Reliable in vitro measurement of nitric oxide released from endothelial cells using low concentrations of the fluorescent probe 4,5-diaminofluorescein

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Abstract 4,5-Diaminofluorescein (DAF-2) and its membrane-permeable derivate DAF-2 diacetate are fluorescent probes that have been developed to perform real-time biological detection of nitric oxide (NO). Their use for intracellular imaging, however, has recently been seriously questioned and data using DAF-2 for extracellular NO detection at low levels, as for example released from endothelial cells, are rare. Here we show that a reliable detection of low levels of NO in biological systems by DAF-2 is possible (a) by using low DAF-2 concentrations (0.1 μM) and (b) by subtracting the DAF-2 auto-fluorescence from the measured total fluorescence. The described method allows easy real-time detection of endothelial NO formation. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Nitric oxide; 4,5-Diaminofluorescein; Fluorescence spectroscopy; Endothelial nitric oxide synthase

1. Introduction

Nitric oxide (NO) is an arginine-derived radical playing a pivotal role in numerous physiologic as well as pathophysiologic processes. The molecule is being implicated in neurotransmission, immune function, hemostasis and vascular tone as well as in pathologic conditions as septic shock, atherosclerosis, ischemia/reperfusion injury, and carcinogenesis [1–3]. Reliable detection and quantification of NO is therefore a subject of significant biomedical interest.

NO is synthesized by NO synthases (NOS). These enzymes are either constitutively expressed (cNOS) synthesizing low levels of NO in the pico- to nanomolar range (NOS I, NOS III) or are induced by, for example, cytokines producing NO in the nano- to micromolar range (NOS II) [1]. The low cellular output from cells expressing a cNOS, like endothelial cells, and the short half-life of NO make its direct measurement extremely difficult.

Several methods have been developed over the past years to detect NO [4]. None of these methods, however, has the sensitivity, specificity and ease of use to be used routinely to measure low-output NO in an average equipped laboratory. Several years ago, Kojima et al. developed fluorescence probes based on the fluorescein chromophore which are able to detect intra- as well as extracellular NO [5–8]. The probe mostly used up to now is 4,5-diaminofluorescein (DAF-2). DAF-2 which itself shows only low fluorescence reacts with NO to the highly fluorescent triazolofluorescein (DAF-2T). DAF-2 is membrane-impermeable. To measure intracellular NO cells can be loaded with the membrane-permeable DAF-2 diacetate (DAF-2 DA) which is hydrolyzed to DAF-2 and thus trapped within the cell. DAF-2 DA was used in a number of studies detecting intracellular NO mainly by fluorescence microscopy and flow cytometry [9–11]. The reliability of this method, however, was recently seriously questioned by Broillet et al. showing that, in the presence of NO, divalent cations like Ca^{2+} increase DAF-2 fluorescence [12]. The Ca^{2+} sensitivity of DAF-2, thus, makes it difficult to distinguish an intracellular Ca^{2+} increase from an increase in intracellular NO. Measurement of extracellular NO released from cells by DAF-2 should be less prone to this problem and thus might offer an alternative to the intracellular bio-imaging by DAF-2 DA. Using the published method employing 10 μM DAF-2 [5–8] we were, however, unable to detect NO released from endothelial EA.hy926 cells. In fact, searching the literature, we found only two publications using DAF-2 or DAF-4 for extracellular NO measurement released from endothelial cells [7,13] implying that this method may be prone to error.

We, therefore, re-examined the use of DAF-2 for the extracellular measurement of low-output NO using the human endothelial cell line EA.hy926. We found that a reliable detection of low levels of NO in biological systems by DAF-2 is possible (a) by using lower DAF-2 concentrations (0.1 μM) and (b) by subtracting the DAF-2 auto-fluorescence from the measured total fluorescence. With these changes in the handling of DAF-2, we found this fluorescent probe indeed suitable to measure NO released from human endothelial cells.

2. Materials and methods

2.1. Chemicals

DAF-2, DAF-2T, the calcium ionophore A23187, N^G-monomethyl-L-arginine (L-NMMA) and the NO donor MAHMA·NONOate (NOC-9) were purchased from Alexis® Biochemicals (Grünberg, Germany). L-Arginine hydrochloride and dimethyl sulfoxide were pur-

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Abbreviations: DAF-2, 4,5-diaminofluorescein; L-NMMA, N^G-monomethyl-L-arginine; NO, nitric oxide; NOC-9, MAHMA·NONOate; PBS, phosphate-buffered saline; PMA, phorbol-12-myristate-13-acetate

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chased from Sigma (Deisenhofen, Germany). Phorbol-12-myristate-13-acetate (PMA) was purchased from Calbiochem (San Diego, CA, USA). Phosphate-buffered saline (PBS) contained 1.47 mM KH_2PO_4, 2.68 mM KCl, 137 mM NaCl, 0.90 mM $CaCl_2$, 0.49 mM $MgSO_4$ and 9.57 mM $NaPO_4$ (pH 7.4).

2.2. Cell culture

The human endothelial cell line EA.hy926 (kindly provided by Dr. Edgell, University of North Carolina, NC, USA) [14] were grown in Dulbecco's modified Eagle's medium without phenol red containing 584 mg/l L-glutamine (BioWhittaker Europe, Belgium) supplemented with 100 U/ml benzylpenicillin, 100 μg/ml streptomycin (PAN Biotech, Germany), HAT supplement (100 μM hypoxanthine/0.4 μM aminopterin/16 μM thymidine) and 10% fetal bovine serum (LifeTechnologies, Germany). For experiments, cells were seeded in six-well plates at a density of 0.4×10^6 cells/well. Assays were performed exactly 4 days after seeding, when cells reached the state of confluence, to ensure equal cell growth conditions for all experiments [15]. As a positive control, cells were stimulated with PMA (2 nM, 18 h) [16].

2.3. Spectrofluorimetric determination of NO released from endothelial cells

Cells were washed with PBS and then pre-incubated with L-arginine (100 μM in PBS, 5 min, 37°C). In some experiments as indicated, L-NMMA was added 5 min before the addition of L-arginine. Subsequently, 1 μM A23187 and DAF-2 at various concentrations as indicated were added and cells were incubated in the dark (37°C, 5 min). Then the fluorescence of the supernatants was measured at room temperature using a spectrofluorimeter (RF 1501, Shimadzu) with excitation wavelength set at 495 nm and emission wavelength at 515 nm. The band width was 10 nm for both excitation and emission. The sensitivity was programmed on *high*.

2.4. Data analysis

Analysis of data (column statistic, linear regression, statistical analysis) was performed using the software GraphPad PRISM® (GraphPad Software, Inc., San Diego, CA, USA).

3. Results and discussion

The measurement of NO by using the fluorescent probe DAF-2 is based on the reaction of DAF-2 with NO in the presence of O_2 under neutral pH, yielding the highly fluorescent DAF-2T (Fig. 1) [7]. Since we were unable to detect NO released from EA.hy926 endothelial cells using the published method [5–8] the following considerations were made: although DAF-2 and DAF-2T differ strongly in their fluorescence output, their absorbance as well as fluorescence maxima are nearly identical [5,6]. Consequently, measurement of DAF-2T includes a simultaneous detection of DAF-2. In high-output NO systems the contribution of the DAF-2 auto-fluorescence to the measured total fluorescence may be negligible due to the high quantum yield of DAF-2T, that is more than 180 times higher than that of DAF-2 [6,7]. In systems with a low output of NO, such as endothelial cells,

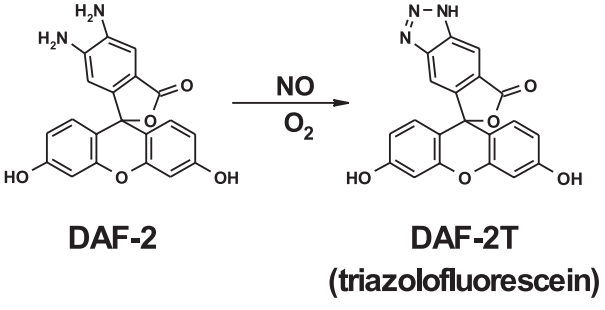

Fig. 1. Structures of DAF-2 and DAF-2T.

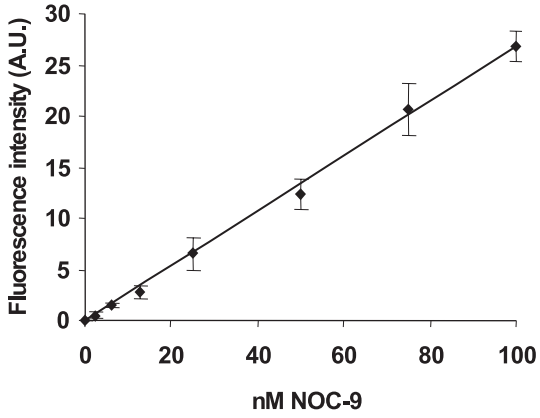

Fig. 2. Dependency of the fluorescence intensity on the NO concentration released from the NO donor NOC-9 using DAF-2 (0.1 μM). Confluent EA.hy926 cells were washed with PBS and incubated with 100 μM L-arginine (5 min). Then 0.1 μM DAF-2 and NOC-9 at various concentrations as indicated were added. The cells were incubated in the dark (37°C, 5 min) and the fluorescence of the supernatants was measured as described in Section 2. The fluorescence obtained from cells without NOC-9 addition was subtracted from each value. All data are mean ± S.D. ($n = 6$).

DAF-2 auto-fluorescence may, however, contribute considerably to the measured total fluorescence. Thus, a small increase in DAF-2T due to low levels of released NO may not be detectable.

We addressed this hypothesis by measuring the auto-fluorescence of various concentrations of DAF-2 in PBS in comparison to the fluorescence obtained from PBS supernatants of A23187-activated EA.hy926 cells containing [DAF-2–DAF-2T]. As expected, the difference in fluorescence intensities of DAF-2 in PBS and [DAF-2–DAF-2T] in supernatants of EA.hy926 cells was hardly detectable at higher DAF-2 concentrations (1–5 μM). In contrast, at low DAF-2 concentrations (0.01–0.1 μM) the fluorescence of EA.hy926 supernatants was significantly increased compared to DAF-2 in PBS (data not shown). This result suggests that detection of NO generated by endothelial cells demands low DAF-2 concentrations.

The use of low DAF-2 concentrations (e.g. 0.1 μM), however, raises the question whether this low amount is still sufficient to trap all NO released e.g. from endothelial cells. Considering the stoichiometry of the reaction between DAF-2 and NO that is proposed to be 1:2 [7], 0.1 μM DAF-2 should be consumed by 200 nM NO. Thus, if NO release reaches the nanomolar range 0.1 μM DAF-2 is likely to be rate limiting for the reaction.

In order to be able to calculate how much DAF-2 is exactly consumed in our system (using 0.1 μM DAF-2 and EA.hy926 cells) we created a standard curve for the DAF-2T fluorescence intensity (slope: 59.09 ± 0.3609; y-intercept: 0.8170 ± 0.1664; r^2: 0.999) and a curve correlating increasing amounts of NO (released from the NO donor NOC-9 in the presence of EA.hy926 cells) to the fluorescence intensity obtained after addition of 0.1 μM DAF-2. Fig. 2 shows that up to 100 nM NOC-9 (corresponding to 200 nM NO [17]) the correlation is linear ($r^2 = 0.997$) suggesting that the reaction, in spite of the above discussed stoichiometry, is not running out of DAF-2. The reason for this becomes clear when the amount of consumed DAF-2 is calculated by use of the DAF-2T standard curve: the highest applied concentration

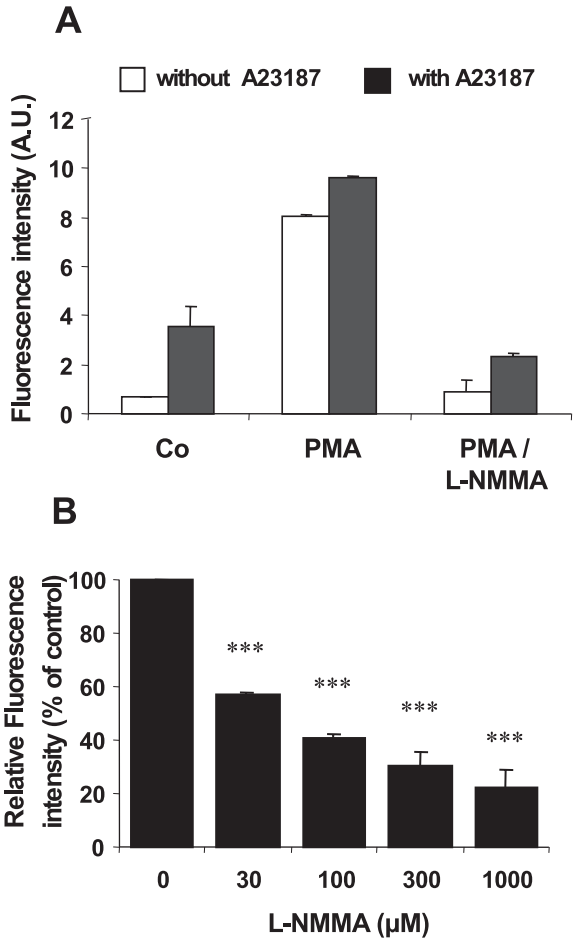

Fig. 3. DAF-2-detected NO released from EA.hy926 cells after PMA treatment in the absence or presence of A23187 or L-NMMA. A: Cells were either kept untreated (Co) or stimulated with PMA (2 nM) for 18 h. Then cells were washed with PBS, where indicated pre-incubated with L-NMMA (1 mM, 5 min), exposed for an additional 5 min to L-arginine (100 μM) and finally incubated with 1 μM A23187 and 0.1 μM DAF-2 (37°C, 5 min). B: Cells were stimulated with PMA (2 nM, 18 h). Then cells were washed with PBS, pre-incubated with L-NMMA as indicated (5 min) and further treated as in (A). Fluorescence of the supernatants was measured as described in Section 2. The auto-fluorescence obtained from PBS/DAF-2 (0.1 μM) was subtracted from each value. In (A) data are presented as mean ± S.D. ($n = 3$ in triplicate). In (B) values are given in % of the fluorescence detected in supernatants in the absence of L-NMMA and data are mean ± S.D. ($n = 3$). Statistical comparisons were made by ANOVA followed by a Bonferroni multiple comparison test. P values < 0.05 were considered significant. ***$P < 0.001$.

of NOC-9 (100 nM, corresponding to 200 nM NO) consumes only 0.44 nM DAF-2. Thus, less than 1% of all employed DAF-2 is converted in fact to DAF-2T.

Explanations for this finding may be, first, that other compounds, such as thiols, present in biological samples may react with NO and its oxidation products and thus influence the yield of DAF-2T [7]. Second, the mechanism of the reaction between NO and DAF-2 is complex: DAF-2 does not react with NO itself but with an active intermediate formed during the oxidation of NO [7]. Thus, the reaction yield of DAF-2T depends not only on the concentration of NO and DAF-2 but also on the oxidation rate of NO.

Taken together, the above results demonstrated that detection of low amounts of NO demands low DAF-2 concentrations and that a concentration of 0.1 μM DAF-2 is not rate limiting for the reaction with NO in the nanomolar range. Moreover, since we showed that less than 1% of DAF-2 is converted to DAF-2T, the background fluorescence formed by DAF-2 stays nearly constant (≥99%) and thus can be subtracted from the measured total fluorescence under the conditions used in our system (0.1 μM DAF-2, 5 min incubation time, 37°C). This will lead to a selective detection of DAF-2T.

In order to test this newly developed DAF-2 method based on (a) the use of low concentrations of DAF-2 (0.1 μM) and (b) the subtraction of DAF-2 auto-fluorescence from the measured total fluorescence, we employed known NOS III activators and inhibitors. Fig. 3A shows the fluorescence intensities of PBS supernatants in the absence or presence of the calcium ionophore A23187 (1 μM) of untreated cells, cells which were treated with the NOS III inductor PMA (2 nM, 18 h) [16] and cells treated with PMA and the NOS III inhibitor L-NMMA (1 μM). Addition of A23187, that increases intracellular free Ca^{2+} and thereby NOS III enzyme activity, increased fluorescence intensities in both untreated and PMA-activated cells. Supernatants of PMA-activated cells showed considerably increased fluorescence intensities compared to control cells in the presence as well as in the absence of A23187. The NOS III inhibitor L-NMMA reduced this fluorescence intensity significantly and dose dependently (Fig. 3B). These data demonstrate that indeed physiological amounts of endothelial NO can be detected using the method described here.

In summary, we optimized the use of the fluorescent probe DAF-2 in order to allow reliable detection of extracellular low-output NO from e.g. endothelial cells. Essential requirements for the detection of NO in the low-nM range were found to be (a) the use of low DAF-2 concentrations (0.1 μM) and (b) the subtraction of the DAF-2 auto-fluorescence from the measured total fluorescence. Our newly developed assay is simple, economical and sensitive and may therefore serve many laboratories as acceptable method for detecting biological low-output NO.

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