Pharmaceutica Analytica Acta
ISSN: 2153-2435

Shintani, Pharm Anal Acta 2013, 4:6
DOI: 10.4172/2153-2435.1000251

Short Communication Open Access

Measurements of Nitric Oxide and Peroxynitrite

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Keywords: Nitric Oxide; Peroxynitrite; Measurement; Macrophage

Introduction

Although there is much interest in NO (nitric oxide) and peroxynitrite (OONO), their measurement in biological media is difficult because of their short lifetimes and low concentrations. Here, the use of an NO-sensitive electrode is first described for direct real-time measurement of NO in endothelial cells. A method for detecting peroxynitrite produced by the macrophage is also given. Brief references to other methods are included in the comments sections.

NO measurement with NO-sensitive electrodes

Calibration [1]

Protocol

1. Prepare KH solution (HEPES-buffered modified Krebs-Henseleit solution), containing NaCI (137 mM), KCI (1.1 mM), $CaCI_2 \cdot 2H_2O$ (0.18 mM), $MgCI_2 \cdot 6H_2O$ (0.1 mM), $NaHCO_3$ (4.2 mM), glucose (5.6 mM), and HEPES (5 mM). After addition of optional reagents adjust pH to 7.4 with NaOH (0.1 M).
2. Immerse NO electrode in KH solution (20 mL) and read basal current after stabilization.
3. Immerse NO electrode in freshly prepared SNAP solution (1 mM, 20 mL) and read NO current after stabilization (waiting for ca 5 min).
4. Repeat steps 2 and 3 three times and calculates mean NO-dependent current.

Although calibration of the electrode can be performed with authentic NO solution, this calibration is not practical because it is difficult and troublesome to maintain the buffer solution anaerobic; and the concentration of authentic NO solution should be determined by other methods, e.g. the oxyhaemoglobin method [2].

Comments

1. NO measurements can be performed with an NO monitor (NO-501, lnter Medical, Japan) with an NO-sensitive electrode (NOE-47, 200 mmφ, Inter Medical, Japan), or with an isolated nitric oxide meter (ISO-NO Mark II, World Precision Instruments, USA) with an NO sensor (ISO-NOP 200, World Precision Instruments, USA). All experiments must be performed with electromagnetic shields, made of an iron mesh screen, to avoid external noise. Plastics which are easily charged with electricity can cause unexpected noise.
2. New electrodes (both working and counter electrodes from Inter Medical) should be immersed for one day in buffer solution before use. It takes more than 30 min to stabilize basal current after first connecting the electrode and turning on the NO monitor. The electrodes are stored with the tips immersed in the buffer.

The Measurement of NO Produced by Endothelial Cells

Protocol

1. Perfuse bovine aortic endothelial cells confluently cultured on a cover slip (15 mmφ), with L-arginine solution (L-arg, MW = 174.2, 50 μM).
2. Place NO electrode as close as possible to the cover slip without making contact. Start recording the basal level of electrode current and wait until the current has stabilized (ca 30 min).
3. Switch the perfusate to 1 mM ATP/50 mM L-arg solution to stimulate endothelial cells. ATP (Mr = 551) should be stored at -20°C.
4. Re-switch the perfusate to 50 μM L-arf solution and confirm that the current returns to the basal level.

Comments

1. Use a perfusion system as shown in Figure 1. It is important to use a hydrostatic pressure of ca 40 cm H_2O so that the flow is constant, because fluctuations of the flow rate cause noise on the electrode current. All perfusate should be prepared by dissolving reagents in KH solution and adjusting the pH to 7.4.

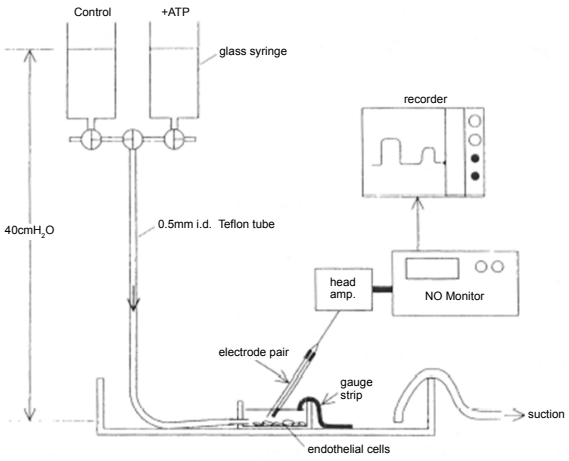

Figure 1: A perfusion system for measuring NO generated from endorhelial vells.

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Received May 20, 2013; **Accepted** June 17, 2013; **Published** June 20, 2013

Citation: Shintani H (2013) Measurements of Nitric Oxide and Peroxynitrite. Pharm Anal Acta 4: 251. doi:10.4172/2153-2435.1000251

Nitric oxide synthases (NOS) which produce NO in biological systems consist of three isoforms. Among these NOS-l (neuronal type) and NOS-3 (endothelial type) transiently produce a relatively small amount of NO in response to external signalling molecules. To elucidate these transient NO responses, highly sensitive and real-time methods such as chemiluminescence methods [3,4] or an electrochemical method [1] are required. On the other hand, spectrophotometric [5] and fluorimetric [6] methods are convenient for determining total NO production from NOS-2, which persistently produces a relatively large amount of NO. These methods can be applied to assay NO_2^-, the end products of NO, accumulated in incubation media for 6 to 48 h.

Quantitation of Peroxynitrite Produced by Macrophages

Protocol

1. Prepare Hank's balanced salt solution (HBSS) by dissolving HBSS powder (Gibco, 450-1201) and $NaHCO_3$ (0.35 g/L) in doubly distilled water (pH 7.4).
2. Prepare a stock solution of phorbol・12・myristate-12 acetate (PMA, Sigma; 1 mg/mL) in DMSO. Divide into 100-mL aliquots, and store below -80°C (in the deep freeze).
3. Incubate macrophages (8×10^6 cells/mL) in HBSS containing PMA (400 ng/mL), 4-hydroxyphenylacetic acid (4・HPA・MW 152.15; 1 mM) and Cu, Zn-superoxide dismutase (SOD; 0.1 mg/mL) for 4 h at 37°C.
4. Centrifuge the solution (1000 rpm for 10 min) to remove macrophages.
5. Acidify supernatant with H_3PO_4 (10%) and add acetonitrile (final con- centration 20% v/v).
6. Pass through a 0. 45-μm membrane filter.
7. Apply the sample to a 4.6 mm×150 mm C-18 (ODS) reversed-phase HPLC column equilibrated with 20:80 (v/v) acetonitrile-phosphate buffer (pH 3.2, 10%).
8. Elute the column with a linear gradient of 20 to 60% acetonitrile over 10 min at a flow rate of 1 mL/min.
9. Monitor UV absorption at 360 nm to assay 4-hydroxy-3-nitrophenyl.acetic acid (NO_2-HPA).

Comments

Peroxynitrite nitrates phenol derivatives in the presence of redox-active metal complexes such as Fe^{3+}-EDTA (1 mM) and Cu, Zn-SOD (0.1g/mL). Because relatively large amounts of Fe^{3+}-EDTA have toxic side-effects and promote reactions of reactive oxygen species, Cu, Zn-SOD is used as the copper catalyst although it scavenges superoxide and might reduce peroxynitrite generation. In this method, peroxynitrite is assayed as a nitrated phenol derivative (NO_2-HPA) and separated by HPLC. It has also been reported that a manganese porphyrin complex has negligible SOD activity, and is thus an excellent catalyst for this nitration reaction [7].

Peroxynitrite formation *in vivo* can also be estimated by measuring nitrotyrosine by means of an HPLC [8,9] or immunohistochemical [10] method. Because there are several routes for nitrotyrosine formation (for example, nitrite + peroxidase + tyrosine), nitrotyrosine itself is not such a specific indicator for peroxynitrite, although the efficiency of peroxynitrite at producing nitrotyrosine is high. Thus, control experiments with NOS inhibitors and scavengers of reactive oxygen species should be performed to determine the origin of nitrotyrosine.

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