Published in final edited form as:

Methods Mol Biol. 2011 ; 704: 81–89. doi:10.1007/978-1-61737-964-2_7.

Detection of Low Levels of Nitric Oxide Using an Electrochemical Sensor

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Abstract

Nitric oxide produced from nitric oxide synthases mediates various physiological and pathological events in biological systems. However, quantitative assessment of nitric oxide from biological sources remains a difficult task. Here we describe a procedure for the quantification of low levels of nitric oxide using a nitric oxide – selective electrochemical sensor. Nitric oxide is oxidized to nitrite and/or nitrate and accumulated in the aqueous media. First, nitrate in biological fluids or culture media is converted to nitrite by an enzymatic method. Nitrite is then chemically converted to equimolar NO in an acidic iodide bath, where nitric oxide is detected by the sensor. Using this method, the present study demonstrates siRNA-mediated suppression of nitric oxide synthase 3 leading to a significant decline of basal nitric oxide production in human umbilical vein endothelial cells. Basal nitric oxide production from HUVECs is also shown to be inhibited by N^G -nitro-L-arginine methyl ester but not by N^G -nitro-D-arginine methyl ester (D-NAME). The analytical method presented here provides a sensitive and convenient tool for measuring basal and stimulated nitric oxide production from biological sources.

Keywords

Nitric oxide; nitrate; nitrite; electrochemical sensor

1. Introduction

The biological importance of nitric oxide (NO) has been well established in the past decades. As a consequence, various methods have been developed to directly or indirectly assess the low levels of NO produced from biological sources. They include spectrophotometric, fluorometric, electrochemical, chemiluminescence, and electron spin resonance methods. Indeed, some of these methods appear to be highly sensitive and selective, but they also require expensive and highly technical equipment making them inaccessible to many potential users.

NO is very unstable with a half-life of 2–30 s in aqueous media and rapidly reacts with molecular oxygen to form nitrite (1). In the presence of oxidizing species such as oxyhemoproteins, nitrite is further oxidized to nitrate (2). Nitrite or nitrate (collectively referred to as NO_x) accumulate in extracellular fluids such as sera, urine, and cell culture media (3). Therefore the detection of NO_x has been a useful method to assess NO production from biological sources (4, 5) when direct monitoring of NO is impractical.

Berkels et al. (6) reported a method to measure NO_x by converting them to NO which is detected by an amperometric NO sensor (6). This method is attractive because it provides a rapid and easy assay while requiring relatively inexpensive instruments that are affordable for laboratories. Furthermore, a new version of the NO sensor with higher sensitivity has become commercially available (7).

Here we present a detailed procedure for the quantification of NO_x using a new version of the NO sensor. The sensor is very specific to NO because the gas membrane eliminates all ions and other compounds except gases, and the applied electrical potential and electrode material eliminate interferences from other gases such as O₂, CO, and CO₂. The sensor used in this study generates 100-fold higher current than the previous version, providing a more stable signal, with a detection limit of 0.1 nM in comparison with 2 nM of the older sensors (*see* ^{Note 1}) (6, 7).

This analytical method is based on the conversion of nitrite to NO in acidic iodide solutions (Fig. 7.1), which can be detected by the sensor. The percent conversion of nitrite to NO in the acidic iodide bath is quantitative as demonstrated by a wide range in linearity between electric current changes and the amount of nitrite added (7).

Nitrate is not converted directly to NO in the acidic iodide bath. Thus, the conversion of nitrate to nitrite is necessary to measure the total amount of NO formed by the cells. The conversion can be achieved by enzymatic or chemical reductions of which the former is much more efficient. Berkels et al. (6) used nitrate reductase for this purpose (6). In the present method, the reaction of nitrate reductase (Fig. 7.1) is coupled to a glucose 6-phosphate dehydrogenase reaction (Fig. 7.1), as in Verdon et al. (8). The conversion of nitrate to nitrite using this coupled enzyme system, termed nitrate reductor (NR), appears to be close to 100% (7). The coupled enzyme system, although its use instead of a single system may look tedious, avoids potential incomplete reduction of nitrate due to NADPH shortage.

A big advantage of the current analytical method is that the total NO release from cells in culture can be measured because the degradation products of NO (nitrate/nitrite) accumulate and are stable in the culture media. In the present study, basal NO production is determined in cultured human umbilical vein endothelial cells (HUVECs). In order to examine the specificity and usefulness of the current method, cells are subjected to molecular, biological, and pharmacological treatments leading to the inhibition of nitric oxide synthase 3 (NOS3) expression or enzyme activity, and the resulting changes in NO production are determined.

2. Materials

2.1. Cell Culture

1. HUVECs and endothelial cell growth medium EBM-2 with endothelial growth supplements.

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¹The apparent sensitivity of the NO sensor used in this study was 1 pA = 15 pM. The detection limit of the sensor was about 0.1 nM which generated 7 pA of current over the noise level (± 2 pA).

2. Fetal bovine serum and antibiotic antimycotic solution.
3. Cell culture plasticware: 100 mm petri dishes and 6-well plates.
4. 0.2% gelatin.
5. Solutions of trypsin (0.25%) with 0.38 g/L of EDTA.

2.2. Small Interfering RNA (siRNA) Transfection

1. Human NOS3 siRNA (1299001, HSS107237) with nucleotide sequences corresponding to the coding region of a human NOS3 gene transcript (NCBI GenBank accession number, NM_000603.3) and a negative control oligoribonucleotide duplex with scrambled sequences. The nucleotide sequences of the NOS3 siRNA are as follows: 5'-GAA GAG GAA GGA GUC CAG UAA CAC A-3' (sense) and 5'-UGU GUU ACU GGA CUC CUU CCU CUU C-3' (antisense).
2. Lipofectamine RNAiMAX and Opti-MEM (Invitrogen).

2.3. Assay for NO Production

1. Dulbecco's modified Eagle's medium (DMEM) without phenol red formulated to contain 8.3 g/L DMEM powder, 1 g/L D-(+)-glucose, 1 g/L L-glutamine, 110 mg/L sodium pyruvate, and 3.7 g/L sodium bicarbonate (NaHCO₃). Sterilize by filtration.
2. 100 mM L-NAME) and D-NAME. Sterilize by filtration (*see* ^{Note 2}).
3. Standard solutions of sodium nitrite (NaOH₂) (0.1–10 μM). Prepare daily from the stock solution (10 mM).
4. Acidic iodide bath (20 mM potassium iodide (KI) in 0.1 M sulfuric acid (H₂SO₄)). Prepare fresh daily.
5. Nitrate reductor (NR) consists of 0.2 units/mL nitrate reductase, 0.4 units/mL glucose 6-phosphate dehydrogenase, 2 mM glucose 6-phosphate, and 2 μM NADPH in 14 mM Na-P buffer (pH 7.4). Stock solutions of each component are prepared in 14 mM Na-P buffer (pH 7.4) and kept frozen at –80°C. Just prior to use, all of these four components are combined in the appropriate volume of Na-P buffer and kept on ice.
6. The NO measuring system: NO model T supplied by Innovative Instruments, Inc. The system comes with software and several types of amperometric NO sensors. The software is installed onto a computer and the NO Model T system is connected to the computer. The AmiNO700 sensor provided has the highest sensitivity for NO detection.

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²L-NAME is a specific inhibitor of nitric oxide synthase while its stereoisomer, D-NAME, is not.

2.4. Western Blotting

1. Cell lysis buffer (10 mM Tris-Cl, 150 mM NaCl, 5 mM EDTA, 0.1% sodium dodecyl sulfate, 1% tritonX-100, 1% deoxycholate pH 7.2) supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail.
2. DC protein assay kit (Biorad).
3. Standard solution of bovine serum albumin.
4. Polyvinylidene difluoride membrane.
5. Tween-tris-buffered saline (TTBS) (10×): 2 M NaCl, 200 mM Tris, pH 7.5, 1% Tween-20.
6. Blocking buffer and antibody dilution buffer: 5% (w/v) skim milk in TTBS.
7. Primary antibodies: monoclonal NOS3 antibody, monoclonal β -actin antibody.
8. Secondary antibody: goat antimouse IgG conjugated to horseradish peroxidase.
9. ECL kit.

3. Methods

3.1. Cell Culture and siRNA Transfection

1. HUVECs are cultured on 0.2% gelatin-coated 100 mm petri dishes at 37°C and 5% CO₂ in complete growth medium: endothelial cell growth medium (EBM-2) supplemented with endothelial growth supplements, fetal bovine serum (10%), and antibiotics (100 U/mL penicillin, 100 μ g/mL streptomycin, and 0.25 μ g/mL amphotericin B).
2. Adhering cells are detached using trypsin/EDTA solution and resuspended in growth medium without antibiotics. Cells are seeded on 6-well plates (2×10^5 cells/well) and grown for 1 day in growth medium without antibiotics.
3. Cells at ~50% confluency are washed with Opti-MEM and treated with 25 nM NOS3 siRNA (or NC) and 2 μ L/mL Lipofectamine RNAiMAX in 1 mL Opti-MEM for 3 h. Then, 1.5 mL of growth medium without antibiotics is added and cells are incubated for 1 day.
4. Cells are washed with DMEM, followed by incubation in the same medium for 24 h. Some medium is incubated in wells without cells to provide control media needed for the correction of the endogenous levels of NOx in the culture media (*see*^{Note 3}).
5. The conditioned media and cells are harvested separately.

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³Due to the sensitivity of the current analytical method, the background NOx level in culture media is easily detected. To correct this value, no cell control media was prepared by incubating media only without cells.

3.2. Treatment of Cells with L-NAME and D-NAME

1. Cells are seeded on 6-well plates at a density of 2×10^5 cells/well and grown for 2 days in complete growth medium.
2. Cells are washed with DMEM and treated with 1 mM D-NAME or L-NAME in the same medium followed by a 24-h incubation. Some medium is incubated in wells without cells to provide control media needed for the correction of the endogenous levels of NO_x in the culture media.
3. After these treatments, the conditioned media and cells are harvested separately.

3.3. Calibration with the Standard Nitrite Solutions

1. Set up the NO measuring system, in NO model T.
2. The NO sensor is polarized by connecting to the NO system and is immersed in water for a few hours or overnight.
3. The sensor is equilibrated in the 10 mL acidic iodide bath until the background current is stabilized.
4. Add an aliquot (50 μ L) of NaNO₂ standard solutions to acidic iodide bath and monitor current changes due to NO generation. In the acidic iodide bath, nitrite is quantitatively reduced to NO which is detected by the sensor. The peak current increases with the amount of nitrite added.
5. Wait until the current reduces to a background value, and then conduct the second addition.
6. Construct a calibration curve, the current peak vs. the amount of nitrite added.

3.4. Determination of NO_x in the Conditioned Media

1. After treatment of the cells, the conditioned medium (including control media) is harvested.
2. An aliquot (100 μ L) of the conditioned medium is mixed with a solution (100 μ L) of NR to reduce nitrate to nitrite.
3. The mixture is incubated at room temperature for 45 min and kept on ice during analysis.
4. A 50–100 μ L sample is added to the acidic iodide bath (10 mL) while stirring with a small magnetic bar.
5. Record the current change generated by an NO sensor (Fig. 7.2).

3.5. Western Blotting

1. After treatments, cells are washed in ice-cold PBS and harvested in 100 μ L/well cell lysis buffer. Cell suspensions are incubated on ice for 45 min and centrifuged at 13,400 rcf for 5 min to obtain clear cell lysates.
2. Protein content of cell lysates is determined using a Bio-Rad DC assay kit.

3. Dilute the portion of protein sample to be analyzed 4:1 (v/v) with 5× Laemmli buffer and heat for 5 min at 95°C in a sealed microcentrifuge tube.
4. Prepare SDS-PAGE gels in an electrophoresis apparatus.
5. Load equal amount of the protein samples (20 µg protein) into wells. Load control wells with molecular weight standards.
6. Connect the power supply and run at 80 V for 40 min and at 120 V for 2 h until the bromophenol blue tracking dye reaches the bottom of the separating gel.
7. When electrophoresis is complete, gels are removed and subjected to electrophoretic protein transfer to a polyvinylidene difluoride membrane at 100 V for 1 h or 30 V for 14 h at 4°C.
8. After protein transfer, the membrane is washed in TTBS and then incubated in a blocking solution.
9. The membrane is incubated with a primary antibody (1:1,000) for 2 h with agitation at room temperature, followed by three washes with TTBS.
10. The membrane is incubated with a secondary antibody (1:3,000) for 1 h with agitation at room temperature, followed by three washes with TTBS.
11. Soak the membrane for 30 s in ECL substrate solution.
12. Remove the membrane, drain, and place face down on a sheet of plastic wrap.
13. In a dark room, place an X-ray film onto the membrane. Expose film for a few minutes.
14. Band intensities are analyzed using the NIH ImageJ program (Fig. 7.3).

3.6. Statistical Analysis

Statistical analysis was performed by Student's *t*-test. A *p*-value of less than 0.05 based on at least three or more independent experiments was considered to be statistically significant.

Acknowledgments

This work was supported by funding from a National Institute of Health grant HL87012, HL75209, and a World Class University Project (HJ) from the Ministry of Science, Technology and Education of S. Korea. It was also supported by Basic Science Research Program through the National Research Foundation funded by the Ministry of Education, Science and Technology, Republic of Korea (2009-0071171) (YB).

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Fig. 7.1.

The conversion of nitrite to nitric oxide in acidic iodide solution (1). For the enzymatic conversion of nitrate to nitrite, nitrate reductase requires the activity of glucose 6-phosphate dehydrogenase (2 and 3).

Fig. 7.2.

The panel represents a typical recording demonstrating the molecular and pharmacological inhibition of basal NO production. HUVECs were transfected with a scrambled sequence (NC) control oligonucleotide or with NOS3 siRNA for a 3-h period followed by a 24-h incubation in complete growth medium. Pharmacological inhibition of NOS3 was achieved following a 24-h exposure to 0.1 nM D-NAME or L-NAME. Controls are blank medium to correct for basal levels of NO_x and conditioned medium removed from the cells.

Quantification of NO production derived from the NO model T recording. Data represent mean $\pm$ SEM ($n = 3$). b's represent significant differences compared with the corresponding a's.

Fig. 7.3.

The cell lysates were analyzed by Western blot using antibodies specific to NOS3 or β -actin to show relative expression levels of NOS3 proteins. Blots shown are representatives of at least three independent experiments.