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Simultaneous monitoring of intra- and extracellular nitric oxide in living cells by means of dual-color fluorescence imaging

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4,4-Difluoro-8-(3,4-diaminophenyl)-3,5-bis(4-methoxyphenyl)-4-bora-3a,4a-diaza-*s*-indacene
Disodium 2,6-disulfonate-1,3-dimethyl-5-hexadecyl-8-(3,4-diaminophenyl)-4,4'-difluoro-4-bora-3a,4a-diaza-*s*-indacene

ABSTRACT

A dual-color fluorescence imaging method for simultaneous monitoring of intra- and extracellular nitric oxide (NO) was developed. Assisted by confocal laser scanning microscope, the intra- and extracellular NO can be successfully visualized by using two selected probes, 4,4-difluoro-8-(3,4-diaminophenyl)-3,5-bis(4-methoxyphenyl)-4-bora-3a,4a-diaza-*s*-indacene (*p*-MOPB) and disodium 2,6-disulfonate-1,3-dimethyl-5-hexadecyl-8-(3,4-diaminophenyl)-4,4'-difluoro-4-bora-3a,4a-diaza-*s*-indacene (DSDMHDAB), which display distinct membrane permeability and show different colors of fluorescence after reaction with NO. Results indicated that intra- and extracellular NO could be fluorometrically detected without mutual interference. The applicability of the proposed method was validated by dual-color imaging of NO on both sides of the plasma membrane in RAW 264.7 murine macrophages and human vascular endothelial (ECV-304) cells. This multi-labeling approach using multi-laser excitation and multi-color fluorescence detection holds great promise for simultaneous analysis of NO as well as other gasotransmitters in living cells with subcellular resolution.

1. Introduction

Nitric oxide (NO) is a gaseous signal molecule in organisms involved in numerous physiological and pathological processes, such as cell differentiation, growth regulation, vasodilation, immune response, and neurotransmission, etc [1–6]. Accumulating evidence suggests that beneficial or deleterious effects of NO in cells are determined by its concentration [7–11]. Therefore, the balance between intra- and extracellular NO levels plays a vital role in maintaining normal life activities [12–14]. In order to understand the physiological and cellular functions of NO comprehensively, it is desirable to simultaneously monitor the NO produced in cells and the NO released to the outside of the cell. However, NO is present at nanomolar levels in the body with high reactivity and diffusibility [15,16], making the simultaneous determination of NO with subcellular resolution more difficult.

In attempts to investigate NO in biological systems, intracellular NO and extracellular NO are usually determined separately with different analytical approaches including chemiluminescence, fluorescence, electron paramagnetic resonance spectroscopy (EPR) and electrochemistry [17–24]. Apparently, the data obtained from different techniques and different samples cannot exactly elucidate the production and the diffusion of NO in the same cell. Up to now, only one combined system for the simultaneous monitoring of intra- and extracellular NO produced by glioblastoma cells has been proposed, using a fluorescent probe for fluorescence imaging of intracellular NO and a chemically modified electrode for sensing extracellular NO [25]. However, this combined system suffer from complicated pretreatment, and the electrochemical determination is just suitable for local monitoring of NO at the electrode tip [26,27]. On account of the high performance characteristics of fluorescent probe-based analytical methods in term of selectivity, sensitivity and flexibility [28–35], the fluorescent probe-based analytical methods offer a potentially attractive way to realize

Abbreviations: NO, nitric oxide; EPR, electron paramagnetic resonance spectroscopy; *p*-MOPB, 4,4-difluoro-8-(3,4-diaminophenyl)-3,5-bis(4-methoxyphenyl)-4-bora-3a,4a-diaza-*s*-indacene; DSDMHDAB, 2,6-disulfonate-1,3-dimethyl-5-hexadecyl-8-(3,4-diaminophenyl)-4,4'-difluoro-4-bora-3a,4a-diaza-*s*-indacene; NOC 13, 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-methyl-1-triazene; JS-K, O^2-(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate; PBS, phosphate buffered saline; BODIPY, borondipyrromethene.

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simultaneous monitoring of intra- and extracellular NO in the same biological sample by means of designing site-specific fluorescent probes for NO. As we reported previously, a robust and facile strategy based on a water-soluble fluorescent probe and a capillary electrophoresis system was proposed to quantify the released and remaining NO from the single cell [36]. Although NO on both sides of the plasma membrane was quantified in the same single cell, they were trapped successively rather than simultaneously which cannot reflect the real-time situation of NO release.

In this work, we presented a simple and efficient method for simultaneous visualization of NO on both sides of the plasma membrane based on the dual-labeling strategy. The membrane-permeable probe, 4,4-difluoro-8-(3,4-diaminophenyl)-3,5-bis(4-methoxyphenyl)-4-bora-3a,4a-diaza-*s*-indacene (*p*-MOPB), was used for intracellular NO and the membrane-targeted one, disodium 2,6-disulfonate-1,3-dimethyl-5-hexadecyl-8-(3,4-diaminophenyl)-4,4'-difluoro-4-bora-3a,4a-diaza-*s*-indacene (DSDMHDAB), was for extracellular NO. With the same NO trapper, *o*-phenylenediamine, these two probes can trap NO released inside and outside of cells almost at the same speed and respond to real-time NO release more accurately. Excited by two lasers with different wavelength, bright turn-on red fluorescence was observed intracellularly upon the production of NO in cells and green fluorescence appeared on the outer surface of the plasma membrane while NO was released out of cells. Using RAW 264.7 murine macrophages and human vascular endothelial (ECV-304) cells as models, the proposed method was successfully applied to dual-color imaging for simultaneous monitoring of intra- and extracellular NO produced in cells.

2. Materials and methods

2.1. Reagents and chemicals

Dulbecco's modified Eagle's medium (DMEM) was purchased from Thermo scientific (Waltham, MA, USA). Roswell Park Memorial Institute (RPMI) 1640 medium was obtained from Gibco. Fetal bovine serum (FBS) was taken from Tianhang Biological Technology (Zhejiang, China). Penicillin, streptomycin and trypsin were obtained from Amresco (Solon, Ohio, USA). 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-methyl-1-triazene (NOC 13) was synthesized in our laboratory according to a literature method [37]. Lipopolysaccharide (LPS) and O^2-(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl) piperazin-1-yl]diazen-1-ium-1,2-diolate (JS-K) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Interferon-γ (IFN-γ) was purchased from ProSpec-Tany (Rehovot, Israel). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was got from Biosharp (Hefei, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA).

Phosphate buffered saline (PBS) solution was consisted of 8.00 g/L NaCl, 0.20 g/L KCl, 0.24 g/L KH_2PO_4, 3.63 g/L $Na_2HPO_4 \cdot 12H_2O$, and pH value was adjusted to 7.4 with 1.0 M HCl and 0.1 M NaOH in this work.

Saturated NO solution (at 25 °C, NO ≈ 2.0 mM) was prepared by bubbling purified NO gas into deoxygenated PBS buffer (pH 7.4) and standard solutions were freshly prepared for each experiment with reference to literatures [38,39]. JS-K was dissolved in dimethyl sulphoxide (DMSO) to give a stock solution of 5 mM and diluted to desired concentration for use. LPS and IFN-γ were dissolved in sterilized PBS solution for use. *p*-MOPB [40] and DSDMHDAB [41] were synthesized in our laboratory and dissolved in DMSO to prepare stock solutions of 1.0 mM and 2.5 mM, respectively.

2.2. Instruments

The pH values of solutions were measured by a FE20 pH meter (Mettler-Toledo, Shanghai, China). The cells were cultured in a Forma Series II 3111 Water-Jacketed CO_2 Incubator (Thermo Scientific, USA). Cytotoxicity was determined on a Thermo Scientific microplate reader. Fluorescence imaging experiments were performed on a Nikon confocal laser scanning microscope (TE2000, Japan). Images in the green channel (515/30 nm) were excited with a laser of 488 nm and that in the red channel (650 LP) were excited with a laser of 543 nm. The objective used for imaging was a 60 × oil-immersion objective (Nikon). Images and merges were obtained with an EZ-C1 software. HPLC analysis was performed on a C_{18} column (5 μm, 150 mm × 4.6 mm i.d., Kromasil, Bohus, Sweden) in a Shimadzu LC-20A HPLC system with RF-10AXL fluorescence detector (FLD).

2.3. Cell culture

RAW 264.7 murine macrophages were obtained from Center for Animal Experiment/ABSL-3 Laboratory of Wuhan University (Wuhan, China) and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin at 37 °C in a 5% CO_2/95% air incubator. Human vascular endothelial (ECV-304) cells were purchased from the China Center for Type Culture Collection (Wuhan, China) and grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS and 1% penicillin and streptomycin at 37 °C in a 5% CO_2/95% air incubator.

2.4. Cell viability studies

RAW 264.7 murine macrophages were grown in 96-well plates at an initial density of 10^5 cells per well. After incubation for 24 h, cell medium was removed and cells were divided into blank groups and experimental groups for further processing. The cells in blank groups were cultured in medium only, while the cells in experimental groups were cultured in medium containing *p*-MOPB and DSDMHDAB at total concentrations ranged from 0.2 μM to 100 μM as well as 0.5% (v/v) DMSO. After incubation for 24 h at 37 °C, MTT assay was performed according to the literature [42,43].

2.5. Dual-color fluorescence imaging of NO in living cells

Two days before experiments, the cells were detached with trypsin-EDTA solution and plated on 35 mm glass-bottomed dishes. For labeling, the cells were washed three times with PBS and incubated with *p*-MOPB for 20 min at 37 °C, and then loaded with DSDMHDAB for 2 min at room temperature. Afterwards, the cells were washed twice with PBS and then incubated with PBS containing NOC 13 or JS-K for a period of time. NOC 13 is an NO donor with a half-life of 13.7 min which can release NO both in the bulk solution and within cells [37,44], while JS-K is a GST-activated NO generator which can only stimulate the production of NO just within cells [45]. Subsequently, cells were subjected to imaging analysis. Experiments of each group were repeated three times at least.

For visualization of endogenous NO in living cells, RAW 264.7 murine macrophages were stimulated with LPS (500 ng/mL) and IFN-γ (500 U/mL) for 12 h. And then the cells were incubated with *p*-MOPB and DSDMHDAB for 20 min at 37 °C. After rinsed twice with PBS, the cells were subjected to imaging analysis.