Salt Sensitivity

Inhibition of Nitric Oxide Synthase 1 Induces Salt-Sensitive Hypertension in Nitric Oxide Synthase 1α Knockout and Wild-Type Mice

Ximing Wang, Kiran Chandrashekar, Lei Wang, En Yin Lai, Jin Wei, Gensheng Zhang, Shaohui Wang, Jie Zhang, Luis A. Juncos, Ruisheng Liu

Abstract—We recently showed that α, β, and γ splice variants of neuronal nitric oxide synthase (NOS1) expressed in the macula densa and NOS1β accounts for most of the NO generation. We have also demonstrated that the mice with deletion of NOS1 specifically from the macula densa developed salt-sensitive hypertension. However, the global NOS1 knockout (NOS1KO) strain is neither hypertensive nor salt sensitive. This global NOS1KO strain is actually an NOS1αKO model. Consequently, we hypothesized that inhibition of NOS1β in NOS1αKO mice induces salt-sensitive hypertension. NOS1αKO and C57BL/6 wild-type (WT) mice were implanted with telemetry transmitters and divided into 7-nitroindazole (10 mg/kg/d)–treated and nontreated groups. All of the mice were fed a normal salt (0.4% NaCl) diet for 5 days, followed by a high-salt diet (4% NaCl). NO generation by the macula densa was inhibited by >90% in WT and NOS1αKO mice treated with 7-nitroindazole. Glomerular filtration rate in conscious mice was increased by ≈40% after a high-salt diet in both NOS1αKO and WT mice. In response to acute volume expansion, glomerular filtration rate, diuretic and natriuretic response were significantly blunted in the WT and knockout mice treated with 7-nitroindazole. Mean arterial pressure had no significant changes in mice fed a high-salt diet, but increased ≈15 mm Hg similarly in NOS1αKO and WT mice treated with 7-nitroindazole. We conclude that NOS1β, but not NOS1α, plays an important role in control of sodium excretion and hemodynamics in response to either an acute or a chronic salt loading. (Hypertension. 2016;67:792-799. DOI: 10.1161/HYPERTENSIONAHA.115.07032.) • Online Data Supplement

Key Words: 7-nitroindazole ▪ arterial pressure ▪ hypertension ▪ sodium ▪ telemetry

Hypertension affects >25% of the American adults and is a major risk factor for cardiovascular morbidity and mortality. More than half of hypertensive patients are salt sensitive and exhibit a significant rise in blood pressure when salt intake is elevated. Abundant evidence from numerous studies both in human and in experimental animal models indicates the significance of kidney in the development of salt-sensitive hypertension. However, the renal mechanisms for salt sensitivity have not been fully elucidated. Increases in glomerular filtration rate (GFR) in response to salt loading may play a vital role in rapid elimination of sodium to maintain salt–water balance. This GFR response is blunted or blocked in human and animal models with salt-sensitive hypertension, but the underlying mechanism is unclear. GFR is normally regulated by tubuloglomerular feedback (TGF). Increases in tubular flow initiate a TGF response, mediated by increased NaCl delivery to the macula densa. This promotes the release of adenosine or ATP, which constricts afferent arterioles and reduces single nephron GFR. Flow and salt delivery in the distal nephron are thus restored. If the increased flow at the macula densa persists, the TGF curve will shift to right; therefore, TGF functions at a higher operating point (higher flow rate) to permit elevation of GFR. The mechanisms responsible for TGF modulation remain to be determined.

NO is one of the most important factors that modulate TGF responsiveness. Three isoforms of nitric oxide synthases (NOS), neuronal NOS (NOS1), inducible NOS (iNOS/NOS2), and endothelial NOS (eNOS/NOS3) exist in mammals. They are all expressed in the juxtaglomerular apparatus of the kidneys. NOS1 is abundantly expressed in the macula densa. NO generated by NOS1 in the macula densa inhibits TGF response. Long-term blockade of NOS1 by 7-nitroindazole (7-NI) leads to hypertension in SD rats and causes salt-sensitive hypertension in Dahl salt-resistant hypertensive rats. 17,18,23,24

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rats, underlining the significance of NOS1 in controlling salt–water balance and blood pressure. However, studies on global NOS1 knockout (NOS1KO) mice have shown that these animals are normotensive, even on a high-salt diet. This potential discrepancy can be partially explained by our recent findings. We have shown that 3 splice variants of NOS1 exist in the macula densa, namely α, β, and γ; among these, NOS1β is the major splice variant and accounts for most of the NO generated by the macula densa. We have also demonstrated the significance of TGF responsiveness in long-term control of sodium excretion and blood pressure by using a tissue-specific knockout mouse strain, in which NOS1 has been specifically deleted from the macula densa. These knockout mice develop salt-sensitive hypertension, associated with enhanced TGF responsiveness and low GFR in response to an acute salt loading. In addition, the global NOS1KO model targets exon-2 and deletes only the NOS1α isoform with an intact NOS1β splice variant. Therefore, we will call this strain NOS1αKO in this study. These mice do not develop hypertension, further suggesting that inhibition of NOS1β in NOS1αKO mice induces salt-sensitive hypertension. In this study, we administered 7-NI to NOS1αKO mice and then measured their blood pressure. In addition, we also tested a hypothesis that NOS1α does not play a significant role in response to an acute sodium load. We determined the significance of NOS1α in control of sodium excretion and renal hemodynamics by comparing kidney clearance function between NOS1αKO and wild-type (WT) mice in response to acute volume expansion. Current pharmacological study further expanded our understanding of the significance of NOS1 in control of volume homeostasis and blood pressure.

Methods

All procedures and experiments were approved by the Institutional Animal Care and Use Committee at the University of South Florida College of Medicine and the University of Mississippi Medical Center. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except as indicated.

Transmitter Implantation and Mean Arterial Pressure Measurement

Similar methods were used as we previously described (see the online-only Data Supplement).

Animal Groups and Treatment

The C57BL/6 and NOS1αKO mice were divided into 7-NI–treated and nontreated groups. Mean arterial pressure (MAP) was measured for 5 days in all the mice fed a normal salt diet (0.4% NaCl), followed by 7 days of a high-salt diet (4% NaCl; days 6–12). From day 13, in addition to the high-salt diet, the mice in 7-NI–treated groups were given 7-NI (10 mg/kg per day) in drinking water as reported for 16 more days. The nontreated groups were maintained on a high-salt diet without 7-NI in drinking water.

GFR Measurement in Conscious Mice

We used a single bolus injection of fluorescein isothiocyanate (FITC)–inulin similar to a previously published method but with modifications for measurement of GFR in conscious mice (see the online-only Data Supplement).

Renal Clearance in Response to Isotonic Volume Expansion

At the end of 7-NI treatment or high-salt diet, kidney clearance function was measured as we described recently (see the online-only Data Supplement).

Measurement of NO in Isolated Perfused Macula Densa

We measured NO production by the macula densa using a fluorescent NO indicator 4-amino-5-methylamino-2′,7′-dichlorofluorescein diacetate in isolated perfused juxtaglomerular apparatus as we described previously (see the online-only Data Supplement).

Isolation of Macula Densa Cells

Laser capture microdissection was used to isolate macula densa cells from frozen kidney slices, as we previously described (see the online-only Data Supplement).

Real-Time Polymerase Chain Reaction

RNA and quantitative polymerase chain reaction analyses were performed similarly as we previously described (see the online-only Data Supplement).

Western Blot to Measure Splice Variants of NOS1

Splice variants of NOS1 were measured in renal cortical with Western blot as we described previously (see the online-only Data Supplement).

Statistical Analysis

Data are presented as mean±SEM unless specified. We tested only the effects of interest, using ANOVA for repeated measures and a post hoc Fisher least significant difference test or a Student paired t test when appropriate. The changes were considered to be significant if P<0.05.

Results

Expressions of NOS1 Splice Variants in the Macula Densa

We compared mRNA expressions of NOS1 splice variants in the macula densa using laser capture microdissection and real-time polymerase chain reaction in normal WT and NOS1αKO mice. We found no significant difference in NOS1β mRNA levels between WT (71.6±4.7 AU) and NOS1αKO mice (75.4±5.9 AU). NOS1β expressions were significantly higher than NOS1α in both strains (P<0.01), whereas NOS1α was undetectable in NOS1αKO mice (n=6; Figure 1).

The protein levels of NOS1 splice variants in renal cortex were measured by Western blot using a C-terminal NOS1 antibody that can detect all the splice variants of NOS1. As shown in Figure 1B and 1C, NOS1α was the primary splice variant expressed in the brain, whereas NOS1β was the major splice variant in renal cortex. NOS1β protein levels in the renal cortex were 580±25 AU in WT mice and 596±34 AU in NOS1αKO mice (P<0.01 versus NOS1α). There was no significant difference between them. NOS1α was undetectable in NOS1αKO mice (n=5 per group).

NO Generation by the Macula Densa

To determine whether there was any difference in NO generation by the macula densa between NOS1αKO and WT mice, we compared TGF-induced NO generation by the macula densa in isolated perfused juxtaglomerular apparatus in
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normal WT and NOS1αKO mice fed a normal salt diet. The NO generation by the macula densa in WT mice increased by 40.6±3.8% (from 86.4±7.5 to 121.5±12.3 U/min) in response to an increase in tubular NaCl concentration from 10 to 80 mmol/L, a maneuver that initiates a TGF response. TGF-induced NO generation increased by 47.5±5.1% (from 81.2±8.7 to 119.8±13.9 U/min) in NOS1αKO mice. There was no significant difference in the NO generation between WT and NOS1αKO mice (n=5; Figure 2A).

To determine whether 7-NI inhibits NO generation by the macula densa, we repeated the above experiments at the end of 7-NI treatment. The TGF-induced NO generation by the macula densa was reduced to 9.4±2.1% in WT mice (n=4) and 7.8±3.5% in NOS1αKO mice (n=4). In the WT mice without 7-NI treatment, TGF-induced NO generation was 54.2±6.5% (P<0.01 versus 7-NI–treated animals, n=5; Figure 2B).

GFR Measurement in Conscious Mice

To determine whether high-salt diet and 7-NI treatment had any effect on GFR, we measured GFR in conscious animals in WT and NOS1αKO mice after 7-NI or high-salt diet treatment. GFR was increased by ≈40% after a high-salt diet in both NOS1αKO and WT mice (P<0.01 versus normal salt diet). There were no significant differences in GFR among all groups during the first period of high-salt diet or second period of high-salt diet, with and without 7-NI treatment (Figure 3, n=8 per group).

Kidney Clearance Function in Response to Acute Volume Expansion in NOS1αKO and WT Mice

To evaluate the significance of NOS1α and further determine whether inhibition of NOS1 affects renal hemodynamics and sodium excretion in response to acute volume expansion, we measured kidney clearance function by intravenous infusion of saline in WT and NOS1αKO animals at the end of 7-NI or high-salt diet treatment. The baseline GFR was similar in the WT and knockout mice. GFR rose by ≈60% (P<0.01 versus basal) in WT and knockout mice without 7-NI treatment during 60 minutes after acute volume expansion. In contrast, GFR increased <40% in the animals treated with 7-NI (P<0.05 versus WT without 7-NI, Figure 4A). Urinary flow...
rate and sodium excretion were similar in WT and knockout mice in basal and increased significantly in all group of animals in the first hour after acute volume expansion. However, the diuretic and natriuretic response were significantly blunted in the WT and knockout mice treated with 7-NI (Figure 4B and C, *P* < 0.05 versus WT without 7-NI, n=5 per group).

Changes in Blood Pressure in Response to a High-Salt Intake Plus 7-NI in NOS1αKO and WT Mice

To determine if inhibition of NOS1 promotes the development of salt-sensitive hypertension, we compared changes in MAP measured by telemetry in WT and NOS1αKO mice. Baseline MAP measured on the normal salt diet averaged 91.7±4.5 mm Hg in all group of animals. After switching to a first period of high-salt diet, the MAP of the mice did not change significantly. The MAP of mice maintained a second period of high-salt diet and treated with 7-NI increased to 16.1±3.5 mm Hg in WT mice (n=5) and 14.7±3.1 mm Hg in NOS1αKO mice (*P* < 0.01 versus basal, n=6), whereas it was not significantly altered in mice without 7-NI (Figure 5, *P* < 0.01 versus 7-NI treated groups, n=5).

Discussion

This study demonstrated that inhibiting NOS1 with 7-NI promoted salt sensitivity of blood pressure to an equal extent in both the NOS1αKO and the WT mice because the expression levels and activity of the NOS1β splice variant in the macula densa are intact in NOS1αKO mice. In response to an acute volume expansion, the diuretic and natriuretic response were blunted in both WT and NOS1αKO mice treated with 7-NI. NOS1α did not play a significant role in control of sodium excretion and renal hemodynamics, whereas NOS1β dominated the function of NOS1 in control of salt sensitivity of blood pressure.

Alternative 5′-end splicing of NOS1 mRNA results in at least 3 different N-terminal NOS1 protein variants, which are NOS1α at ≈155 kDa, NOS1β at ≈145 kDa, and NOS1γ at ≈125 kDa. NOS1α exhibits full enzymatic activity, NOS1β has ≈80%, whereas NOS1γ has only ≈2% catalytic activity compared with that of NOS1α. The predominant splice variant in brain is NOS1α, which accounts for >95% of NOS1 activity. In the kidney, splice variants of NOS1 have been found in both cortex and medulla. NOS1 is a predominant isoform expressed in the macula densa cells. Recently, we found that macula densa expresses α, β, and γ splice variants of NOS1. Mice with deletion of NOS1 specifically from the macula densa developed salt-sensitive hypertension. This previous study using the macula densa–specific knockout model clearly demonstrated the significance of NOS1 in the macula densa and TGF response in long-term control of volume homeostasis.
we also investigated whether NOS1α plays an important role in control of sodium excretion and renal hemodynamics in response to an acute salt loading.

Similar to our previous findings,31 we confirmed in this study that NOS1β was the primary splice variant expressed in the macula densa and accounts for most of the NO generated by the macula densa. We found that a high-salt diet enhanced NO generation by the macula densa to a similar level in both NOS1αKO and WT mice. These data provided additional evidence indicating NOS1β is a salt-sensitive splice variant. In addition, we observed that a high-salt diet enhanced NOS1β activity as we previously reported.30,31

Our findings about salt intake and NO generation were in agreement with previous studies. Rats on a high-salt diet had higher plasma levels, increased renal excretion rates of nitrite/nitrates,50–53 and increased cGMP levels,50 suggesting that NO activity was higher during high-NaCl intake. Inhibition of NOS1 in vitro augmented TGF responses to a greater extent in animals on a high-salt diet,23,54 whereas inhibition of NOS1 in vivo had a greater effect on renal blood flow, GFR, and renal vascular resistance in animals fed a high-salt diet,30,52,55 also indicating a higher NO generation in response to a high-salt diet. Similar findings have been reported in clinical trials in normal and hypertensive humans. A high-salt diet was associated with an elevation in GFR, renal blood flow, sodium, and cGMP excretion compared with that in a low-salt diet,56,57 and these effects were significantly enhanced after L-arginine administration, suggesting that they were possibly because of the increased NO production.

In this study, we found that the NO generation by the macula densa was inhibited in mice treated with 7-NI, confirming an effective NOS1 inhibition. In response to a salt loading, GFR increased significantly in both conscious NOS1αKO and WT mice. Similar findings have been reported in animals10,13,58–60 and humans,61–65 which is considered as an important mechanism for rapid elimination of a salt load, possibly modulated by TGF responsiveness.31 No differences were found in GFR in mice fed a high-salt diet with or without 7-NI. The reason that 7-NI inhibited NO generation but did not alter GFR may be because of the increased blood pressure in 7-NI–treated mice. For GFR measurement in conscious mice, completely intravenous injection of FITC-inulin without leaking is critical for accurate measurement. We found that injection via penile vein is a easy and reliable way for FITC-inulin injection.

Accurate measurement of salt–water balance in mice on a high-salt diet is notoriously difficult. Therefore, to determine whether chronic inhibition of NOS1 with 7-NI impairs sodium excretion; we measured kidney clearance function in response to an acute volume expansion in anesthetized mice. We found that elevations of GFR in response to an acute volume expansion was significantly blunted in mice treated with 7-NI, for both NOS1αKO and WT mice. The inhibition of GFR increase may be mediated by inhibition of NO generation by the macula densa with 7-NI, which enhances TGF response. Similarly, sodium excretion rate in response to an acute volume expansion was also significantly lower in mice treated with 7-NI. These data indicated that one of the mechanisms underlying the effects of 7-NI...
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was mediated by inhibition of NO in the macula densa, enhancement of TGF responsiveness, and inhibition of GFR increases after salt loading. These data also indicated that NOS1α did not play a significant role in control of sodium excretion and renal hemodynamics.

To determine whether 7-NI–induced impairment of sodium excretion promotes a development of salt-sensitive hypertension, we measured blood pressure in mice fed a high-salt diet plus 7-NI. We found that a high-salt diet did not significantly increase blood pressure in NOS1+KO and WT mice, indicating that neither of the strains are salt sensitive. The findings are agreement with previous studies. However, a high-salt diet plus 7-NI similarly elevated MAP =15 mm Hg in both NOS1−KO and WT mice. These data indicate that inhibition of NOS1 with 7-NI enhanced salt sensitivity, mediated by NOS1β. These data also indicated that NOS1α did not play a significant role in control of sodium excretion and renal hemodynamics.

7-NI shows little isoform selectivity in vitro. Its IC50 is ranging from 0.5 to 0.8 μmol/L similarly for both purified enzymes of NOS1 and NOS3. However, 7-NI shows high selectivity in tissue and in vivo for the NOS1. 7-NI at 100 μmol/L failed to inhibit endothelium-dependent relaxation of the rabbit isolated aorta in response to acetylcholine. In contrast, L-NAME (N-G-nitro-L-arginine methyl ester) at 1.5 and 15 μmol/L produces ≈20% and 70% inhibition of the response to the rabbit aorta to acetylcholine. Several studies have demonstrated that acute intraperitoneal or intravenous administration of 7-NI did not affect MAP in either anesthetized or conscious mice or rats. These studies demonstrated that 7-NI is a highly selective inhibitor for NOS1 in vivo. The mechanisms for the notable differences between in vitro and in vivo effect of 7-NI have not been fully clarified. The basis of selectivity of 7-NI seems to lie in the differential uptake of the inhibitor into cells express NOS1 versus NOS3.

Perspectives

In summary, we found that the NOS1β splice variant was intact in both expression and function in NOS1−KO mice. Increases of GFR and sodium excretion in response to acute salt loadings were blunted in mice treated with 7-NI in both NOS1−KO and WT mice. A high-salt diet did not increase blood pressure, but adding 7-NI elevated blood pressure to a similar level both in NOS1−KO and in WT mice. Taken together, this study demonstrated that NOS1β, but not NOS1α, plays an important role in control of sodium excretion and salt sensitivity of blood pressure.

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What Is New?

• Nitric oxide synthase 1α (NOS1α) does not play an important role in control of sodium excretion and hemodynamics in response to acute salt loading. NOS1β is the primary isoform that regulate sodium excretion and blood pressure.

What Is Relevant?

• We determined the role of NOS1 in control of hypertension.

Summary

NOS1β, but not NOS1α, plays an important role in control of sodium excretion and salt sensitivity of blood pressure.
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Inhibition of NOS1 induces salt-sensitive hypertension
in NOS1α knockout and wild type mice

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Methods:
Transmitter implantation and mean arterial pressure (MAP) measurement: NOS1αKO and C57BL/6 WT mice (25-30 g, male, Jackson Lab) were anesthetized with inhaled isoflurane (Butler chemicals, UK). A small incision was made in the middle of the neck for insertion of the telemetry transmitter (PA-C10, Data Sciences International). The catheter of the transmitter was placed in the left carotid artery and advanced down to the aortic arch. The body of the transmitter was placed subcutaneously in the right ventral flank of the mice, as we previously described. The mice were allowed to recover for 10 days. MAP was measured for 10 sec every minute for 4 hours from 1PM-5PM.

Animal groups and treatment: The C57BL/6 and NOS1αKO mice were divided into 7-NI treated and non-treated groups. MAP was measured for 5 days in all of the mice fed a normal salt diet (0.4% NaCl), followed by 7 days of a high salt diet (4%NaCl; days 6-12). From day 13, in addition to the high salt diet, the mice in 7-NI treated groups were given 7-NI (10mg/kg/day) in drinking water as reported for 16 more days. The non-treated groups were maintained on a high salt diet without 7-NI in drinking water.

GFR measurement in conscious mice: We used a single bolus injection of FITC-inulin, similar to a previously published method but with modifications for measurement of GFR in conscious mice. Dialyzed FITC-inulin (5% in saline, 3.74µL/g body weight) was injected via penile vein. The mice were lightly anesthetized with isoflurane during injection and awakened within a minute. Blood (20 µL) was collected into heparinized capillary tubes at the following times: 3, 7, 10, 15, 35, 55, 75, and 90 minutes by tail vein. The fluorescence of FITC-inulin in the plasma was detected by the plate reader and GFR was calculated with GraphPad Prism.

Renal clearance in response to isotonic volume expansion: Similar methods were used as we described recently. At the end of 7-NI treatment or high salt diet, the mice were anesthetized with ketamine (30 µg/g) and inactin (50 µg/g), and a catheter was placed in the femoral vein for an intravenous infusion of 2% BSA and FITC-inulin (2mg/ml) in a 0.9% NaCl solution at a rate of 0.5 ml/hour. Another catheter was inserted into the left ureter for urine collection. After surgery, urine and plasma were collected during a 30-min period after a 30-min equilibration period. This was followed by a bolus infusion of saline (3% body weight) and the infusion rate was maintained at 0.5 ml/hour with FITC-inulin afterwards. Urine and plasma were collected during a 60-min period and a 60-90 min period after volume expansion. At the end of the experiment, the left kidney was removed and weighed, and the concentration of Na+, and inulin in the urine and plasma sample were determined.

Measurement of NO in isolated perfused macula densa: We measured NO production by the macula densa using a cell permeable fluorescent NO indicator 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) in isolated perfused JGA as we described previously. Briefly, once the thick ascending limb was perfused, the macula densa cells were loaded with 10 µM DAF-FM DA in 0.5% dimethyl sulfoxide plus 0.1% pluronic acid from the tubular lumen for 30 to 40 min, then washed for 10 min. DAF was excited at 490 nm with a xenon light, and the emitted fluorescence was recorded at wavelengths of 510 to 550 nm. Square-shaped regions of interest
(ROIs) were set inside the cytoplasm of macula densa cells and mean intensity within the ROI's was recorded every 5 seconds for 5 minutes. NO production was calculated based on the rate of increase of the intensity of DAF at units/min.

**Isolation of macula densa cells:** Laser capture microdissection (LCM) was used to isolate macula densa cells from frozen kidney slices, as we previously described. Kidneys from mice were removed and snap-frozen in Tissue-Tek Optimal Cutting Temperature Compound (Thermo Fisher Scientific, Waltham, MA). Frozen sections (8 µm) were prepared and stained and dehydrated using an Arcturus Histogene frozen section staining kit (Life Technologies, Carlsbad, CA). The macula densa cells were dissected using a Laser Capture Microdissection System (Arcturus, Grand Island, NY).

**Real-Time PCR:** RNA was extracted from the LCM isolated macula densa cells using a PicoPure RNA isolation kit (Life Technologies, Carlsbad, CA). Quantitative PCR analysis was performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and CFX96 Real-Time Detection System (Bio-Rad, Hercules, CA). The Real-Time PCR reactions contained 1µl of the RT reaction and 0.1µM of the forward and reverse primers in 25µl volume. The cycling conditions were: 1 cycle at 95°C for 3min, followed by 40 cycles at 95°C for 30s, 60°C for 30s, and 72°C for 30s. The relative expression of the NOS1 splice variants was compared using the delta CT method. The following primers were used to amplify the various splice variants: NOS1α-F: 5'-GGCTCGGCAGCAGCTCCAGGTA-3', NOS1α-R: 5'-TCAAGGGTTCACAGGAGACAGC-3'; NOS1β-F: 5'-GGGCTCGGCAGCAGACACCTC-3'. GAPDH was used as a housekeeping gene and the primers sequences were: GAPDH-F: 5'-TGATGACATCAAGAAGGGGAA-3', GAPDH-R: 5'-TCCTTGGAGGCCATGTGGGCC-3'.

**Western blot to measure splice variants of NOS1:** Renal cortical protein extracts (50µg/lane) were separated on a 7.5% SDS-PAGE gel. After blocking for 1 hour at room temperature with 5% skim milk, the membranes were incubated overnight at 4 °C with a C-terminal NOS1 antibody (Mouse polyclonal IgG, 1:3000, Bd Biosciences, San Jose, CA). After incubation of the membranes with horseradish peroxidase conjugated secondary antibody (goat anti-mouse, IgG, 1:300000, Bio-Rad, Hercules, CA), the immunoreactive bands were revealed by enhanced chemiluminescence detection on Hyperfilm (Amersham Pharmacia Biotech, Piscataway, NJ) and normalized by β-actin.
Supplementary Reference List


