Real Time Microelectrode Measurement of Nitric Oxide in Kidney Tubular Fluid *in vivo*

David Z. Levine[*] **and Michelle Iacovitti**

Division of Nephrology, The Kidney Research Centre, Ottawa Health Research
Institute and University of Ottawa, 451 Smyth Road, Room 1333, Ottawa, Ontario, CANADA
K1H 8M5, Tel: (613) 562-5800, ext 8240, Fax: (613) 562-5487

*Author to whom correspondence should be addressed. E-mail: dzlevine@uottawa.ca

Abstract: In this review we summarize our experience using a microelectrode to measure nitric oxide concentrations [NO] in living rat kidney tubules. In the anaesthetized living rat, the abdomen can be opened, and the kidney can be placed in a cup such that one can puncture a surface single tubular segment, 1-2 mm long, connected to one of 30,000 filtering glomeruli. The tubular segment can be viewed with a stereo microscope and punctured using sophisticated micromanipulators. The segment, ranging in diameter from about 15 - 35 um contains freely flowing RBC-free fluid, electrolytes, O_2, pCO_2 and NO gas concentrations, and a host of other known and unknown substances. After a "pre" puncture with a 7-10 um beveled glass pipette, intratubular [NO] can be directly determined by inserting, into the tubular lumen, the tip of a specially modified amperometric integrated electrode (WPI P/N ISO-NOP007). We review our *in vivo* experience with this electrode, emphasizing optimal practice to ensure appropriate calibration, stability, and selectivity for *in vivo* use. The electrode is highly selective for NO, and, despite fragility, with appropriate precautions, it can provide reproducible and highly sensitive NO measurements in the 40-1000 nM range.

Key words: Nitric oxide, kidney tubules, in vivo NO measurements

Introduction

Hundreds of publications have appeared addressing the myriad of kidney effects of nitric oxide (NO), but most of these reports have depended on indirect methods of measuring [NO]. Direct intrarenal [NO] measurements are needed to characterize and quantify effects of nitric oxide synthase inhibition, or NO donors, in order to track short term changes–seconds to minutes–of [NO]. In studies of reduced blood flow to the kidney, it is agreed NO plays an important role, but unfortunately conflicting results have emerged which, as has been already pointed out by Weight and Nicholson [8], can only be resolved by direct intrarenal NO measurements. Of course, different approaches to NO measurement have been reported for more than a decade with the electrochemical and chemiluminescence methods attracting most attention, as previously reviewed by our laboratory and others [2, 6]. Nonetheless, Noiri et al impaled a 30 μm diameter NO sensitive electrode 1 mm into the kidney cortex of control and ischemic rats showing clear differences in tissue NO release in their preparations [4], while Majid et al [3], using coated platinum/iridium amperometric electrodes of 200 :m diameter, impaled their electrodes 5 mm into the cortex of dog kidneys, and, they demonstrated clear current increases in response to intrarterial injection of SNAP, despite a varying baseline.

However, to better understand the short term effects of NO on kidney vessels and tubular transport and signaling, it would be desirable to measure, quantitatively, real time [NO] in structures such as tubules and the glomerular capsule. Accordingly, we modified the WPI amperometric NO electrode (WPI P/N ISO-NOP007), with an integrated Ag/AgCl reference system, so that its reactive length is approximately 5-15 μm and could be completely inserted into a tubule (Figure 1). The basics of this technique, and the specificity, sensitivity, and other electrochemical characteristics have already been described by Zhang et al [9]. The purpose of the present review is to describe, for the non-kidney investigator, the technique of real time in vivo measurements, with emphasis on calibration issues, and precautions related to detecting integrity of the electrode membrane during its traumatic in vivo use.

Structure of the NO electrode: (*World Precision Instruments (WPI P/N ISONOP007*).

The basic electrode design, and details of its performance have already been published [1,9]. Electrodes, specially modified for our in vivo use, were constructed (Figure 1), with glass insulation to absolutely preclude NO reactivity. Reactive length was ~ 5-15 μm, and the tip diameter usually about 5-7 μm .

Calibration

a. *Zero point:* After calibration in vitro, a zero point is needed between repeated punctures. We have chosen to use saline on the surface of the rat kidney to be that point. Thus, after an "extratubular" zero, the puncture is made along the "pre-puncture" track, and the pA response is taken from that "zero". We have also considered the possibility of NO leaking from the tubule, but have determined that is not a significant concern: samples of surface fluid were pipetted into the copper sulfate solution without any significant response, even after many tubular segments were punctured.

"Intratubular Zero Point". We presumed that if the pA signal was solely due to intratubular NO, the signal should be abolished by high flow saline perfusion around the electrode. Indeed, 40 nl/min saline diminished the pA current in a repeated and reproducible fashion (Figure 2) to values similar to that obtained from surface fluid.

b. *Mechanical effects:* Most would agree that optimal calibration should simulate as closely as possibly the environment in which the sensor is being used. Accordingly, calibration in aqueous solution may not reflect conditions encountered when the sensor is surrounded by living tissue: the tissue per se, may have electro mechanical effects which may influence response. In the work describe herein, the electrode tip is in an aqueous biological fluid–tubular fluid filtered from blood in the living animal–and
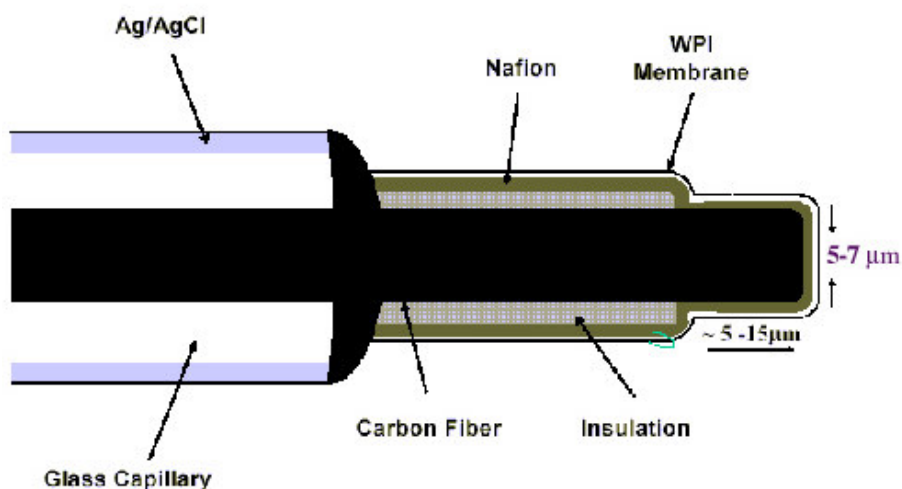

Figure 1. Modified WPI integrated amperometric NO sensing electrode. The basic features of the electrode structure are shown. Arrow pointing to insulation layer refers to either epoxy or glass coatings. The NO sensitive length, coated with Nafion and a WPI membranes was approximately 5 -15 µm; the tip diameter varied from 5 to 7 µm. See Methods for additional details.

it is calibrated in an aqueous copper sulphate solution, with SNAP as the NO donor. Nonetheless, the mechanical effect of entering the tubule–despite the "pre" puncture with a larger glass pipette --see above–still is a departure from the simple aqueous calibration, and may theoretically cause transient or prolonged changes in response. Indeed, at least in the short term, that is seconds, in many tubules, immediately after inserting the tip of the electrode, a spike was observed (Figure 3), but the pA readout immediately dropped to a more stable trajectory, at a higher current reflecting intratubular [NO]. This spike seemed to be associated with bending of the tip of the coated carbon electrode shaft fibre, and could be reproduced, at times, by bending it on the kidney capsule. According, we assumed this spike effect to be mechanical in origin.

c. *Intratubular response to NO solutions:* Assuming then that the aqueous intratubular milieu is not dramatically different from the in vitro calibration conditions, what assurance can we have that some unknown filtered substance could behave similarly to NO? This issue, of course, is virtually unresolvable, since it's formal resolution would require identifying every possible biological

component of tubular fluid–an impossible task. As a first step, we attempted to address a related question. Would the electrode respond in an expected manner, *within the tiny tubule*, when different concentrations of NO were perfused upstream, using two nanolitre perfusion pumps, through separate ~7 μm tipped glass pipettes, flows ranging from zero to 40 nl/min. Figure 2 shows an intratubular zero point, and then appropriate step-wise increment and decrement of response when an NO containing saline solution was mixed with an NO free solution, without altering flow rate.
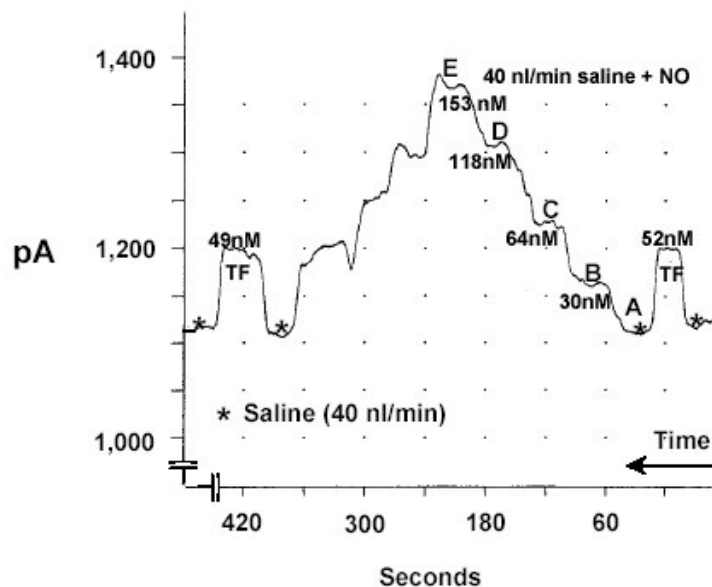

Figure 2. Intratubular recording of [NO] with perfusion of saline and/or saline equilibrated with NO. The record should be read from right to left. First, a tubule is punctured, the electrode inserted, and [NO] is measured in tubular fluid (TF). Next, an intratubular "zero" is taken (A) after perfusion at 40 nl/min of NO free saline. Then, stepwise increments of the saline/NO solution is perfused with corresponding reduction of the saline perfusate to maintain an approximately constant 40 nl/min flow rate. B, C, D, E, denote 10, 20, 30, 40 nl/min of the saline/NO perfusate.

Selectivity

As reported by Zhang et al [9] 50 uM ascorbic acid or 50 uM nitrite do not elicit a response from this sensor, with an approximate 1000:1 selectivity ratio. Nonetheless, it is appropriate to know under specific experimental conditions what the concentrations of these substances could be in kidney tubular fluid, and , of course, whether they might change in the course of experimental maneuvers and be misconstrued as changes in [NO]. Plasma ascorbic acid in humans may be in the 80 uM range (7) while nitrite/nitrate concentrations in the rat range about 2 uM in plasma and 50 uM in urine (5). If it is correct to assume that nitrate and ascorbic acid levels would be below 1000 uM with a selectivity ratio of 1:1000, and not change, then there would be no anticipated problem. With respect to urea, we have determined that even 1 M concentrations have no effect.
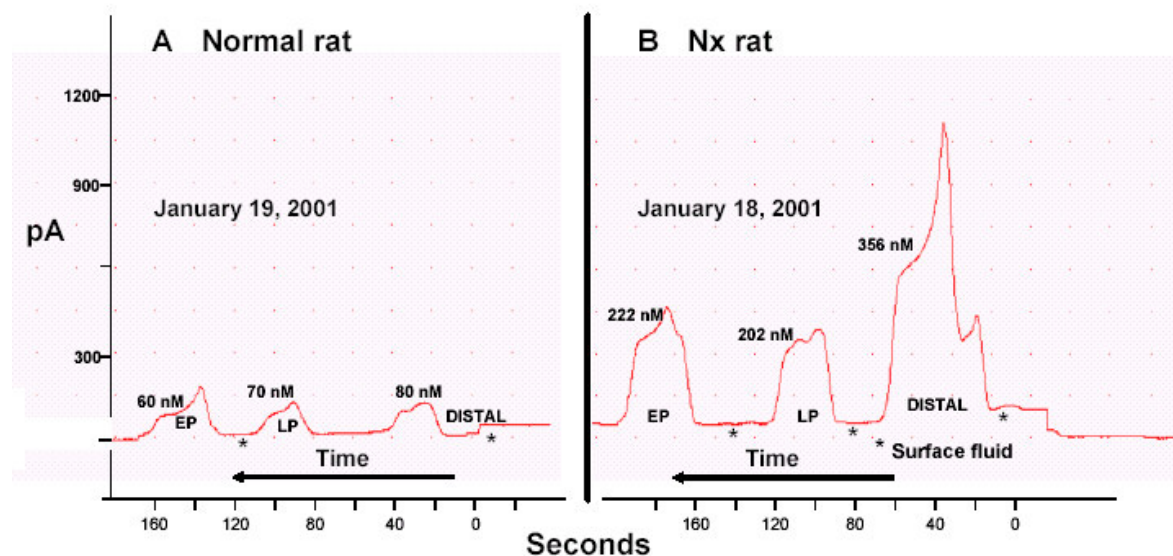
Figure 3 A, B. Intratubular recordings from normal and Nx rats using the same glass insulated electrode on successive days. A. Recording showing NO measurements from a distal, late proximal, and early proximal tubule from the same nephron of a normal rat kidney. The surface fluid (asterix) is the zero point taken immediately before inserting the electrode into the nephron segment. The pA difference is taken from the surface fluid to the shoulder of the response. The time scale is compressed so that stable and more level shoulders are apparent in real time. B. Corresponding measurements from an Nx rat using the same electrode. See also Results.

Detecting electrode deterioration

We emphasize that *in vivo* micropuncture, and intratubular [NO] measurements require considerable technical skill, and sophisticated equipment. We surmise without substantial experience in similar in vivo preparations, the obstacles in working with breathing animals, and pulsing kidneys would be formidable. While it is true we have occasionally used one electrode for several experiments involving perhaps 50 punctures, breakage is not uncommon. We now take the following precautionary steps with each set of new electrodes, and during every experiment. Electrodes are checked on arrival for appropriate response to SNAP calibration, and insensitivity to 100 uM acorbic acid or potassium nitrite. In addition, in the course of an experiment, we recalibrate after several tubular fluid [NO] measurements, because of concern that trauma could alter sensitivity. We now periodically recheck against acorbic acid and potassium nitrite, to be certain that after several experiments electrode integrity is maintained. We also note the manufacture's caution that there is a deterioration of membrane integrity with time of even unused electrodes. While the majority of our electrodes in the last three years have performed well, we have occasionally found unexpectedly high [NO] values in some rats, and these have occasionally, but not always, been associated with defective electrodes.

Experimental design can counter methodological uncertainty

As noted above, with quantitative work such as ours, it is possible this and many other methods, may be subject to interfering substances, calibration errors, false zero points, etc. Clearly, the most secure approach, in addition to attempting to simulate the calibration conditions outlined above, is to try to use two kinds of experimental controls. With the same electrode, try to introduce the perturbation so that each tubule acts as its own control [2]. The flaw here would only be an interfering substance which would move in parallel with [NO]. The other approach needed, when different animal preparations are compared–ie the study of tubular fluid [NO] in intact versus rats with reduction of renal mass by surgery [1], would be to use the same electrode(s) in animals of both groups. Presumably membrane disruption and loss of selectivity would apply to both groups, albeit theoretical flaws still exist.

Future Directions

While the current modified 5-7 µm electrode should be quite satisfactory for tissue culture or isolated *in vitro* microscopic kidney structures*,* the fragility remains a burden for in vivo use. Perhaps, a core of tungsten, or a parallel tungsten shaft–not in electrochemical continuity with the rest of the combined electrode circuit, could add strength the to the shaft. This, combined with hardier membrane assemblies, would be welcome, and is undoubtedly the subject of ongoing research and development.

Acknowledgments

References

1. Levine, D. Z., M. Iacovitti, K. D. Burns, and X. Zhang. Real-time profiling of kidney tubular fluid nitric oxide concentrations in vivo. *Am J Physiol Renal Physiol.* **2001,** 281, F189-94.
2. Levine, D. Z., and M. Iacovitti. Nitric oxide concetrations in distal tubular fluid is modulated in vivo by loop perfusion of NaCl in normal rats. *Proc American Soc Neph*. **2002,** F-PO465.
3. Majid, D. S., S. A. Omoro, S. Y. Chin, and L. G. Navar. Intrarenal nitric oxide activity and pressure natriuresis in anesthetized dogs. *Hypertension* **1998,** 32, 266-72.
4. Noiri, E., T. Peresleni, F. Miller, and M. S. Goligorsky. In vivo targeting of inducible NO synthase with oligodeoxynucleotides protects rat kidney against ischemia. *J Clin Invest.* **1996,** 97, 2377-83.
5. Roczniak, A., J. N. Fryer, D. Z. Levine, and K. D. Burns. Downregulation of neuronal nitric oxide synthase in the rat remnant kidney. *J Am Soc Nephrol.* **1999,** 10, 704-13.
6. Schnermann, J and D. Z. Levine. Paracrine factors in tubuloglomerular feedback: adenosine, ATP, and nitric oxide. *Annu. Rev .Physiol.* **2003**, 65, 501-509.

7. Seghieri G, L Martinoli, M. Miceli, et al. Renal excretion of ascorbic acid in insulin dependent diabetes mellitus. *Int J Vitam Nutr Res.* **1994**, 64(2), 119-24.

8. Weight, S. C., and M. L. Nicholson. Nitric oxide and renal reperfusion injury: a review. *Eur J Vasc Endovasc Surg.* **1998,** 16, 98-103.

9. Zhang, X, L. Cardosa, M. Broderick, H. Fein, and Jie Lin. An integrated nitric oxide sensor based on carbon fiber coated with selective membranes. *Electroanalysis* **2000**, 12, 113-117.

Sample Availability: Available from the authors.