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Detection of Nitric Oxide Formation in Primary Neural Cells and Tissues

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Abstract

Nitric oxide (NO) is a free radical molecule with a short half-life (<5 s). Because its synthesis from L-arginine by constitutive NO synthase (NOS) is low in many cell types, including neurons and endothelial cells, direct detection of NO in biological systems is a difficult task. During pathological conditions in the CNS, the inducible form of NOS (iNOS or NOS2) is expressed in activated astrocytes and microglial cells and can result in higher levels of NO. However, it may still be difficult to detect NO in these cell types using typical spectrophotometric methods. Of particular note, NO is readily oxidized to nitrite and nitrate (relatively stable products) in cells and medium, which can be measured as a valid indicator of NO synthesis. The conversion of NO to peroxynitrite leads to the formation of stable protein adducts that can be detected by immunohistochemical or immunofluorescence methods. Additionally, intracellular levels of NO can be detected in real time using fluorescence imaging and NO-specific, cell permeable indicator dyes.

Keywords

Nitric oxide; Peroxynitrite; HPLC; Immunofluorescence

1. Introduction

To circumvent the problem of detecting low levels of NO in neural cells and tissues, we present several methods based upon analytical, immunohistochemical, and fluorescence imaging approaches. The analytical approach describes a simple, rapid, sensitive, and specific high performance liquid chromatography (HPLC) method for detecting picomole levels of nitrite and nitrate in biological samples (1). In this method, nitrite reacts with 2,3-diaminonaphthalene (DAN) under acidic conditions to yield 2,3-naphthotriazole (NAT), a highly fluorescent product, which is stable in alkaline solution (2). Reversed-phase HPLC effectively separates NAT from DAN and other fluorescent compounds in samples, permitting fluorescence detection of NAT. Separation of these products is critical to overcoming issues surrounding poor limits of detection inherent in spectrophotometric analysis of DAN/NAT.

NO rapidly reacts with superoxide to form peroxynitrite, a potent electrophile that readily forms covalent adducts with proteins, leading to profound mitochondrial inhibition and neurotoxicity (3). Immunohistochemical detection of 3-nitrotyrosine (3-NTyr) protein

adducts thus enables region- and cell-specific detection of NO production relevant to CNS pathology. Immunofluorescence detection of 3-NTyr protein adducts offers the additional advantage of employing antibodies to identify particular cells or structures in conjunction with 3-NTyr adducts, thus allowing a finer level of discrimination of the precise cell type in which NO/peroxynitrite adducts are being formed. We have employed this technique to identify protein adducts in both neurons in vivo (4) and astrocytes in vitro (5).

Finally, a method is presented for the detection of intracellular production of NO in real time using fluorescence imaging. The application of cell permeable NO-specific fluorescent indicator dyes, such as 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM), permit the detection of NO in live cultured cells. The fluorescence signal of DAF-FM is low until reaction with NO, which produces a stable covalent benzotriazole product with much greater quantum yield of emitted fluorescence (6). Using this approach, steady-state increases in NO caused by inflammatory stimuli or rapid increases in NO caused by drugs or endogenous agonists can be reliably detected in real time (7, 8).

2. Materials

2.1. Analytical Determination of NO by High Performance Liquid Chromatography

1. Nitrate reductase (Roche).
2. 2,3-Diaminonaphthalene (DAN, Sigma).
3. C₈ column (15 cm × 4.6 mm, 5 μm) and C₁₈ column (5 cm × 4.6 mm, 40 μm) (both from Supelco, Bellefonte, PA).
4. HPLC-grade methanol and HPLC-grade water (both from Fisher Scientific, Houston, TX).
5. Waters HPLC apparatus including a Model 600E Powerline multisolvent delivery system with 100-μl heads, a Model 712 WISP autosampler, a Model 474 fluorescence detector, and a Millennium-32 Workstation (Waters, Milford, MA).

2.2. Determination of NO/ONOO Formation by Immunofluorescence in Fixed Cells

1. Cell culture plates and compatible thin microglass coverslips (e.g., 24-well culture plate and 12-mm round glass cover slips).
2. Wash buffer: phosphate-buffered saline (PBS) 10× stock containing 1.37 M NaCl, 0.027 M KCl, 0.01 M Na₂HPO₄, and 0.02 M KH₂PO₄; adjust the pH to 7.4 with HCl. Stock may be sterilized by filtration or autoclave if desired. 1× Solutions may then be easily prepared from this stock.
3. 100% Methanol, stored at −20°C.
4. Triton X-100, diluted to 0.1% v/v in PBS.
5. Blocking and antibody dilution buffer: 1% w/v bovine serum albumin (BSA) in PBS.
6. Primary antibody (e.g., anti 3-nitrotyrosine, Chemicon, Temecula, CA).

7. Secondary antibody (e.g., Alexa Fluor-labeled secondary antibody, Molecular Probes, Eugene OR).
8. Microscope slides.
9. Coverslip mounting media (Vectashield mounting media with DAPI, Vector Laboratories, Burlington, CA).
10. Clear nail polish.
11. Fluorescence microscopy detection and quantification capabilities (see Note 1).

2.3. Qualitative Real-Time Detection of NO Formation in Live Cells Using Fluorescence Imaging

1. Round glass coverslips for microscopy, 30 mm diameter (Warner Instruments, Hamden, CT). These are suitable for use with the Zeiss POC Mini incubation chamber (Carl Zeiss Microimaging, Thornwood, NY). Alternately, adherent cells may be subcultured onto chambered coverglass slides; typically 4-well chamber slides are used (Nalge Nunc International, Rochester, NY).
2. 2 mM DAF-FM diacetate (4-amino-5-methylamino-2',7'-difluorofluorescein diacetate; Invitrogen, Carlsbad, CA); working stock solution in DMSO (see Note 2).
3. Imaging Medium: Hank's buffered salt solution (HBSS), with HEPES: 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.3 mM CaCl₂, 1.0 mM MgSO₄, and 10 mM HEPES (pH 7.4). Do not add sodium bicarbonate to this solution.
4. 10 mM Adenosine triphosphate, sodium salt (Sigma-Aldrich, St. Louis, MO); working stock solution in 18 Ω MilliQ water. Make fresh and keep on ice.
5. 1–10 mM *S*-nitroso-*N*-acetylpenicillamine (SNAP), (Invitrogen, Carlsbad, CA); working stock solution in DMSO at concentrations appropriate for the specific experiment to be performed.
6. Phosphate-buffered saline (PBS), pH 7.4.
7. Fluorescence microscopy detection and quantification capabilities (see Note 1).

¹Fluorescence images may be acquired by wide field or confocal microscopy using either an upright or inverted microscope equipped with analysis software suitable for the quantitation of individual cellular fluorescence in multiple channels. The system used to collect the images presented here consists of a Zeiss Axiovert 200M inverted microscope with 20–100 $\times$ Plan Apochromat objectives, ORCA-ER CCD camera (Hamamatsu), DG-4 xenon flash lamp rapid filter changer (Sutter), and a motorized xy stage (Proscan stage by Prior). The software used for both acquisition and analysis is Slidebook (v5.0, Intelligent Imaging Innovations, Denver, CO).

²Fluorescent indicator dyes for live cell imaging are typically easily oxidized and very light sensitive. It is, therefore, highly recommended to purchase the catalog number listed for DAF-FM, which is 1 mg total solid material packaged in ten individual 50 μ g amounts. DMSO is then added immediately before the experiment and the vial should be wrapped in aluminum foil to protect from light. It is acceptable to leave this stock solution at room temperature during the experiment. If the working stock solution is to be used for more than one experiment, it should be stored at -20°C . If the working stock solution is to be stored for longer than 1 week, it is helpful to gently flush the headspace with nitrogen and then wrap the tightly capped tube in parafilm before storing at -20°C .

3. Methods

3.1. Analytical Determination of NO by High Performance Liquid Chromatography

1. Filter all samples to be tested through 10-KDa cutoff ultrafilters to remove large molecular weight proteins. Wash filters four times with deionized and double-distilled water (DD-H₂O) prior to use. Dilute filtered samples with DD-H₂O (e.g., 2–3 times for medium from neuron or endothelial cell culture medium) depending on concentrations of nitrite and nitrate.
2. Reaction of nitrite with DAN. This is performed as follows: 100 µl of diluted sample, diluted blank medium, or sodium nitrite standard (0–2 µM) is incubated with 100 µl of DD-H₂O and 20 µl of 316 µM DAN (in 0.62 M HCl) at room temperature for 10 min, followed by the addition of 10 µl of 2.8 M NaOH. This solution is vortexed before analysis by HPLC.
3. Analysis of the nitrite-DAN derivative (NAT; see Fig. 1). 15 µl of the nitrite-DAN derivatization solution is directly injected into a 5-µm C₈ column guarded by a 40-µm C₁₈ column for chromatographic separation of NAT. The mobile phase (1.3 ml/min) is 15 mM sodium phosphate buffer (pH 7.5) containing 50% methanol (1 l of 30 mM Na₂HPO₄ and 125 ml of 30 mM NaH₂PO₄ mixed with 1.125 l of 100% methanol) (0.0–3.0 min), followed sequentially by 100% HPLC-grade water (3.1–5.0 min), 100% methanol (5.1–8.0 min), 100% HPLC-grade water (8.1–10.0 min), and the initial 15 mM sodium phosphate buffer (pH 7.5)–50% methanol solution (10.1–15.0 min). All chromatographic procedures are carried out at room temperature. Fluorescence is monitored with excitation at 375 nm and emission at 415 nm. NAT is rapidly eluted from the column and its retention time is approximately 4.4 min (Fig. 2).
4. Analysis of nitrate. Nitrate is converted to nitrite as follows: 200 µl of diluted sample or nitrate standard (0–2 µM), 10 µl of 1 U/ml nitrate reductase, and 10 µl of 120 µM NADPH are mixed and incubated at room temperature for 1 h. This solution is then used directly for nitrite analysis as described for step 3, above. (The conversion of nitrate to nitrite is 98% as determined with known amounts of both standards).

3.2. Determination of NO/ONOO Formation by Immunofluorescence in Fixed Cells and Tissues

1. Remove the cell culture media and rinse the coverslips with 1× PBS by adding 0.5–1 ml of PBS, and then aspirating (see Note 3).
2. Fix the cells by covering the glass coverslip with methanol prechilled to –20°C, and store at –20°C for 10 min. This step will precipitate proteins and remove lipids.
3. Remove the methanol and wash the cells by adding 0.5–1 ml of 1× PBS.

³These instructions are optimized for primary cortical astrocytes subcultured onto 12-mm coverslips and grown to confluence in a standard 24-well cell culture plate. This protocol is easily adapted to different cell types and coverslip sizes; however, reagent volumes and antibody dilutions will need to be empirically optimized. Unless otherwise noted, all steps may be performed at room temperature.

4. Remove the PBS and permeabilize the membranes by adding 0.1% Triton X-100 for 30 s.
5. Remove the Triton X-100 and perform three, 5 min washes using 1× PBS with gentle agitation using a plate shaker.
6. Remove the final PBS wash, add 1% BSA (w/v) in PBS, and incubate with gentle agitation for 1 h to block.
7. Remove the blocking solution, add the primary antibody, and incubate overnight at 4°C with gentle agitation. We have found our optimal dilution with this antibody to be 1:600; however, this will likely vary based on cell type and other parameters. Furthermore, using a standard 24-well cell-culture plate, 250 ml of antibody solution is typically sufficient to coat the coverslip, provided the plate is agitated using a rotary shaker rather than a plate rocker.
8. Remove the primary antibody and perform three, 5-min washes using 1× PBS.
9. Remove the final wash, add the secondary antibody, and incubate for 1 h in a light-proof container. Because the fluorescent label on the secondary antibodies is light sensitive, limit the exposure to ambient light. The secondary antibody incubation and all subsequent wash steps should be conducted in a light-proof container, such as foil-wrapped plastic ware.
10. Perform three, 5 min washes in 1× PBS, using a light-proof container.
11. One standard microscopy slide can easily accommodate two 20 mm coverslips. Place a small drop (e.g. 3–5 µl) of mounting media on the slide, and place the coverslip cell-side down on top of the drop, taking care to minimize air bubbles.
12. Blot excess mounting media gently with a Kimwipe, and seal the edge of the coverslip by gently applying a bead of clear nail polish around the perimeter of the coverslip.
13. Place the slides in a slide box, and store at 4°C, allowing several hours (overnight) for the nail polish to dry.
14. Fluorescent imaging is conducted using a Zeiss 20× air Plan Apochromatic objective (Carl Zeiss, Inc., Thronwood, NY) using Slidebook v 5.0 (Intelligent Imaging Innovations, Inc., Denver, CO). Regardless of the imaging system employed, image saturation, or photobleaching should be avoided by using the smallest possible exposure time. An example of immunofluorescence detection of 3-NTyr adducts is shown in Fig. 3a.
15. Multiple approaches regarding signal quantification may be employed; however, our laboratory prefers to record the total signal per field, normalized to the number of cells, thus giving a representation of immunofluorescence signal per cell. In our experience, this method has proven more reliable than quantifying mean signal per field, as this latter approach may not accurately represent the robustness, or number of cells adversely affected, by a given treatment. An example of quantifying the fluorescence intensity of 3-NTyr adducts is shown in Fig. 3b.

3.3. Qualitative Real-Time Detection of NO Formation in Live Cells Using Fluorescence Imaging

1. Prior to imaging, allow cells cultured on microscopic grade cover glass to reach approximately 75% confluency.
2. Add DAF-FM (from the 2 mM working stock solution) to 2 μ M final concentration in culture medium and incubate 15 min at 37 C, 5% CO₂.
3. Replace the dye incubation solution with Imaging Medium prewarmed to room temperature. All subsequent steps and imaging may be performed at room temperature. Do not wash the cells; simply removed the dye incubation solution and replace directly with Imaging Medium.
4. Mount the coverslip holder or chambered slide on the stage of the inverted microscope and focus using a 20 $\times$ objective.
5. Imaging parameters for detecting steady-state production of NO:
 - Filter set – fluorescein/FITC (490 nm excitation/515 nm emission). For confocal imaging, a 488 nm laser line is used for excitation. If imaging using a confocal system, attenuate the laser power as much as possible to avoid photobleaching the dye.
 - To minimize exposure time, resolution is typically reduced, e.g., 2 $\times$ 2 binning on a charge-coupled device (CCD)-based wide field system. Exposure times should be limited to 20–50 ms if at all possible.
 - Set up parameters for multiple exposures over time to collect one image per minute for 20 min, which allows a steady state to be reached between evolution of NO and reaction with DAF-FM to yield the highly fluorescent benzotriazole derivative.
 - Initiate the imaging sequence as rapidly as possible after mounting cells on the microscope stage to avoid saturation of the DAF-FM signal.
 - An example of steady-state imaging of NO production in astrocytes induced by the NO donor, SNAP, is shown in Fig. 4a.
6. Imaging parameters for detecting rapid increases in NO induced by treatment with drugs or agonists:
 - Filter set – fluorescein/FITC (490 nm excitation/515 nm emission). For confocal imaging, a 488 nm laser line is used for excitation. If imaging using a confocal system, attenuate the laser power as much as possible to avoid photobleaching the dye.
 - To minimize exposure time, resolution is typically reduced, e.g., 2 $\times$ 2 binning on a CCD-based wide field system. Exposure times should be limited to 20–50 ms if at all possible.
 - Set up parameters for multiple exposures over time to collect one image per second for 60–300 s.

- Initiate the imaging sequence and add drug or agonist at the desired time, typically after 10 s (per the parameters given here, this would be the tenth exposure after initiation of the imaging sequence). It is important to collect images for at least the first five exposure times before adding drug or agonist to achieve a steady baseline of fluorescence signal.
 - An example of detecting rapid increases in NO in astrocytes induced by treatment with ATP is shown in Fig. 4b.
7. Analysis. Using the masking feature of the imaging software, place a mask on each cell in the microscopic field to define individual regions of interest for analysis. Export data for the background-subtracted fluorescence intensity of each region of interest. Data are expressed as the mean fluorescence intensity of each region of interest relative to the baseline image for that region. This value is expressed as F/F_0 , where F is the fluorescence intensity of a region of interest (e.g., a single cell) at any given exposure time divided by F_0 , the fluorescence intensity of the same region of interest in the first image collected for the series, the “time zero” or baseline image. Data can then be expressed as the fold-change in DAF-FM signal from the first to the last image in the series, or from the first image to whichever image represents the appropriate point of steady-state production of NO, based upon the shape of the curve (see Note 4).

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⁴When calculating F/F_0 , it is useful to represent the baseline fluorescence intensity as the average of the fluorescence intensity of each region of interest of the first three images in the sequence. This helps to correct for variations in fluorescence signal that often occur when performing single cell imaging. To calculate the F/F_0 value, divide the fluorescence intensity of a given region of interest at time (t) by the average intensity of the same region for the first three images in the sequence.

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Fig. 1.
Reaction of nitrite with 2,3-diaminonaphthalene (DAN) to form 2,3-naphthotriazole (NAT)
under acidic conditions. Reprinted from ref. 1, with permission from Elsevier.

Fig. 2.

Analysis of nitrate in biological samples using the HPLC-DAN method. Nitrate was reduced to nitrite by nitrate reductase. Nitrite reacts with 2,3-diaminonaphthalene (DAN) to yield under acidic conditions. DAN was separated from NAT by reversed-phase HPLC followed by fluorescence detection at 375 nm excitation wavelength and 415 nm emission wavelength. (a) 200 nM nitrate standard; (b) endothelial cell culture medium; (c) plasma; (d) urine. Reprinted from ref. 1 (with permission from Elsevier).

Fig. 3.

Inflammatory stimulation astrocytes increase total cellular protein nitration. Astrocytes were exposed for 8 h to 10 μ M 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in combination with the cytokines tumor necrosis factor alpha (TNF- α , 10 pg/ml) and interferon gamma (IFN- γ , 1 μ g/ml). **(a)** Immunofluorescence detection of 3-nitrotyrosine adducts was performed in conjunction with antibodies against the astrocyte-specific marker glial fibrillary acidic protein (GFAP) to confirm the identity of the cell type. Nuclear morphology was detected using the fluorescent DNA stain, 4',6-diamidino-2-phenylindole

(DAPI, 10 μ M in mounting medium) to permit cell counting. **(b)** Total 3-nitrotyrosine signal per field was normalized to the number of cells by counting nuclei and presented as a ratio. Quantitative analysis demonstrates an elevated presence of adducts following the inflammatory stimulation, and prevention of this response with the selective neuronal nitric oxide synthase inhibitor 7-nitroindazole (7-NI). Reprinted from ref. 5 (with permission from Elsevier).

Fig. 4.

Real-time detection of NO in live primary cultured astrocytes. (a) Primary cortical murine astrocytes were loaded with DAF-FM and examined for increases in NO following addition of increasing concentrations of the NO donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP). (b) Rapid changes in intracellular NO were detected using DAF-FM imaging in astrocytes following the addition of ATP to stimulate purinergic receptor-mediated calcium transients.