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) was used at a concentration of 3 μmol/L to perfuse 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 was 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 ± 5.57 μmol/L, n = 10) than in the cortex (31 ± 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 the cortex and medullary NO, with nitrite/nitrate levels of 10 (μg/kg per minute) by 33% in the renal cortex and by 46% in the renal medulla. We have also compared under the same conditions the degradation products of NO, nitrite, and nitrate in the renal cortex and medulla using in vivo microdialysis combined with microtiter plate colorimetry. Nitrite/nitrate concentration was significantly higher (P < 0.05) in the medulla (2.7 ± 0.6 μmol/L, n = 4) than in the cortex (2.1 ± 0.2 μmol/L, n = 4). Infusion of L-arginine increased cortical and medullary nitrite/nitrate by 55% and 39%, respectively. L-NAME reduced cortical and medullary nitrite/nitrate by 18% and 23%, respectively. The results indicate that the OxyHb-MetHb trapping technique is a highly sensitive in vivo method for detecting regional tissue NO concentration and changes in the NO synthase activity in the kidney. These studies have shown 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 a rise of arterial blood pressure in rats. Measurement of cGMP indicated that NO production in the renal medulla is greater than in the renal cortex, and 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 venous cannulas were inserted into the renal capsule and cortex with a 23-gauge needle, after cessation of bleeding, through the renal capsule. 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 urine nitrate 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 e = A/eb, where e is MetHb or NO concentration, A is absorbance increase at 401 nm, eb 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 = NO
met-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

A calibration curve was constructed in vitro with the use of a saturated solution of NO, which was prepared as previously described. Briefly, 5 mL PBS was pipetted into a glass vial (6-mL-thick wall) with a rubber seal and deoxygenated by repeatedly vacuumizing and bubbling with argon for 1.5 hours. Pure NO gas passed through a column of KOH pellets was injected as a fine stream into the vial. The concentration of this NO stock solution was 1.5 to 18 mmol/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 (e), which is the calculated absorbance of a 1 mmol/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 the difference 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 mL 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 nitrite-metabolizing enzyme (0.1 U) were added to each well. 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 a 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 Duncan'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⁻¹·cm⁻¹ with a correlation coefficient of 0.998. 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 dialyzed 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:  \[ c = A / \epsilon \]  where  \( c \) is concentration,  \( A \) is absorbance, and  \( \epsilon \) 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 37.1 ± 5.6 nmol/L compared with 31.2 ± 5.7 nmol/L in the renal cortex (P < 0.05). Intravenous infusion of L-NAME decreased NO by 33% in the renal cortex and by 46.5% in the renal medulla.

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 2  NO release from SNP in vitro in the absence or presence of SOD and catalase (Cat) in cuvettes. Difference spectrum of OxyHb versus MetHb was recorded every 3 minutes. NO concentrations were calculated as described in Fig 1.

Fig 3  Effect of intravenous infusion of L-NAME (10 μg/kg per minute) on renal cortical and medullary NO concentrations. *P < 0.05 indicates a significant difference from the control value.

Fig 4  Effect of intravenous infusion of L-arginine on renal cortical and medullary NO concentrations. *P < 0.05 indicates a significant difference from the control value.
Nitrite/nitrate 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 nitrite/nitrate concentration by 18% in the renal cortex and 23% in the renal medulla, and intravenous infusion of L-arginine increased cortical and medullary nitrite/nitrate 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 proteaceous 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 metalloporphyrines 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 nitrite/nitrate 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 nitrite/nitrate 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 nitrite/nitrate is produced not only in the kidney, and the renal reabsorption and excretion of nitrite/nitrate is complicated, making measurement of nitrite/nitrate 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 levels in the renal cortex and medulla.
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 nitrite/nitrate was smaller than hemoglobin-trapped NO. These results indicate that the microdialysis and hemoglobin-trapping technique can be used in the kidney for the in vivo detection of regional concentration of NO.

Because free radicals such as superoxide can be formed in the kidney and NO itself can also catalyze the production of the oxidant superoxide,28 we were concerned whether in vivo superoxide anions could be dialyzed and directly influence the formation of MetHb, thereby producing an artifact 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 obtained 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 the dialysate in the presence of SOD and catalase further demonstrated that the oxyhemoglobin trap assay is sufficiently specific for nitric oxide.

The existence of endothelium-derived relaxing factor is important to note that peroxymetrone, a product of NO metabolism, may be partly derived from peroxymetrone. Therefore, we cannot exclude the possibility that MetHb changes of OxyHb identical with those effected by NO.29

These results indicate that the microdialysys and hemoglobin-trapping technique can be used in the kidney for the in vivo detection of regional concentration of NO.

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