Nitric Oxide 70 (2017) 59–67

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

Real-time visualization of distinct nitric oxide generation of nitric oxide synthase isoforms in single cells

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ARTICLE INFO

Article history:
Received 15 June 2017
Received in revised form
23 August 2017
Accepted 3 September 2017
Available online 4 September 2017

Keywords:
eNOS
geNOps
iNOS
Live-cell fluorescence imaging
Nitric oxide
nNOS

ABSTRACT

The members of the nitric oxide synthase (NOS) family, eNOS, nNOS and iNOS, are well-characterized enzymes. However, due to the lack of suitable direct NO sensors, little is known about the kinetic properties of cellular NO generation by the different nitric oxide synthase isoenzymes. Very recently, we developed a novel class of fluorescent protein-based NO-probes, the geNOps, which allow real-time measurement of cellular NO generation and fluctuation. By applying these genetic NO biosensors to nNOS-, eNOS- and iNOS-expressing HEK293 cells we were able to characterize the respective NO dynamics in single cells that exhibited identical Ca^{2+} signaling as comparable activator of nNOS and eNOS. Our data demonstrate that upon Ca^{2+} mobilization nNOS-derived NO signals occur instantly and strictly follow the Ca^{2+} elevation while NO release by eNOS occurs gradually and sustained. To detect high NO levels in cells expressing iNOS, a new ratiometric probe based on two fluorescent proteins was developed. This novel geNOp variant allows the measurement of the high NO levels in cells expressing iNOS. Moreover, we used this probe to study the L-arginine-dependency of NO generation by iNOS on the level of single cells. Our experiments highlight that the geNOps technology is suitable to detect obvious differences in the kinetics, amplitude and substrate-dependence of cellular NO signals-derived from all three nitric oxide synthase isoforms.

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1. Introduction

In higher organisms all three nitric oxide synthase (NOS) isoforms, neuronal, endothelial, and inducible NOS (nNOS, eNOS, iNOS), produce nitric oxide (NO) [1,2] that serves as important signal molecule for many cellular processes and biological functions [3–5]. The nNOS and eNOS isoenzymes are activated by Ca^{2+}/calmodulin [6]. In contrast, iNOS is constitutively active [7] and the amount of iNOS-derived NO generation is predominately controlled on the transcriptional level [8]. Based on their distinct expression profile in particular organs and cell types eNOS-, nNOS- and iNOS-derived NO is involved in either the control of vascular tone [9], neurotransmission [10], or the immune response [11], respectively. While NO rather freely diffuses through bio-membranes [12], the susceptibility of this radical for oxidation clearly restricts its area of action [13]. The rather local intra-, endo- and paracrine actions of NO are mainly mediated by NO-induced nitrosylation of heme-containing proteins and enzymes [14,15] like the soluble guanylate cyclase (sGC) that represents a well-characterized, highly NO-sensitive downstream target [16]. However, due to its chemical reactivity NO may also modify other biomolecules [17] and modulate diverse signaling pathways or, at very high concentrations, initiate cytotoxic damage [18]. In addition to the classical NO-sensitive mechanisms of vasorelaxation, neurotransmission, and immune defense, NO is further discussed to control many other cellular processes in (patho)-physiology [19–21]. Considering the short half-life [22] and limited diffusion [23] of NO, its various physiological activities are mainly determined by its local concentration and spatiotemporal distribution. Most of the current NO detection methods are indirect providing an end-point

Abbreviations: geNOps, genetically encoded fluorescent nitric oxide probes; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; IP_3, inositol 1,4,5-trisphosphate; NO, nitric oxide; FP, fluorescent protein; LNNA, N^G-nitro-L-arginine; $[Ca^{2+}]_{cyto}$, cytosolic Ca^{2+} concentration; $[NO]_{cyto}$, cytosolic NO concentration; WT, wild type.

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http://dx.doi.org/10.1016/j.niox.2017.09.001
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quantification of NO in cell populations [24] and deliver only limited information about the spatiotemporal patterns of cellular NO kinetics and fluctuation. Due to these technical limitations, the visualization of NO signals from distinct NOS isoforms in real-time and on the level of individual cells has not been comprehensively investigated. In this study we have exploited and refined recently developed genetically-encoded NO probes, the geNOps [25–27], to investigate NO dynamics in single cells. To ensure identical Ca^{2+} signaling for comparable activation of nNOS and eNOS, we utilized HEK-293 cells expressing either eNOS or nNOS. Our data highlight fast and pulsatile NO signals upon the activation of nNOS in single cells that appear strictly coupled to the cytosolic Ca^{2+} signal. In contrast, Ca^{2+}-activated eNOS showed a delayed NO generation and slowly increasing NO levels that remained elevated even after Ca^{2+} levels return to basal values. Cells expressing iNOS (HEK293 cells) showed strong and stable NO production that was merely dependent of extracellular L-arginine supplementation. Our experiments emphasize that the different NOS isoenzymes provoke distinct patterns of cellular NO signals, which most likely shape the activity of tissue-specific downstream signaling pathways and related organ functions.

2. Materials and methods

2.1. Reagents and buffers

Dulbecco's modified Eagle's medium (DMEM) and Nω-nitro-L-arginine (LNNA) were obtained from Sigma-Aldrich (Vienna, Austria). Fura-2-acetoxymethyl ester (fura-2/am), tetramethylrhodamine methyl ester perchlorate (TMRM) was purchased from Invitrogen (San Diego, CA, USA). TransFast™ transfection reagent was obtained from Promega (Mannheim, Germany). Antibodies against eNOS and nNOS were obtained from BD Transduction Laboratories™ (Schwechat, Austria), alpha-tubulin was from Cell Signaling Technology® (Cambridge, UK). Adenosine-5′-triphosphate (ATP) and L-arginine were purchased from Roth (Karlsruhe, Germany). Ionomycin was obtained from Abcam (Cambridge, UK). NOC-7 was from Santa Cruz (San Diego, CA, USA). The geNOps probes and the Iron(II) booster solution were from Next Generation Fluorescence Imaging GmbH - NGFI, Graz, Austria (www.ngfi.eu). Fetal Calf Serum (FCS), 100× Penicilin/Streptomycin, and Amphotericin were purchased form GIBCO (Invitrogen, Austria). Geneticin (G418) was purchased form Sigma Aldrich (Vienna, Austria).

2.2. Molecular cloning

In brief, cloning of the geNOp consisting of seCFP and tagRFP was performed according to standard procedures and all constructs were verified by sequencing. The rigid alpha-helical linker was synthesized by Genscript (Piscataway, NJ, USA) including the restriction sites *Bam*HI at the N-terminus and *Eco*RI at the C-terminus: GAGGCCGCCGCCCGGGAGGCCGCCGCCAGAGAGGC-CGCCGCC-AGGGAGGCAGCAGCCCGCGAGGCAGCAGCCCGGGAGGCTGCTGCCA-GAGAGGCTGCTGCCAGGGAGGCCGCCGCCCGCGAGGCTGCTGCCCGG GAGGCTGCAGCCAGA. Plasmid DNA encoding for the NO sensitive geNOps was provided from NGFI, Graz. cDNA encoding the double FP based ratiometric geNOp was subcloned into a pcDNA3.1(−) vector via the restriction sites *Xho*I (N-terminus) and *Hin*dIII (C-terminus).

2.3. Cell culture

Human embryonic kidney (HEK293) cells were cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. HEK293 cells stably expressing nNOS [28], eNOS or iNOS were cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1.25 μg/ml amphotericin, and 1 mg/ml geneticin (G418) in humidified atmosphere (95% O_2/5% CO_2) at 37 °C. Culture medium of EA.hy926 cells additionally contained 1% HAT (5 mM hypoxanthin, 20 μM aminopterin and 0.8 mM thymidine). Prior to transfection cells were plated on 30 mm glass cover slips. At 60–80% confluence cells were transfected with 1 ml of serum- and antibiotic-free medium containing 1.5 μg of the appropriate plasmid DNA and 2.5 μg of TransFast transfection reagent (Promega, Madison USA). Cells were maintained in a humidified incubator (37 °C, 5% CO_2, 95% air) for 16–20 h before replacing the culture media against DMEM containing antibiotics and FCS. All experiments were performed either 24 or 48 h after transfection.

2.4. Fluorescence imaging

For cytosolic Ca^{2+} recordings using fura-2, cells were incubated in storage buffer containing 3.3 μM fura-2/AM for 40 min as previously described [29]. For NO imaging cells were loaded with iron(II) fumarate solution for 20 min and subsequently incubated in a storage buffer composed of 138 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, 1 mM HEPES, 2.6 mM $NaHCO_3$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 10 mM D-glucose, 0.1% vitamins, 0.2% essential amino acids, and 1% penicillin/streptomycin, pH 7.4 for 1 h prior to experiments. All imaging experiments were performed using a semi-automatic gravity-driven superfusion system. During experiments cells were superfused with a HEPES-buffered solution (HBS) containing 138 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 $MgCl_2$, 10 mM D-glucose, 10 mM HEPES, pH 7.4 (NaOH or HCl adjusted) as previously described [29]. For Ca^{2+}-free conditions HBS containing 1 mM EGTA instead of $CaCl_2$ was used. Fluorescent recordings were taken on an advanced wide-field fluorescent microscope (Till Photonics, Graefling, Germany) equipped with a motorized sample stage, a polychrome V monochromator (Till Photonics), a 40× objective (alpha Plan Fluar 40×, Zeiss, Göttingen, Germany) and a charge-coupled device camera (AVT Stingray F145B, Allied Vision Technologies, Stadtroda, Germany). Fluorescence of C-geNOp and C-geNOp^{mut} transfected cells was recorded at 480 nm upon 430 nm excitation. For detecting NO dynamics of CR-geNOp, expressing cells were alternately excited at 430 and 570 nm and the emissions were collected at 480 and 590 nm, respectively. Multichannel imaging experiments for simultaneous recording of cytosolic Ca^{2+} and NO, and G-geNOp expressing cells loaded with fura-2/am were alternately excited at 340, 380 and 480 nm and emission was collected at 515 nm (515dcxr). SypHer fluorescence was sequentially excited at 430 and 500 nm. Data acquisition and control was carried out by the Live Acquisition 2.0.0.12 software (Till Photonics).

2.5. Image analysis

The background values recorded by the respective channels were subtracted from the emission of the probe to obtain F and calculate F_0 (reflecting the function of fluorescence of the probe over time without stimulation) using an appropriate equation e.g. $F_0 = F_{inital} * exp(-K * Time) + F_{plateau}$ in case of a fluorescence decrease reflected by a one exponential decay. To normalize the geNOp signals over time the formula $1\text{-}F/F_0$ was used for calculation, whereby F is defined as the background subtracted raw fluorescence over time.

2.6. Immunoblotting

Wild-type HEK293 cells or HEK293 cells stably expressing either

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