Nitric Oxide in Renal Cortex and Medulla
An In Vivo Microdialysis Study

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Abstract This study examined the production of nitric oxide (NO) in the renal cortex and medulla through the use of an in vivo microdialysis technique. Oxyhemoglobin (OxyHb) at a concentration of 3 μmol/L was perfused through the dialysis system to trap tissue NO Methemoglobin (MetHb), which was formed by NO oxidation of OxyHb in the dialysate, was spectrophotometrically assayed at 401 nm. Because the oxidation of OxyHb to produce MetHb is stoichiometric with NO, the production of NO can be determined by the rate of MetHb formation. We found that NO concentration was significantly higher (P < 0.05) in the medulla (57 ± 5.8 μmol/L, n = 10) than in the cortex (31.2 ± 5.7 μmol/L, n = 9). The minimal detectable NO level of this assay is ~10 μmol/L. Intravenous infusion of L-arginine (3 mg/kg per minute) for 30 minutes produced a twofold to threefold increase in cortical and medullary NO. No-nitro-L-arginine methyl ester (L-NAME) (10 μg/kg per minute) decreased NO by 33% in the renal cortex and by 46% in the renal medulla. We have also determined that NO concentration was significantly higher (P < 0.05) in the medulla (57.1 ± 5.57 μmol/L, n = 10) than in the cortex (31.2 ± 5.7 μmol/L, n = 9). The minimal detectable NO level of this assay is ~10 μmol/L. Intravenous infusion of L-arginine (3 mg/kg per minute) for 30 minutes produced a twofold to threefold increase in cortical and medullary NO. No-nitro-L-arginine methyl ester (L-NAME) (10 μg/kg per minute) decreased NO by 33% in the renal cortex and by 46% in the renal medulla. We have also determined that NO concentration is higher in medullary tissue than in the cortex (Hypertension. 1997;29[part 2]:194-198).

Key Words • spectrophotometry • rats • nitric oxide • microdialysis • kidney

There is evidence that NO formation may play an important role in the regulation of renal function. Recently, we have found that endogenous NO participates in the control of renal medullary blood flow, water and sodium excretion, and arterial blood pressure. Chronic inhibition of endogenous NO in the renal medulla markedly decreased medullary blood flow and sodium excretion and consequently resulted in an increase of arterial blood pressure in rats. Measurement of cGMP indicated that NO production in the renal medulla is greater than in the renal cortex. Western blot analysis demonstrated a greater expression of NOS in the renal medulla. However, direct measurement of NO concentration in various regions of the kidney has not been carried out, and because NO is the final effector stimulus in this pathway, it is important to be able to measure its concentration in the regions of functional interest. Here we report the use of an in vivo microdialysis hemoglobin-trapping technique to determine NO levels in the renal medulla and cortex and to examine the effect of L-arginine and its analogue on renal cortical and medullary levels of NO.

Methods

In Vivo Microdialysis

In vivo microdialysis studies were performed as described by Baranowski and Westenfelder. Briefly, the rats (Sprague-Dawley, weight, 290 to 310 g) were anesthetized with ketamine (30 mg/kg IM) and Inactin (40 mg/kg IP), and body temperature was maintained at 37°C. The trachea, femoral artery, and vein were cannulated. We exposed and immobilized the left kidney by placing it dorsal side up in a kidney cup. A small channel was made in the renal capsule and cortex with a 23-gauge needle, after cessation of bleeding, the microdialysis probe with a 0.5-mm-tip diameter and a 20-KD transmembrane diffusion cutoff (L-NAME) (10 μg/kg per minute) was inserted into the renal medulla (5 mm in depth). The cortical probe was then perfused at a rate of 2 μL/min with PBS containing NaH2PO4 50 mmol/L, NaH2PO4 50 mmol/L, NaCl 145 mmol/L, and OxyHb 0.003 mmol/L (pH 7.4). The medullary probe was perfused at the same perfusion rate with the same solution except that NaCl of a greater osmolality was used (NaCl 500 mmol/L). During an 1-hr equilibration period, the animal received an intravenous infusion of 2% bovine serum albumin in a 0.9% NaCl at a rate of 1 mL/hr per 100 g body wt, while microdialysis probes were perfused continuously at a rate of 2 μL/min. This equilibration time was to allow the tissue to recover from the insertion of the microdialysis probe. After the equilibration period, dialysate fluid was collected at 30-minute intervals for a 1-hour control measurement period. L-arginine was infused intravenously at a dose of 3 mg/kg per minute for 2 hours (n = 6 rats), and the dialysate was collected after 30 minutes of L-arginine infusion. The dose of L-arginine was based on that used in a previous study, in which L-arginine markedly increased urinary nitrite excretion and improved renal function. These dialysate samples were analyzed to record absorbance spectra of MetHb as described below. MethHb or NO concentration was calculated according to the equation c = A/eb, where c is MetHb or NO concentration, A is absorbance increase at 401 nm, e is extinction coefficient of MetHb, and b is light path in centimeters.

In a separate group of rats, L-NAME was infused intravenously at a dose of 10 μg/kg per minute for 2 hours (n = 6 rats), and the dialysate was collected over 30-minute intervals after a 1-hour control period. This dose of L-NAME has been reported to decrease renal blood flow and increase arterial blood pressure.
Selected Abbreviations and Acronyms

L-NAME = N\textsuperscript{\textregistered}o-nitro-l-arginine methyl ester
MetHb = methemoglobin
NO = nitric oxide
NOS = nitric oxide synthase
OxyHb = oxyhemoglobin
PBS = phosphate-buffered saline
SNP = sodium nitroprusside
SOD = superoxide dismutase

in anesthetized rats\textsuperscript{11} The collected dialysate samples were analyzed as described below.

In another series of experiments, we included SOD (200 U/mL) and catalase (600 U/mL) in the microdialysis perfusate. At these concentrations, they completely blocked the effects of xanthine/xanthine oxidase (a superoxide-producing system) on the spectrum of hemoglobin. These experiments were designed to exclude the possible effect of superoxide on the spectrum of MetHb. The dialysates were collected, and spectrophotometric analysis was performed as described below.

Effect of Renal Medullary Infusion of SNP on NO Concentration

To further confirm that MetHb in the microdialysate is derived from NO-mediated oxidation in renal tissue, the effect of renal medullary infusion of the NO donor SNP on measured MetHb or NO concentration was examined. These experiments were performed on 5 rats surgically prepared as described above. In addition, a small extruded polyethylene catheter with a 100-μm diameter tip was implanted into the interstitium of the renal outer medulla as previously described\textsuperscript{16} to allow for administration of SNP directly into the renal medullary interstitium. After implantation, a 0.9% solution of NaCl as the vehicle control solution for drug delivery was continuously infused at a rate of 0.5 mL/h. Microdialysis probes were implanted into the renal medulla as described above, and dialysate and catalase were included in the microdialysis perfusate. After a 1-hour equilibration period and a 1-hour control collection period, SNP at a concentration of 1 mmol/L (0.5 mL/h) was infused into the renal medulla. After 1-hour infusion, the dialysate was collected for MetHb spectrum analysis.

Spectrophotometry Assay of NO-Induced MetHb Formation

The spectrophotometric hemoglobin-NO trapping technique is based on the rapid oxidation of ferrous OxyHb (Fe\textsuperscript{2+}) to MetHb (Fe\textsuperscript{3+}) by NO. The oxidation of ferrous hemoglobin is stoichiometric with NO and occurs in <100 ms. Because the oxidation of OxyHb to MetHb is characterized by changes in its absorbance that occurred between 401 and 411 nm, the difference in absorbance between 401 and 411 nm can be used to calculate MetHb or NO concentration according to the extinction coefficient of MetHb. Because the isosbestic point for the conversion of OxyHb to MetHb occurs at 411 nm and should not change, the assay was simplified by measuring the increase in absorbance at 401 nm\textsuperscript{18,19}. This approach has been used widely for the quantitative determination of NO and kinetic studies of NO metabolism in vitro and in biochemical experiments\textsuperscript{18}.

Hematocrit (hemoglobin (ferrous) (Sigma)) was used to trap NO. It was prepared in PBS, which contained Na\textsubscript{2}HPO\textsubscript{4}, 50 mmol/L, NaH\textsubscript{2}PO\textsubscript{4}, 50 mmol/L, and NaCl 145 mmol/L as a 25 mg/2 mL solution. This stock OxyHb solution (300 μmol/L) was quickly frozen at −80°C in small aliquots and used for 2 weeks. For the assay procedure, OxyHb was diluted to a working solution to provide the appropriate concentrations. A Du-640 Beckman spectrophotometer was used to determine changes in absorbance of MetHb and OxyHb at 401 and 411 nm and to record the spectra or differences in spectra of MetHb.

A calibration curve was constructed in vitro with the use of a saturated solution of NO, which was prepared as previously described\textsuperscript{17}. Briefly, 5 mL PBS was pipetted into a glass vial (6-mL-thick wall) with a rubber seal and deoxygenated by repeatedly vacuumizing and inhaling with argon for 1 hour. Pure NO gas was passed through a column of KOH pellets injected as a fine stream into the vial. The concentration of this NO solution was 1.5 to 18 μmol/L. Different volumes of saturated NO solution were injected into the reaction mixtures with the use of gas-tight syringes to obtain various concentrations of NO. The molar extinction coefficient (ε) is the calculated absorbance of a 1 μmol/L solution of MetHb with a 1-cm light path, was obtained by quantitative oxidation of increasing amounts (0.75 to 12 μmol/L) of OxyHb by NO and measurement of absorbance increase at 401 nm.

To determine the applicability of SNP as an in vivo NO donor, we first examined in vitro the release of NO from SNP using the OxyHb trapping technique. SNP (1 mmol/L) was added to the reaction mixture containing 3 μmol/L OxyHb. NO release was evaluated in the presence or absence of SOD (200 U/mL) and catalase (600 U/mL), and differences spectra of MetHb were continuously recorded for 30 minutes. The addition of SOD and catalase blocked the possible effects of free radicals such as superoxide on the MetHb formation. We found that the concentrations of SOD and catalase used in these experiments completely inhibited the effect of superoxide in our preliminary experiments (data not shown).

Colorimetric Assay of Nitrite/Nitrate

Nitrite/nitrate concentration in the dialysate was measured with a colorimetric nitric oxide assay kit (Oxford). A microplate was used to perform enzyme reactions in vitro. For spectrophotometric assay of nitrite with Griess reagent, 80 μL MOPS (50 mmol/L)/EDTA (1 mmol/L) buffer and 5-μL dialysate samples were added to wells in duplicate. Nitrate reductase (0.01 U) and 50 μL NADH (2 mmol/L) were added to the reaction mixture and the plate was shaken for 20 minutes at room temperature. Color reagents, sulfanilamide, and N-(1-Naphthyl) ethylenediamine dihydrochloride were added, and absorbance values at 540 nm were read in an microtiter plate reader (Bio-Rad model 3550). The concentration of nitrite/nitrate was estimated from a standard curve, which was constructed with the use of standard reagents included in the assay kit.

Statistical Analysis

Data are presented as mean±SE. The significance of differences within and between multiple groups was evaluated with the use of ANOVA for repeated measures followed by a Dunn’s multiple range test. Statistical differences between two groups were determined by Student’s t or paired t test. A value of P<0.05 was considered statistically significant.

Results

Characteristics of NO-Induced MetHb Formation

Addition of NO-saturated solution into the OxyHb reaction mixture produced a concentration-dependent increase in absorbance at 401 nm, indicating the formation of MetHb (Fig 1A). By oxidizing increasing amounts (0.75 to 12 μmol/L) of OxyHb with the use of NO-saturated solution, we calculated the molar extinction coefficient of MetHb as 112 000 M\textsuperscript{-1} cm\textsuperscript{-1}, with a correlation coefficient of .998. There was a highly linear correlation between calculated NO concentration by MetHb formation and NO-saturated solution (r=0.999) (Fig 1B).

SNP released NO and oxidized OxyHb in a time-dependent manner. Maximal MetHb formation was observed at 30 minutes. SOD and catalase had no effect on the MetHb formation when added into the reaction mixture (Fig 2). SNP-released NO can be effectively diazylized through the
Fig 1. Difference spectra of OxyHb versus increasing amounts of MetHb (A) and correlation of standard and estimated NO concentrations (B) (Abs indicates absorbance). 18 mmol/L NO-saturated solution was used to determine the difference spectra of OxyHb versus MetHb. The extinction coefficient of the MetHb increase was 112 000 mol⁻¹ cm⁻¹. The estimated concentration of NO was calculated according to following equation \( c = \frac{A}{b} \), where \( c \) is concentration, \( A \) is absorbance, and \( b \) is extinction coefficient.

Use of a microdialysis probe in vitro at a perfusion rate of 2 μL/min A 97% relative recovery was observed (data not shown).

**Microdialysis Study In Vivo**

The effects of intravenous infusion of L-NAME on NO concentration in the interstitial fluid of the renal cortex and medulla are presented in Fig 3. The basal concentration of NO was significantly higher in the renal medulla, averaging 37 ± 3.6 nmol/L compared with 31.2 ± 5.7 nmol/L in the renal cortex (\( P < .05 \)). Intravenous infusion of L-NAME decreased NO by 33% in the renal cortex and by 46.5% in the renal medulla.

Fig 3. Effect of intravenous infusion of L-NAME (10 μg/kg per minute) on renal cortical and medullary NO concentrations. *\( P < .05 \) indicates a significant difference from the control value.

In contrast to the effect of L-NAME, intravenous infusion of L-arginine produced a twofold to threefold increase in cortical and medullary NO (Fig 4). Addition of SOD and catalase to the microdialysis perfusate did not alter baseline NO levels or the effect of L-NAME and L-arginine on cortical and medullary NO concentration (Figs 3 and 4).

**Fig 4.** Effect of intravenous infusion of L-arginine on renal cortical and medullary NO concentrations. *\( P < .05 \) indicates a significant difference from the control value.
Nitrate/nitrite concentration was 2.7 ± 0.6 μmol/L in the renal medulla and 2.1 ± 0.2 μmol/L in the renal cortex. Intravenous infusion of L-NAME reduced renal interstitial nitrate/nitrite concentration by 18% in the renal cortex and 23% in the renal medulla, and intravenous infusion of L-arginine increased cortical and medullary nitrate/nitrite concentrations by 65% and 39%, respectively (Fig 5).

**Effect of Renal Medullary Infusion of SNP on NO Concentration**

Changes in NO concentrations in the renal medulla during medullary infusion of SNP are presented in Fig 6. When SNP (1 mmol/L) was infused into the renal medullary interstitium, the concentration of dialyzed NO from the renal medulla was significantly increased. Cortical NO concentrations were not measured in the study.

### Discussion

The present study provides the first direct evidence that NO concentration in the renal medulla is higher than in the renal cortex. The responsiveness of the technique was demonstrated by L-arginine and SNP stimulation and L-NAME inhibition of NO release. These studies demonstrated that NO trapping through the use of OxyHb in combination with in vivo microdialysis is a practical and useful method for the detection of tissue NO in the renal cortex and medulla.

There have been a number of approaches used in efforts to indirectly provide indexes of tissue NO production and release. Assay of guanylyl cyclase activity and measurement of citrulline conversion rate have been used to determine NOS activity, but NO levels cannot be measured directly by these methods. Detection of luminescence of products of ozone with NO has been used to estimate NO levels. However, since this technique measures NO in the gas phase, the stripping of NO from a liquid biological sample for release into the gas phase is a complicated process, in which proteinaceous material foams dramatically during stripping. This results in coating of the photomultiplier tube for luminescence measurement and diminishes its sensitivity.

Amperometric or voltammetric microprobes have been developed recently to determine NO from intact tissues or single cells. A glass Clark-like microelectrode with a platinum cathode shows current alteration at 0.9 V, which is specific for NO, but these microprobes exhibit a large variation in sensitivity. A second, more sensitive microprobe is based on the observation that metalloporphyrins catalyze the oxidation of NO and consequently generate electrical current. A carbon fiber is coated with a thin polymeric porphyrin layer, and this electrode can be used to measure NO as the electrical current produced at 0.63 V. Although an additional coat of the carbon fiber with a negatively charged material, perfluorinated ionomer (Nafion) makes the microprobe impermeable to anions such as NO₃⁻, catecholamines can still react with carbon fiber and produce the current. These probes are also quite fragile and problematic for measurements with deep tissue regions such as the renal medulla. Therefore, variable sensitivity, limited specificity, as well as high fragility of microprobes for NO plague the usefulness of these electrodes in vivo systems.

The measurements of nitrate/nitrite as NO end products have been used to determine NO levels in different samples and preparations, but this assay is only adequate as a qualitative or semiquantitative measure of NOS-related activity. Urinary nitrate/nitrite has been used to represent NO levels in the kidney, but such measurements cannot distinguish regional differences between cortical and medullary NO production. In addition, urinary nitrate/nitrite is produced not only in the kidney, and the renal reabsorption and excretion of nitrate/nitrite is complicated, making measurement of nitrate/nitrite very limiting in defining the role of NO in the regulation of renal function.

NO trapping technique using OxyHb has been used to study NO release from pharmacological NO donors, NOS activity, enzyme kinetics, and NO production in isolated perfused organs and cultured or fresh cells. Recent studies indicated that a microdialysis probe using a hemoglobin-trapping technique possesses adequate sensitivity to determine the basal levels of NO in the brain and changes in NO level induced by a neurotoxin, kainic acid. In the present study, we used in vivo microdialysis in combination with a hemoglobin-trapping technique to examine NO production.
levels in the renal cortex and medulla and the effect of L-arginine and its analogue, L-NAME, on NO levels in both regions. We found that NO concentrations in the renal cortex and medulla were 312 and 571 nM/L, respectively, in anesthetized rats. A higher NO level in the renal medulla may be associated with greater expression of NOS in this region. Studies in our laboratory and others have demonstrated that the expression of NOS including eNOS, nNOS, and iNOS in the renal medulla was much greater than in the renal cortex.10 Intravenous infusion of L-arginine as a substrate of NO formation produced a marked increase in cortical and medullary NO, and infusion of L-NAME reduced NO levels in both regions. Using the nitrite/nitrate colorimetric assay, we found that nitrite/nitrate concentration in the dialysate from the renal medulla and cortex was altered in the same direction as NO concentration when L-arginine and L-NAME were infused intravenously. However, the magnitude of alterations in nitrate was smaller than that of nitrite.

Because free radicals such as superoxide can be formed in the kidney and NO itself can also catalyze the production of the oxidant superoxide,24 we were concerned whether in vivo superoxide anions could be dialyzed and directly influence the formation of MetHb, thereby producing an artificial change in measured NO concentrations. To examine this issue, we included a high concentration of SOD and catalase in the dialysate perfusate to effectively scavenge superoxide and H2O2. The values of NO in the renal cortex and medulla were compared with those found in the absence of these scavengers of free radicals. We found that MetHb formation (characterized by increase in absorbance at 401 nm) in both medullary and cortical dialysates was not altered by removal of superoxide anions by SOD and catalase, suggesting that superoxide anions and H2O2 did not influence the MetHb formation in the dialysate from the renal medulla and cortex. The finding that local medullary administration of the NO donor SNP markedly increased MetHb concentration in both medullary and cortical regions compared with the control suggests that endogenous formation of NO in the medulla and cortex can effectively scavenge superoxide and H2O2. The values of SOD and catalase in the dialysis perfusate to 1000 U/ml were higher than those of SOD and catalase in the local medullary administration of the NO donor SNP, which may be the reason for the increase in absorbance at 401 nm in the renal medulla and cortex.

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In summary, the in vivo microdialysis and hemoglobin trapping technique is useful for determining tissue concentration of NO in the kidney. NO concentrations in the renal medulla are much higher than in the renal cortex. A high level of medullary NO may play an important role in the control of vascular tone and tubular function in the renal medulla.

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