Available online at www.sciencedirect.com

SCIENCE DIRECT

Free Radical Biology & Medicine 38 (2005) 356–368

www.elsevier.com/locate/freeradbiomed

Original Contribution

Performance of diamino fluorophores for the localization of sources and targets of nitric oxide☆

Juan Rodriguez[a], Victoria Specian[a], Ronald Maloney[a], David Jourd'heuil[b], Martin Feelisch[a,*]

[a]*Department of Molecular and Cellular Physiology, LSU Health Sciences Center, Shreveport, LA 71130, USA*
[b]*Center for Cardiovascular Sciences, Albany Medical College, Albany, NY 12208, USA*

Received 12 August 2004; revised 15 October 2004; accepted 22 October 2004
Available online 23 November 2004

Abstract

An emergent approach to the detection of nitric oxide (NO) in tissues relies on the use of fluorescence probes that are activated by products of NO autoxidation. Here we explore the performance of the widely used NO probe 4,5-diaminofluorescein diacetate (DAF-2 DA) for the localization of sources of NO in rat aortic tissue, either from endogenous NO synthesis or from chemically or photolytically released NO from targets of nitrosation/nitrosylation. Of importance toward understanding the performance of this probe in tissues is the finding that, with incubation conditions commonly used in the literature (10 μM DAF-2 DA), intracellular DAF-2 accumulates to concentrations that approach the millimolar range. Whereas such high probe concentrations do not interfere with NO release or signaling, they help to clarify why DAF-2 nitrosation is possible in the presence of endogenous nitrosation scavengers (e.g., ascorbate and glutathione). The gain attained with such elevated concentrations is, however, mitigated by associated high levels of background autofluorescence from the probe. This, together with tissue autofluorescence, limits the sensitivity of the probe to low-micromolar levels of accumulated DAF-2 triazole (DAF-2 T), the activated form of the probe, which is higher than the concentrations of most endogenous nitrosation/nitrosylation products found in tissues. We further show that the compartmentalization of DAF-2 around elastic fibers further limits its potential to characterize the site of NO production at the subcellular level. Moreover, we find that reaction of DAF-2 with $HgCl_2$ and other commonly employed reagents is associated with spectral changes that may be misinterpreted as NO signals. Finally, UV illumination can lead to high levels of nitrosating species that interfere with NO detection from enzymatic sources. These findings indicate that while DAF-2 may still represent an important tool for the localization of NO synthesis, provided important pitfalls and limitations are taken into consideration, it is not suited for the detection of basally generated nitrosation/nitrosylation products.

Keywords: Nitric oxide; Fluorescence; DAF; DAR; Ascorbate; Glutathione; Nitrate; UV; Free radicals

Introduction

The discovery in 1987 that nitric oxide (NO) is the signaling molecule responsible for so diverse biological effects, such as endothelium-dependent smooth muscle relaxation, nerve cell communication, and antimicrobial defense in macrophages, precipitated a flood of interest in unraveling its full spectrum of actions in physiology and pathophysiology [1–3]. According to the PubMed database, >60,000 scientific studies have already addressed the involvement of NO in biology and medicine. Despite the enormous attention given to the ubiquitous role that this

Abbreviations: NO, nitric oxide; DAFs, diaminofluoresceins; DARs, diaminorhodamines; Asc, ascorbate; DAF-2 DA, 4,5-diaminofluorescein diacetate; DAF-2 T, DAF-2 triazole; L-NIO, N^{ϖ}-(1-iminoethyl)-L-ornithine dihydrochloride; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; IBMX, isobutyl methylxanthine; TCA, trichloroacetic acid; BSO, buthionine sulfoximine.

☆ This work was supported by grants CA 89366 (to D.J.) and HL 69029 (to M.F.) from the National Institute of Health.

* Corresponding author. Boston University School of Medicine, Whitaker Cardiovascular Institute, 650 Albany Street, X-305, Boston, MA 02118, USA. Fax: (617) 414 8151.

E-mail address: feelisch@bu.edu (M. Feelisch).

0891-5849/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2004.10.036

molecule plays in living systems, the mechanisms by which it fulfills these roles often remain unclear. Among the challenges involved in clarifying these mechanisms is the localization of NO sources and targets within a cell. This will require not only a molecular probe with high sensitivity and specificity for NO, but also resistance to the chemical and physical interventions often employed to free NO from its biological targets.

Recently, a family of fluorescence dyes became available commercially for the detection of NO, including diaminofluoresceins (DAFs) and diaminorhodamines (DARs) that are purported to deliver low nanomolar sensitivity. These indicators contain a benzoic group attached to a fluorophore that possesses a charge transfer state located energetically between the ground and excited states of the fluorophore moiety, which serves as an effective deactivation pathway for the highly fluorescent excited state [4,5]. When nitrosated by reactive nitrogen oxides such as N_2O_3 at one of its vicinal amino moieties, an internal diazotation reaction takes place that leads to formation of the corresponding triazole. This chemical modification of the dye by a secondary product of NO is associated with a lowering of the energetic charge-transfer state below the ground state of the fluorophore, thus removing the quenching effect of the benzoic structure and allowing the fluorophore to emit efficiently. The high yield of fluorescence of the triazole forms of DAF and DAR makes these indicators suitable for the detection of NO production in biological systems, a property that has been exploited by now in many studies using fluorometry [6–17], flow cytometry [18,19], high-pressure liquid chromatography (HPLC) [20], and fluorescence microscopy [4,14,20–58]. Furthermore, when used in conjunction with other probes, diamino fluorophores theoretically provide the option to test for colocalization of NO formation with other signaling events, such as a change in intracellular calcium concentration using Fura-2 [12,16,38], or its association with certain cell organelles by, e.g, the use of MitoTracker to specifically stain for mitochrondria [26,50].

The original aim of this study was to assess the viability of the widely used probe DAF-2 to locate and quantify biological targets of NO in a tissue previously characterized by chemical means, namely in aortic tissue [59,60]. The cellular products, which include nitrite, nitrate, RSNO, RNNO, and NO-heme species, are all known to release NO when exposed to light of appropriate wavelengths [60,61]. We therefore reasoned that exposure to light of wavelengths specific to individual forms of bound NO, in conjunction with DAF-2 incubation, would permit us to image the locations and concentrations of each of these species in intact tissues, with subcellular resolution and nanomolar sensitivity. As we struggled with this seemingly straightforward proposition, it became apparent that, while the development of diamino fluorophores represents a great analytical advance in NO bioimaging, there are a number of unresolved questions regarding the performance of these probes in biological tissues that impact their utility for the localization of sources and targets of NO. One important issue is that their sensitivity may be significantly limited in biological milieus by reducing agents such as ascorbate (Asc) and reduced glutathione (GSH), since these are known to scavenge nitrosating products such as N_2O_3, or their precursor NO_2. Several studies [6,46,66] have demonstrated this effect in buffer solutions, where substantial drops or even a total abolishment of formation of the fluorescent triazole form DAF-2 T were observed. Yet, only modest inhibition of DAF-2 activation is observed within cultured cells containing comparable levels of reducing agents [66]. Given this and the number of studies [6–58] now reporting the successful activation of DAF-2 in cell-cultures and tissues, it remains unclear how these probes can be effective in a cellular environment when they seem to perform so poorly in chemical systems of similar composition. A second issue concerns the artifactual activation of DAF-2 by NO photolysed from natural products of NO synthesis, including nitrosothiols, nitrosamines, nitrite, and nitrate [59]. The photolysis of these products is particularly effective with UV light [60,61], a problem of relevance to experimental setups utilizing illumination optics that unintentionally allow transmission of UV light, or intentionally utilize UV illumination to excite secondary probes, such as Fura-2, for the colocalization of concomitant biochemical events. A third issue relates to the reactivity of the probe with substances commonly used in the NO field to identify targets of nitrosation or nitrosylation, such as mercuric chloride and ferricyanide. A final question, central to the localization of sources/targets of NO, is whether the patterns of fluorescence observed with DAF-2 correspond to sites of activation or compartmentalization of the probe.

To address these issues, we investigated the nitrosation of the diamino fluorophore DAF-2 after loading of rat vascular tissue with its membrane-permeable diacetate analogue, DAF-2 DA, using optical measurements and HPLC analysis. These investigations were complemented with organ bath studies to investigate the functional integrity of dye-loaded tissues, assays for cGMP production to corroborate the production of NO, as well as control measurements performed in isolated buffer solutions. The results presented here help explain how DAF-2 nitrosation can compete for N_2O_3 with Asc or GSH and provide novel insight into its limits of sensitivity for the bioimaging of NO production. Moreover, we demonstrate that UV illumination can lead to significant probe nitrosation through the photoactivation of an NO-related metabolite of hitherto assumed biological inertness, and provide evidence for the chemical origin of the photoactivated species. We also show that substances such as glutathione, mercuric chloride, and ferricyanide elicit spectral changes in the probe that could be misinterpreted as fluorescence intensity changes. Finally, we demonstrate that the DAF-2 T patterns obtained in vascular tissue are not necessarily correlated to subcellular compartmentaliza-