Evaluation of basic performance and applicability of a newly developed in vivo nitric oxide sensor

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Abstract

Direct measurement of nitric oxide (NO) is of great importance and value for both in vitro and in vivo studies on dynamic NO bioactivity. Here, we evaluated the basic performance of a newly developed NO sensor (Innovative Instruments, Inc.). Unlike other NO sensors, the new NO sensor has a highly durable, gas-permeable coating and is affected much less by electrical interference due to its integrated structure where working and reference electrodes are combined in a single element. Calibration with NO gas showed high sensitivity of about 580 pA per nmol-NO l⁻¹ (the detection limit 0.08 nmol-NO l⁻¹, S/N = 3). This sensor also showed high selectivity (25 000 times and more) to NO, compared with NO-related reagents such as L-arginine, N⁵-monomethyl-L-arginine, acetylcholine, nitroglycerin (NTG) and tetrahydrobiopterin as well as dissolved oxygen. As an in vivo application, the sensor was located in the anaesthetized rat abdominal aorta to measure NTG-derived plasma NO. Intra-aortic infusion of 0.5 mg NTG caused a measurable increase in plasma NO level (2.0 ± 2.2 nmol l⁻¹, mean ±SD, n = 3). In conclusion, the new NO sensor demonstrated a satisfying performance for both in vitro and in vivo applications.

Keywords: in vivo measurement, nitric oxide (NO), sensor
1. Introduction

Nitric oxide (NO) plays many physiological roles including vasodilation, neurotransmission and immunological reaction, and thus quantitative \textit{in vivo} NO measurement is a key to understand its dynamic actions (Lancaster 1996).

There are various NO measurement methods including chemiluminescence, fluorescence and electron spin resonance (Feelisch and Stamler 1996). As a high-temporal resolution method, electrochemical measurement methods of NO, i.e. NO sensors, have been developed by several groups (Shibuki 1990, Malinski and Taha 1992, Ichimori \textit{et al.} 1994). These NO sensors enable us to evaluate dynamic changes of NO concentration in solutions and tissues in response to agonists, NO-generating reagents and physical stimuli (Vallance \textit{et al.} 1995, Pinsky \textit{et al.} 1997, Mochizuki \textit{et al.} 1999, 2001). However, electrical interference through a power line, vibration, poor durability of sensor-tip coatings and other factors make \textit{in vivo} NO measurement very difficult. To overcome these drawbacks, a new NO sensor of novel design including built-in structure of both working and reference electrodes with a highly gas-permeable enclosure instead of fragile coatings has been developed. This study is aimed at evaluating the basic performance and applicability of the new NO sensor.

2. Materials and methods

2.1. Sensor

NO sensors (amiNO-IV, 700 µm in diameter, 150 mm in length; Innovative Instruments, Inc., Tampa, FL, USA) were evaluated in the present study. Figure 1 shows a schematic diagram of the sensor. An integrated electrochemical cell (anode, cathode and electrolyte) comprising the sensing element is miniaturized and encased in a cylindrical gas permeable membrane to isolate the sensing element from the sample solution to assure the selectivity and stability of the sensor. This isolation results in the exclusion of artifacts resulting from any of the variations in sample conditions such as pH, ionic strength, etc. The sensor tip is made of sharp stainless steel to aid the insertion of the sensor into samples. The sensor structure contains an electrical shield to eliminate different types of noise. The sensor was connected to inNO-T (Innovative Instruments), an NO-measuring system including a current monitor and a data acquisition software with a temperature compensation function that automatically compensates sample temperature fluctuation. The electric signal due to the oxidation of NO at the sensing element was recorded.

2.2. Calibration

NO-saturated pure water was prepared by bubbling pure NO gas in oxygen-free pure water for 15 min resulting in a saturated NO solution of 1.9 mmol-NO l$^{-1}$ at 20 °C. Using a gas-tight
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syringe 1, 3 and 5 µl was injected into well-stirred saline solution (50 ml) in which an NO sensor was immersed (final NO concentration: 38, 114 and 190 nmol l⁻¹).

To evaluate the sensor response to changes in NO levels, superoxide (•O₂⁻), a well-known NO scavenger, was generated in situ to consume NO. Superoxide was produced using xanthine and xanthine oxidase mixture. An NO sensor was immersed in 50 ml saline containing 100 µmol l⁻¹ xanthine, NO-saturated pure water was injected first, and then after reaching the maximum steady level 1 ml of 2 U ml⁻¹ xanthine oxidase was added.

All solutions were kept at 20 ± 1 °C. Prior to all measurements, each sensor was exposed to a slight temperature increase of 1–2 °C by adding heated saline for temperature compensation calibration.

2.3. Evaluation of basic performance (effects of dissolved reagents and oxygen)

To evaluate possible interferences of reagents commonly used in NO studies, 100 µmol l⁻¹, final concentration, of L-arginine (a substrate of NO synthase: NOS), N⁰-monomethyl-L-arginine (L-NMMA; a NOS inhibitor), acetylcholine (ACh; a NOS stimulator), nitroglycerin (NTG; a nitrosovasodilator) or 50 µmol l⁻¹ tetrahydrobiopterin (BH₄; a NOS cofactor) was added to a well-stirred Krebs–Henseleit buffer solution. All solutions were equilibrated with air.

The effect of dissolved oxygen was evaluated by bubbling a solution (50 ml; saline or Krebs–Henseleit buffer solution) with 95% N₂ and 5% CO₂ under a well-stirred condition and then switching to 95% O₂ and 5% CO₂.

All solutions were kept at 20 ± 1 °C. Here we repeated all the protocols using three sensors and the results were presented as means ± SD.

2.4. Animal preparation and experimental protocol

The experimental procedures were approved by the Animal Research Committee of Kawasaki Medical School and conform with the standards for use of laboratory animals established by the Institute of Laboratory Animal Resources, US National Academy of Sciences, USA.

Wistar-Kyoto rats (age: 13 weeks, body weight: 395–425 g, n = 3) were initially sedated by inhalation of diethyl ether and anaesthetized by intra-abdominal administration of thio-butabarbital (100 mg kg⁻¹). A catheter for NTG infusion was located in the aortic arch from the common carotid artery and the NO sensor was inserted through the left femoral artery and located in the abdominal aorta. Recently, we have reported direct measurement of NTG-derived NO in the vascular wall of canine femoral arteries with another type of NO sensor (Mochizuki et al 2001) using our own approach (Mochizuki et al 1999). Thus, for comparison, we measured NTG-derived NO in the present study. NTG of 0.5 mg ml⁻¹ was infused at a rate of 1 ml min⁻¹ for 1 min and plasma NO concentration in the abdominal aorta was monitored. Here we used the same sensor for all three rats, and the results were presented as mean ± SD.

2.5. Chemicals

L-arginine was supplied by Nacalai Tesque (Kyoto, Japan), Krebs–Henseleit buffer and L-NMMA by Sigma Chemicals (MO, USA), ACh by Daiichi Pharmaceutical (Tokyo, Japan), NTG by Nippon Kayaku (Tokyo, Japan) and BH₄ by BIOMOL Research Laboratories (PA, USA).
3. Results

3.1. Response profile and calibration of the NO sensor

Figure 2 shows a typical response of one of the three sensors to the successive additions of 190, 114 and 38 nmol l\(^{-1}\) NO. The current increased immediately after the NO injection, reflecting fast response of the sensor (initial response rate: \(34 \pm 3\) pA s\(^{-1}\) per nmol-NO l\(^{-1}\) for three sensors, mean \(\pm\) SD). Figure 3 shows the relationship between the NO concentration (38, 114 and 190 nmol l\(^{-1}\)) and the averaged peak current of the sensor used for the study in figure 2 (repeated three times for each concentration). This linear relationship was observed in all sensors (\(r^2 = 0.98\)–0.99). From the result in figure 3, the sensitivity (slope) was calculated to be 580 pA per nmol-NO l\(^{-1}\) (580 \(\pm\) 10 pA per nmol-NO l\(^{-1}\) for three sensors, mean \(\pm\) SD). Using the mean value of 580 pA per nmol-NO l\(^{-1}\), the detection limit of this sensor was calculated (\(S/N = 3\)) as 0.08 nmol-NO l\(^{-1}\) using the noise level (15 pA).
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Figure 4. Time course of the measured current. (i) NO-saturated pure water was added and (ii) superoxide was then produced in the solution by xanthine (100 µmol l⁻¹) and xanthine oxidase (40 mU ml⁻¹) to quickly scavenge NO.

When NO (114 nmol l⁻¹) was reacted with the superoxide produced by xanthine (100 µmol l⁻¹) and xanthine oxidase (40 mU ml⁻¹), the measured NO current decreased quickly (figure 4), demonstrating that NO instantaneously reacted with the superoxide and the sensor measures changes in NO concentration at high selectivity and temporal resolution.

No significant changes in the sensitivity were noted over a period of a few weeks or after using the sensor in biological fluids. Moreover, no notable change in the baseline current was observed with and without solution mixing, suggesting no direct effect of fluid (blood) motion.

3.2. Effects of dissolved reagents and oxygen on measured current

In order to evaluate the possible electrochemical effects of NO-related reagents on the baseline current, L-arginine, L-NMMA, ACh, NTG or BH₄ was injected into a Krebs–Henseleit buffer solution. Only small changes by L-arginine, L-NMMA, ACh and NTG with relatively larger changes by BH₄ were observed (table 1). The irreproducible small effects of these reagents might indicate that these are non-Faradic (non-analytical) effects such as localized temperature change and/or the conversion of nitrogen-containing compound impurities to NO. Further studies are needed to understand these phenomena.

In some experimental protocols, hypoxic conditions would be applied, and thus effects of dissolved oxygen level on the measured current were investigated. When the injected oxygen-gas level was shifted from 0% (95% N₂, 5% CO₂) to 95% (5% CO₂), the measured current increased (table 1).

The effects on the baseline current were also compared as the values per mol l⁻¹ and are listed in table 1. Compared with NO (5.8 ± 0.1 × 10⁸ nA per mol l⁻¹), other compounds including oxygen showed much smaller changes in the current, resulting in the high selectivity to NO (25 000 times and more).

3.3. In vivo NO measurement

Figure 5 shows a representative time course of the measured NO current (presented as NO concentration) in the rat abdominal aorta in vivo. At the onset of NTG infusion, the NO level started increasing and reached a maximum level of about 1 nmol l⁻¹ (2.0 ± 2.2 nmol l⁻¹).
Figure 5. Time course of the nitroglycerin-derived plasma NO concentration measured in the rat abdominal aorta (0.5 mg-nitroglycerin min\(^{-1}\) for 1 min).

Table 1. Effects of dissolved reagents and oxygen on the baseline current.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Changes in current (pA)</th>
<th>Changes in current (nA per mol l(^{-1}))</th>
</tr>
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<tbody>
<tr>
<td>L-arginine</td>
<td>31 ± 90 (100 µmol l(^{-1}))</td>
<td>310 ± 900</td>
</tr>
<tr>
<td>NG-monomethyl-L-arginine</td>
<td>77 ± 125 (100 µmol l(^{-1}))</td>
<td>770 ± 1250</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>3 ± 27 (100 µmol l(^{-1}))</td>
<td>30 ± 270</td>
</tr>
<tr>
<td>Nitroglycerin</td>
<td>22 ± 74 (100 µmol l(^{-1}))</td>
<td>220 ± 740</td>
</tr>
<tr>
<td>Tetrahydrobipterin</td>
<td>219 ± 157 (50 µmol l(^{-1}))</td>
<td>4 370 ± 3150</td>
</tr>
<tr>
<td>O(_2) (saline)</td>
<td>30 000 ± 13 000</td>
<td>23 000 ± 9600</td>
</tr>
<tr>
<td>O(_2) (Krebs buffer)</td>
<td>27 000 ± 11 000</td>
<td>21 000 ± 8200</td>
</tr>
<tr>
<td>NO</td>
<td>5.8 ± 0.1 × 10(^8)</td>
<td></td>
</tr>
</tbody>
</table>

\(n = 3\), which is far above the detection limit (see section 3.1, 0.08 nmol l\(^{-1}\)). No significant changes in the sensitivity were observed after the in vivo measurement, compared with the pre-use sensitivity.

4. Discussion

The new NO sensor showed high sensitivity (580 ± 10 pA per nmol-NO l\(^{-1}\)) and high selectivity to NO, compared with various NO-related reagents. In addition, we evaluated successfully the dynamic changes in plasma NO level in rats, when NTG was administered intra-aortically. The sensor showed satisfying performance and durability even after several uses.

Compared with the sensor that we applied previously (0.7 pA per nmol-NO l\(^{-1}\); Ichimori et al 1994, Mochizuki et al 1999, 2001), this new NO sensor (580 ± 10 pA per nmol-NO l\(^{-1}\)) showed about 800-fold sensitivity. In addition, in the previous sensor, special cautions including electromagnetic shielding were required in applications to exclude the possible electrical interference through a power line, vibration and other factors and to protect the fragile detection tip. This new sensor has a single structure where both working and reference electrodes are built in together, and thus we see much smaller effect of electrical
interference, which is the most significant feature of this new sensor. Another feature is
that this sensor has high selectivity to NO and does not respond to most of the dissolved
NO-related reagents (table 1). However, in some cases, some of these compounds may affect
measurements, and thus some precaution is needed especially when exogenous BH4 is applied
and/or the oxygen level changes significantly, e.g. ischaemia and reperfusion. It is well known
that electrochemical detection is highly sensitive to temperature changes. The new monitoring
system is equipped with a temperature compensation mode, automatically cancelling out the
temperature effect on the sensor sensitivity. In fact, when we evaluated the effects of dissolved
NO-related reagents without temperature compensation, there were some effects (shift of the
baseline current) mainly due to the heat of mixing.

As for the shortcomings of the sensor, the sensor diameter is 700 µm. Thus, accessibility
is limited especially when one is interested in NO measurement in limited locations such as
microcirculation. In fact, smaller electrodes can be fabricated, although their performance
needs to be evaluated thoroughly.

Vallance et al (1995) measured the agonist-stimulated increase in endogenous NO level
in vivo in the human hand vein using their hand-made NO sensor (Malinski and Taha 1992)
and observed an increase of about 130 nmol-NO l⁻¹ by infusion of bradykinin (a NOS
stimulator; 80 pmol min⁻¹ for 3 min). However, in their study, saline or bradykinin was
infused continuously for 10–15 mm anterogradely from the end of the catheter for the sensor,
and thus the NO-scavenging effect of haemoglobin was much less than that in the present
study. In the present study, dilution of red blood cells (a decrease in haematocrit) is considered
negligible and we observed rather low NO levels. Another contributing factor might be the
lack of a gas-permeable membrane in the previous sensor structure which limits the selectivity
to NO and extends the response to NO-related products.

In our previous study, the NTG-derived NO concentration measured in the vascular
media of isolated canine femoral arteries was about 90 nmol l⁻¹ for 100 µmol l⁻¹ NTG
(Mochizuki et al 2001), whereas in the present study the NTG-derived NO concentration in
plasma was about 2 nmol l⁻¹ (estimated plasma NTG concentration 70 µmol l⁻¹). NTG is
metabolized in the vascular media and thus NTG-derived NO is produced in the media which
diffuses into the surrounding tissues. Therefore, NTG-derived NO concentration measured
in the media is much higher than in the lumen where NO is diluted and oxidized by blood
(oxyhaemoglobin). It should also be noted that the sensor location (near the endothelium or
in the middle of lumen) might have affected the measured NO level due to different diffusion
distances and oxidation of NO by dissolved oxygen and oxyhaemoglobin.

The present study promises that the newly developed NO sensor can be a valuable tool for
the evaluation of in vivo NO dynamics. Pharmacological evaluation of drugs that release NO,
stimulate NO production and/or inhibit NO production can also be quantitatively performed
in vivo. The recent study by Perticone et al (2001) suggested that forearm endothelial
dysfunction is a marker of future cardiovascular events in hypertensive patients. Thus, it
may also be possible to apply this sensor to clinical diagnosis of endothelial dysfunction, i.e.
reduced endothelium-derived NO availability in the cardiovascular system.

5. Conclusion

The new NO sensor demonstrated high sensitivity, selectivity and durability. Applicability to
in vivo measurement was also proved in the rat study.
Acknowledgments

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