaporin-2 in kidney membrane fraction were significantly decreased in the triply $NOS^{-/-}$ mice as compared with the wild-type mice (Fig. 5*B*). Immunostaining also demonstrated reduced expression of aquaporin-2 in the apical plasma membrane of collecting duct in those mice (Fig. 5*C*).

Renal cGMP and Prostacyclin Levels. Renal cGMP levels were significantly lower in the 1-week-old triply $NOS^{-/-}$ than in the wild-type mice (4.9 ± 0.7 vs. 9.4 ± 1.0 fmol per mg of tissue; $P < 0.05$, $n = 5$–6). Renal prostacyclin levels (measured as 6-keto-prostaglandin $F_{1\alpha}$) were significantly higher in the 1-week-old triply $NOS^{-/-}$ than in the wild-type mice (98 ± 10 vs. 60 ± 8 ng per mg of tissue; $P < 0.05$, $n = 5$–6).

Discussion

The findings of the present study were as follows: (*i*) mice deficient in all three NOS isoforms were successfully developed; (*ii*) the triply $NOS^{-/-}$ mice had markedly reduced survival and fertility rates and showed polyuria, polydipsia, and failure of renal responsiveness to vasopressin; and (*iii*), in the kidney of those mice, reduced cAMP production, defective membrane aquaporin-2 expression, and tubuloglomerular lesion formation were noted. These results provide evidence that the complete deletion of the whole NOS system causes the development of nephrogenic diabetes insipidus in mice *in vivo*.

NO has been implicated in many biological phenomena (1–6). Therefore, when we started this project, we expected that triply $NOS^{-/-}$ mice could be embryo-lethal, which, however, was not the case. The triply $NOS^{-/-}$ mice were viable and devoid of all NOS protein expression and total NOS enzymatic activity. Because alternatively spliced forms of nNOS and iNOS have been identified (29), we paid special attention to the possible remaining NOS

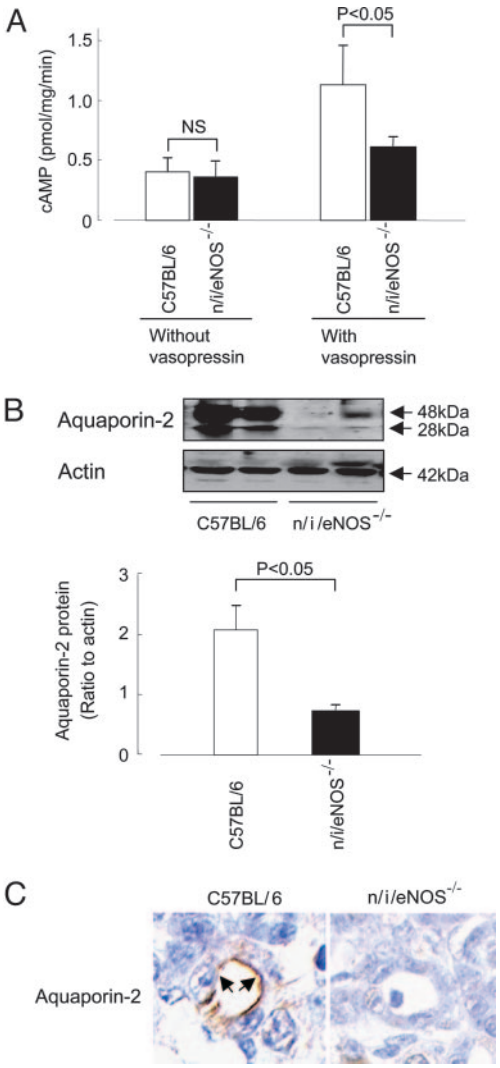

Fig. 5. cAMP level and aquaporin-2 expression in renal collecting duct of triply $NOS^{-/-}$ mice. (*A*) Basal and vasopressin-stimulated (1 μM; for 20 min) cAMP production in collecting duct ($n = 6$ each). NS, not significant. (*B*) Western blot analysis for aquaporin-2 (48 and 28 kDa) in kidney membrane fraction ($n = 6$ each). Actin served as an internal control. (*C*) Immunostaining for aquaporin-2 in renal collecting duct (arrows, brown staining). (Scale bars, 10 μm.)

expression and function. However, we did not detect any expression or enzymatic activity of nNOS or iNOS in the brain, the heart, or the kidney of triply $NOS^{-/-}$ mice either before or after administration of LPS. Thus, the NOS system is completely disrupted in our triply $NOS^{-/-}$ mice.

We next investigated whether systemic NO production was also completely defective in the triply $NOS^{-/-}$ mice. As expected, plasma NOx concentrations and urinary NOx excretion, markers of systemic NO production, were extremely low (≈3% of normal value) in the triply $NOS^{-/-}$ mice, indicating that most of endogenous NO is derived from NOSs. The remaining NO production in the triply $NOS^{-/-}$ mice could be due to a NOS-independent mechanism. Indeed, bacteria, especially enterobacterium, synthesize NOx by means of active nitric acid metabolism, and myoglobin in turn generates NO from NOx (30). In addition, catalase could also produce NO from hydroxylamine or sodium azide (31).

The triple $NOS^{-/-}$ mice were alive, indistinguishable from wild-type mice in phenotype, and were able to mate. However, the survival and fertility rates were markedly reduced in the triply $NOS^{-/-}$ mice compared with the wild-type mice, suggesting an essential role of the NOS system in survival and fertility. In this study, the survival and fertility rates were significantly decreased in accordance with the number of disrupted NOS genes, suggesting that each NOS isoform individually plays an important role in survival and fertility.

Given the location of nNOS, iNOS, and eNOS in the cardiovascular system, NO produced by each NOS isoform could alter blood pressure (32). We thus performed a noninvasive measurement of hemodynamic variables under conscious conditions. The triply $NOS^{-/-}$ mice were significantly hypertensive as compared with the wild-type mice. However, the degree of hypertension in the triply $NOS^{-/-}$ mice was similar to that in the eNOS gene-disrupted singly and doubly $NOS^{-/-}$ mice. These results suggest that hypertension is a common characteristic of eNOS gene disruption and is caused by the lack of endothelium-derived NO with the resultant increase in peripheral vascular resistance (32). Heart rate was significantly lower in the triply $NOS^{-/-}$ than in the wild-type mice, and the degree of bradycardia in the triply $NOS^{-/-}$ mice was also equivalent to that in the eNOS gene-disrupted singly and doubly $NOS^{-/-}$ mice, indicating that bradycardia also is a common phenotype of eNOS gene deletion. Although there is no conclusive explanation for the decreased heart rate in association with eNOS deletion, previous studies revealed that eNOS-derived NO could affect baroreflex resetting or could be involved in establishing the baroreceptor set point (32).

We next examined body water balance in the triply $NOS^{-/-}$ mice. Interestingly, the triply $NOS^{-/-}$ mice showed prominent polyuria, polydipsia, and dehydration as compared with the wild-type mice. Regarding the pathogenesis of the polyuria, osmotic diuresis or water diuresis should be differentiated (33). In the triply $NOS^{-/-}$ mice, the urine was hypotonic, low ionic, and without glucose, excluding osmotic diuresis due to natriuresis and diabetes mellitus. Water diuresis is caused by central or nephrogenic diabetes insipidus or psychogenic polydipsia (33). Psychogenic polydipsia can be excluded because the triply $NOS^{-/-}$ mice had dehydration. Next, to distinguish between central and nephrogenic diabetes insipidus, we examined the release of and the renal sensitivity to vasopressin (33). In the triply $NOS^{-/-}$ mice, renal responsiveness to exogenous vasopressin was markedly attenuated, whereas vasopressin release was unaltered, identifying the presence of the nephrogenic origin of diabetes insipidus in those mice. Nephrogenic diabetes insipidus is classified into two major categories: hereditary (or congenital) and acquired. Acquired nephrogenic diabetes insipidus is induced by electrolyte abnormalities (e.g., hypercalcemia or hypokalemia) or renal insufficiency of any cause (34–36). In the triply $NOS^{-/-}$ mice, plasma Ca^{2+} and K^{+} concentrations were normal, and renal tubular apoptosis and regeneration, glomerulosclerosis, and glomerular thrombi were noted. These results suggest that the deletion of the NOS system is the primary cause of renal structural changes that result in nephrogenic diabetes insipidus.

Polyuria, polydipsia, dehydration, and renal pathological changes were all exacerbated in accordance with the number of disrupted NOS genes. Total renal NOS activity as well as plasma and urinary NOx levels also were decreased in the same manner. We confirmed the expression of all three NOS isoforms in the kidney of wild-type mice but not in the kidney of triply $NOS^{-/-}$ mice. It is therefore possible that the observed abnormalities in the kidney of $NOS^{-/-}$ mice are caused primarily by impaired NO production.

The extent of tubular ischemic injury by glomerular thrombi is more severe in proximal tubules than in distal or collecting tubules. However, tubular lesions in the triply $NOS^{-/-}$ mice were detected predominantly in distal and collecting tubules rather than in proximal tubules. It is therefore conceivable that the tubular lesions are not caused by glomerular thrombi-induced ischemia. Although we checked whether thrombus formation could also be found in other organs of triply $NOS^{-/-}$ mice, it was not the case. Thus, it is possible that the kidney is more supersensitive to defective NO production and that the dysfunction in other organs becomes more

evident in later stages. The possible abnormalities of other organs remain to be examined in future studies.

Under physiological conditions, vasopressin stimulates adenylate cyclase, increases cAMP production, and activates cAMP-dependent protein kinase by means of V_2 receptor in renal collecting duct principal cells. Phosphorylation of aquaporin-2 by the kinase in turn leads to translocation of aquaporin-2 from cytoplasmic vesicles to the apical plasma membrane, thereby increasing water permeability and reabsorption (37, 38). Nephrogenic diabetes insipidus is characterized by an inability to concentrate urine despite normal or elevated plasma concentrations of vasopressin. In inherited nephrogenic diabetes insipidus, the renal response to vasopressin is impaired by abnormalities of the vasopressin V_2 receptor or those of the vasopressin-regulated water channel, aquaporin-2 (35, 37–39). To further clarify the mechanism involved, we examined cAMP accumulation and localization of aquaporin-2 in collecting ducts of 1-week-old, triply $NOS^{-/-}$ mice before the development of structural abnormalities. Vasopressin-stimulated cAMP accumulation, but not basal cAMP accumulation, was significantly reduced in collecting ducts of the triply $NOS^{-/-}$ mice, as compared with wild-type mice. Furthermore, expression of aquaporin-2 in collecting duct plasma membrane was more significantly decreased in the triply $NOS^{-/-}$ than in the wild-type mice. These results suggest that reduced cAMP production and decreased aquaporin-2 expression are also involved in the pathogenesis of nephrogenic diabetes insipidus in the triply $NOS^{-/-}$ mice. Consistent with our notion, previous studies revealed that NO stimulates cAMP production by means of cGMP-dependent activation of adenylate cyclase in isolated rat kidney (40, 41) and that NO promotes the membrane insertion of aquaporin-2 in collecting duct (37, 42). In this study, renal prostacyclin levels were significantly higher in the triply $NOS^{-/-}$ than in the wild-type mice, suggesting that the increased prostacyclin levels may compensate for the loss of NO/cGMP pathway and may account for the normal basal cAMP levels in collecting ducts of the triply $NOS^{-/-}$ mice. Importantly, the occurrence of functional abnormalities preceded that of structural abnormalities. Thus, the triply $NOS^{-/-}$ mice appear to possess the feature of inherited, rather than acquired, nephrogenic diabetes insipidus.

The severity of nephrogenic diabetes insipidus in the triply $NOS^{-/-}$ mice is moderate as compared with aquaporin-2 knock-in mutant mice, which died by day 6 after birth with renal failure, with urine and serum osmolalities of 221 ± 9 and 450 ± 50 milliosmol, respectively (43), and with vasopressin V_2 receptor$^{-/-}$ mice, which died during the first week after birth because of hypernatremic dehydration, with urine osmolality of 235 ± 12 milliosmol (44). Because urinary osmolality of the triple $NOS^{-/-}$ mice was reduced to ≈1,000 milliosmolal, NO may be a physiological way to further increase urine osmolality in response to vasopressin. NO may also protect the ischemic medulla during dehydration and intense water reabsorption.

In patients with inherited nephrogenic diabetes insipidus, ≈90% are males with X-linked recessive inheritance with an incidence of 8.8 per 1 million who have mutations and dysfunction of the vasopressin V_2 receptor (34–36, 45). Most of the remaining 10% of the patients have an autosomal-recessive or autosomal-dominant mode of inheritance and mutations and dysregulation of the vasopressin-sensitive aquaporin-2 (34–36, 45). Although there are few studies addressing the prognosis of untreated patients with inherited nephrogenic diabetes insipidus, mental and physical retardation and renal dysfunction are frequently observed (34–36, 45), which could cause poor prognosis. Indeed, the triply $NOS^{-/-}$ mice had significantly reduced survival rate, and postmortem examination indicated that the disorder was the direct cause of death in at least three (38%) of eight triply $NOS^{-/-}$ mice that died during the study.

In conclusion, we were able to demonstrate that the deletion of the whole NOS system causes nephrogenic diabetes insipidus associated with increased mortality, demonstrating a pivotal role of NO in maintaining homeostasis, especially in the kidney.

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