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Original Contribution

On the specificity of 4-amino-5-methylamino-2′,7′-difluorofluorescein as a probe for nitric oxide

Aneta Balcerczyk[a,*], Mirosław Soszynski[a], Grzegorz Bartosz[a,b]

[a]*Department of Molecular Biophysics, University of Lodz, Banacha 12/16, 90-237 Lodz, Poland*
[b]*Department of Biochemistry and Cell Biology, University of Rzeszow, Rzeszow, Poland*

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Abstract

The specificity of 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) for nitric oxide was evaluated in in vitro systems. The probe was found fairly specific for nitric oxide. Potential sources of artifacts include the autoxidation of DAF-FM, potentiated by light, and its oxidation by sources of superoxide and peroxyl radicals, leading to fluorescence spectra indistinguishable from those of the nitric oxide adduct. Although DAF-FM reacts with peroxynitrite, this reaction seems to be of secondary importance under quasi-physiological conditions. On the other hand, a simultaneous presence of a nitric oxide source and a superoxide or hydrogen peroxide decreases or increases the fluorescence of DAF-FM, respectively, resulting in biased estimates of nitric oxide production.

Keywords: DAF-FM; Nitric oxide; Peroxynitrite; Xanthine oxidase; Superoxide; Hydrogen peroxide

Introduction

The involvement of nitric oxide in a plethora of vital biological functions in the cardiovascular, nervous, reproductive, and immune systems makes its detection and quantification of great interest. Among various methods of measurement of the production of nitric oxide in cellular systems, those based on the use of fluorogenic probes have been gaining popularity due to their simplicity and sensitivity. Probably the most successful indicator for nitric oxide has been 4,5-diaminofluorescein diacetate (DAF-2 diacetate) and FM 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA). These probes are membrane permeant and are deacetylated by intracellular esterases to 4,5-diaminofluorescein (DAF-2) and 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM), respectively. DAF-2 and DAF-FM are essentially nonfluorescent until they are nitrosylated by products of oxidation of nitric oxide, to form fluorescent heterocycles, which become trapped in the cytoplasm (Scheme 1). DAF-FM and (DAF-FM-DA) are even more useful than DAF-2 and DAF-2 diacetate since DAF-FM is more sensitive for NO than DAF-2 and the spectra of the NO adduct of DAF-FM are essentially independent of pH above pH 5.5 [1,2].

However, the specificity of DAF-2 for nitric oxide has been the subject of controversy. It has been suggested that

Abbreviations: ABAP, 2,2′-azobis(2-amidinopropane) hydrochloride; DAF-FM, 4-amino-5-methylamino-2′,7′-difluorofluorescein; DAF-FM-DA, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; DETA NONOate, (*Z*)-1-[*N*-(2-aminoethyl)-*N*-(2-amonioethyl)amino]diazen-1-ium-1,2-diolate; ECGS, endothelial cell growth supplement; GSH, glutathione; HBSS, Hank's buffered salt solution; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; HUVECs, human umbilical vein endothelial cells; MAHMA NONOate, 1-hexanamine, 6-(2-hydroxy1-methyl-2-nitrosohydrazino)-*N*-methyl NONOate; NO, nitric oxide; ONOO-, peroxynitrite; PMA, phorbol myristate acetate; RNS, reactive nitrogen species; ROS, reactive oxygen species; SIN-1, 3-morpholinesyndonimine acid; S-NAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitropruside; SOD, superoxide dismutase; Sulfo-NONOate, diazenesulfonic acid, hydroxy-, 1-oxide.

* Corresponding author. Fax: +48 42 6354476.
E-mail address: abal@biol.uni.lodz.pl (A. Balcerczyk).

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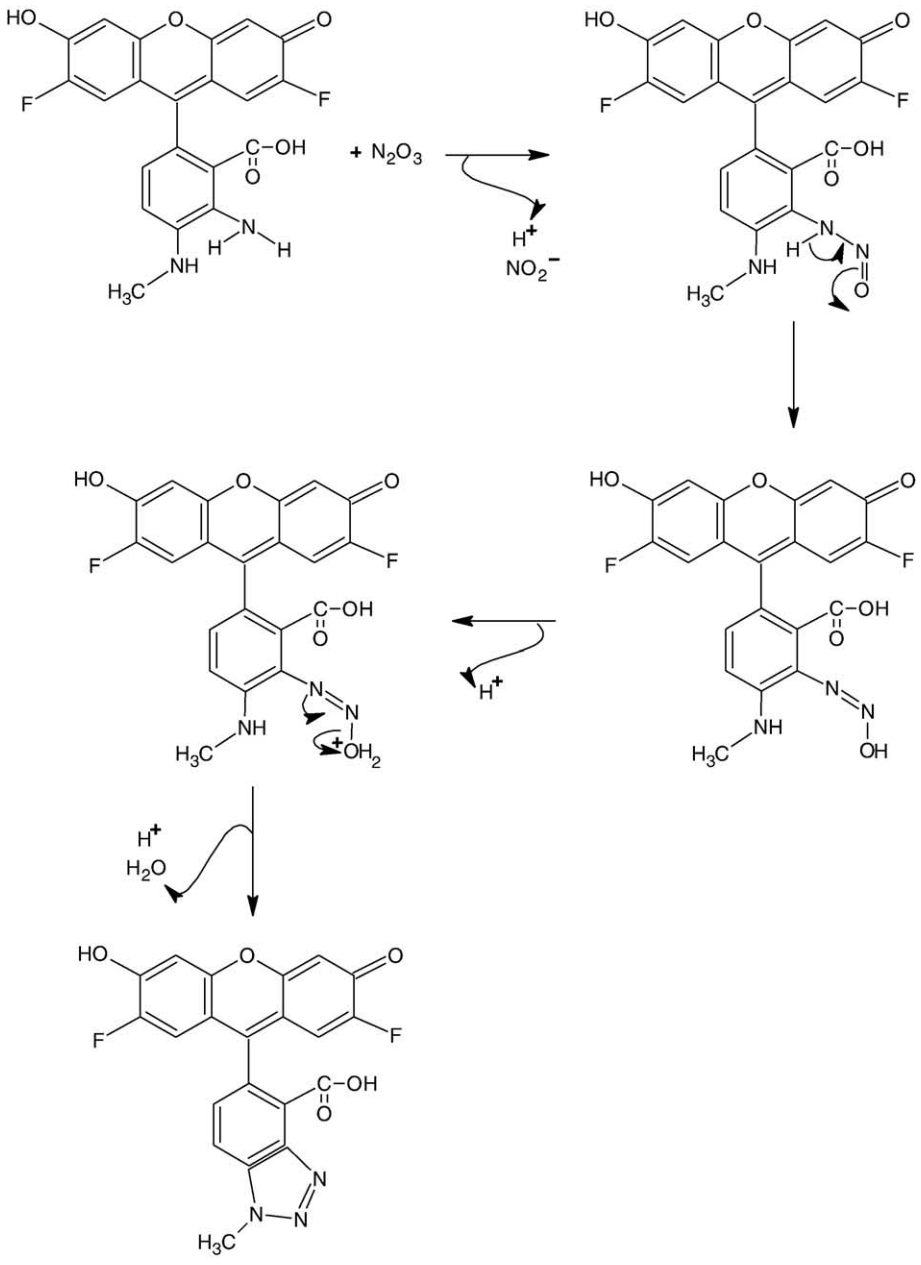

Scheme 1. Reaction of DAF-FM with oxidation products of nitric oxide.

DAF-2 reacts with peroxynitrite rather than nitric oxide [3], and that it reacts with dehydroascorbic acid and ascorbic acid, yielding products of very similar fluorescence emission profiles [4]. It has been reported [5] and rebutted [6] that DAF-2 is sensitive to the presence of divalent cations, especially calcium, in the medium, and that the rate of NO adduct formation is enhanced upon illumination.

This study was aimed at an evaluation of the specificity of reaction of DAF-FM with nitric oxide, in particular at an assessment of its reactivity with peroxynitrite vs nitric oxide and on its sensitivity to oxidants and autoxidation.

Materials and methods

Materials

Cell culture medium 199, fetal bovine serum, collagenase II, and antibiotics were purchased from Invitrogen (Paisley, UK). DAF-FM diacetate was from Molecular Probes (Leiden, Netherlands). 2,2′-Azobis(2-amidinopropane) hydrochloride (AAPH) was from Polysciences (Warrington, PA). NONOate donors were purchased from Cayman (Ann Arbor, MI). Endothelial cell growth supplement (ECGS) and other reagents were from Sigma (Deisenhofen, Germany).