Direct evidence of nitric oxide production from bovine aortic endothelial cells using new fluorescence indicators: diaminofluoresceins

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Abstract The measurement of nitric oxide (NO) is important for direct examination of the regulatory roles of NO in various biological systems. Diaminofluoresceins (DAFs), new fluorescence indicators for NO, were applied to detect the release of NO from bovine aortic endothelial cells (ECs). DAFs react with NO to yield the corresponding green-fluorescent triazolofluoresceins, which provide the advantages of specificity, sensitivity and a simple protocol for the direct detection of NO. Using these DAFs, we could detect the generation of NO not only from inducible NO synthase expressed in macrophages, but also from constitutive NO synthase expressed in ECs.

Key words: Nitric oxide; Fluorescence; Diaminofluorescein; Endothelial cell; Macrophage

1. Introduction

Nitric oxide (NO), which is biologically generated through conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) [1], mediates many physiological and pathophysiological processes [2]. At least two distinct isoforms of NOSs have been identified, constitutive NOS (cNOS) and inducible NOS (iNOS). The cNOS, which is constitutively expressed in endothelial cells and central and peripheral neuronal cells, requires both calcium and calmodulin for its activation. Cells expressing cNOS generally produce small amounts of NO, because of their low levels of cNOS protein. On the other hand, iNOS, which is induced in cells stimulated with endotoxins, produced larger amounts of NO. Because of the short half-life, it is difficult to detect NO in situ directly, especially from cells expressing cNOS.

Several NO detection systems have been developed: a chemiluminescence assay system based on the reaction of NO with ozone [3], a spectrophotometric assay which measures the product of a nitrite azo-coupling reaction [4–7], or the oxidation product of HbO_2 [8], EPR analysis [9], voltammetric assay with an electrode [10], and fluorometric assay [11]. However, due to the limitations of low sensitivity, poor specificity, and complicated apparatus, none of these systems can be used for real-time and continuous assay of NO production from living cells. The colorimetric assay of azo-coupling was used with macrophages [4–7], but was not successful for NO detection from endothelial cells (ECs), which express cNOS.

In fluorometric assays, 2,3-diaminonaphthalene (DAN) has been used as a fluorescent indicator of NO. Although several improved fluorometric methods have been developed [12–14], DAN assay was only useful for cells expressing iNOS, such as macrophages. The DAN assay was not sensitive enough to detect the lower concentration of NO released from ECs.

Recently, we designed and synthesized diaminofluoresceins (DAFs) as novel fluorescent NO indicators [15]. The reaction of NO and DAFs yields the corresponding bright green-fluorescent triazolofluoresceins. Using these newly developed NO-reactive fluorescent indicators, it is feasible to detect the generation of NO not only from macrophages, but also from bovine aortic ECs by means of a simple protocol.

2. Materials and methods

2.1. Materials

DAF-2 and DAF-4 were synthesized according to the literature [15]. Bradykinin was purchased from Peptide Institute (Osaka, Japan). Recombinant mouse interferon-γ (IFN-γ) was purchased from Genzyme (Cambridge, MA, USA). Dimethyl sulfoxide (DMSO), L-arginine, N^G-monomethyl-L-arginine (L-NMMA), and N^G-monomethyl-D-arginine (D-NMMA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). lipopolysaccharide (LPS) (*E. coli* OIII: B4), N^G-Nitro-L-arginine methyl ester (L-NAME), N^G-nitro-D-arginine methyl ester (D-NAME), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Krebs-Ringer phosphate buffer (KRP) consists of 120 mM NaCl, 4.8 mM KCl, 0.54 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, and 15.9 mM sodium phosphate (pH 7.2). Phosphate-buffered saline (PBS) consists of 137 mM NaCl, 2.68 mM KCl, 0.90 mM $CaCl_2$, 0.49 mM $MgSO_4$, 1.47 mM KH_2PO_4, and 9.57 mM sodium phosphate (pH 7.4).

2.2. Cell culture

RAW 264.7 mouse macrophages were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 13 mM $NaHCO_3$, 20 mM glucose, 100 U/ml benzylpenicillin, 100 μg/ml streptomycin, and 10% (v/v) fetal bovine serum (Intergen, Purchase, NY, USA) at 37°C in a humidified atmosphere of 5% CO_2/95% air (1×10^5 cells/well).

Primary cultured ECs from bovine pulmonary arteries were passaged in DMEM containing 10% fetal bovine serum and were grown to confluence. Cultured cells were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air on a 24-well microplate (1.17 mg total protein/well).

2.3. Determination of NO from macrophages

RAW 264.7 macrophages were plated in wells of a 96-well microplate (1×10^5 cells/well), and were cultured for 6 h to make macrophages adherent. Cells were subsequently cultured for 16 h in 200 μl of fresh medium containing an appropriate concentration of LPS. In some experiments, IFN-γ (10 U/ml) was also added. The activated

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; DAF, diaminofluorescein; DAN, 2,3-diaminonaphthalene; L-NMMA, N^G-monomethyl-L-arginine; L-NAME, N^G-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; IFN-γ, interferon-γ; EC, endothelial cell

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cells adherent in wells were washed twice with KRP, and then 10 μM DAF-2, 1 mM L-arginine, and an appropriate concentration of L-NMMA or D-NMMA dissolved in 200 μl of KRP were added. After incubation for further 2 h, the supernatants were transferred to black microplates and the fluorescence was measured with a fluorescence microplate reader (Titertek Fluoroscan II, Flow Laboratories, McLean, VA, USA) calibrated for excitation at 485 nm and emission at 538 nm.

2.4. Determination of NO from endothelial cells

The culture medium was changed to serum-free medium 24 h before the experiment and the cells were further incubated at 37°C. After another 12 h, the ECs were washed twice with PBS and PBS containing 1 μM DAF-4, 100 μM L-arginine, 1 μM bradykinin, and an appropriate concentration of L-NAME or D-NAME was added. After further incubation for 30, 60, and 120 min, the supernatants were transferred to black microplates and the fluorescence was measured with a fluorescence microplate reader calibrated for excitation at 485 nm and emission at 538 nm.

2.5. Protein determination

Total protein concentrations of ECs were determined by BCA protein assay using BSA as the standard [16].

2.6. HPLC analysis

The HPLC unit consisted of a pump (PU-980, JASCO, Tokyo, Japan), a detector (FU-920, JASCO) and a column (Finepak SIL C18-5, 6.0×250 mm, JASCO). The eluent was 10 mM sodium phosphate buffer (pH 7.4)-acetonitrile (94:6, v/v). The flow rate was 1.0 ml/min. Fluorescence was monitored with excitation at 495 nm and emission at 515 nm.

2.7. Griess assay

The macrophages were stimulated with 200 μl of DMEM containing 10 ng/ml LPS and 10 U/ml IFN-γ for 48 h. Cultured supernatant (100 μl) was sampled, and immediately mixed with 100 μl of Griess reagent (1% sulfanilamide, 0.1% naphthalene-ethylenediamine dihydrochloride in 5% H_3PO_4). After 15 min, the product formation was colorimetrically (550 nm) determined. As a standard, $NaNO_2$ was reacted with Griess reagent.

3. Results

The fluorescence assay in this report was based on the reaction of DAFs with NO in the presence of dioxygen under neutral pH, yielding highly green-fluorescent triazolofluoresceins (Fig. 1). The fluorescence quantum efficiency is increased more than 180 times after the transformation of DAFs by NO to the triazole form [15].

A standard curve for DAF-2 T and DAF-4 T fluorescence intensity was calculated. The fluorescence intensity showed

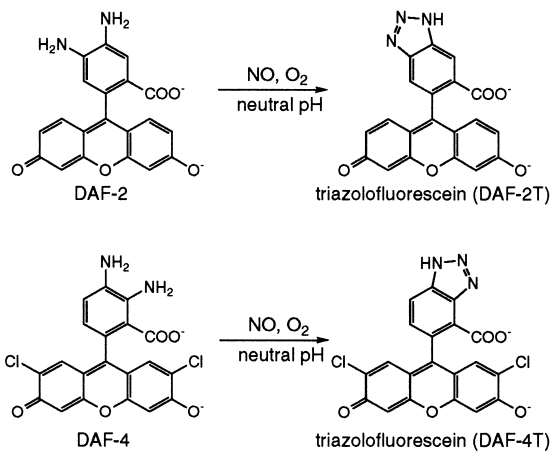

Fig. 1. Structures of DAF-2, DAF-4, DAF-2 T, and DAF-4 T.

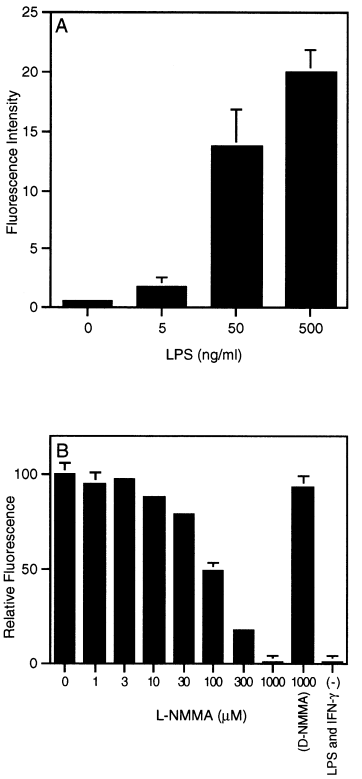

Fig. 2. Increase of fluorescence intensity according to NO production from the mouse macrophage cell line RAW 264.7. A: Dose-response of macrophages to LPS. Macrophages were stimulated with LPS (0, 5, 50, 500 ng/ml). All data are mean ± S.D. ($n = 6$). B: Effects of various concentrations of L-NMMA and D-NMMA on the augmentation of fluorescence intensity of DAF-2 by NO released from macrophages. Macrophages were stimulated with 10 ng/ml LPS and 10 U/ml IFN-γ. Relative fluorescence is expressed as the ratio (%) to the control values obtained in the absence of L-NMMA (0 μM). All data are mean ± S.D. ($n = 3$).

linear correlation with DAF-2 T and DAF-4 T concentration (nM), the slope and y-intercept were 0.148 (DAF-2 T), 0.152 (DAF-4 T) and 0.584, 0.336 (no deviation), respectively (correlation coefficient 1.000, 1.000, 10 data points each). The detection limit of triazolofluorescein is less than 2–5 nM, and a linear correlation was observed up to around 1000 nM.

The detection of NO from activated macrophages has been achieved by using DAN as a fluorescence indicator for NO [12]. In order to confirm the level of NO released from activated macrophages, we first applied the assay with DAF-2 to the mouse macrophage cell line RAW 264.7. The supernatant of macrophages stimulated with LPS produced fluorescent products upon incubation with DAF-2. The amount of products that represent NO production was positively correlated with the concentration of LPS (Fig. 2A). Furthermore, L-NMMA, a known inhibitor of NOS, concentration dependently blocked the increase of fluorescence intensity, but D-NMMA did not (Fig. 2B). Further, the fluorescence intensity did not increase in the absence of activation by LPS or IFN-γ. NO trapping efficiency by DAF-2 was calculated 9.6 ± 2.3% ($n = 6$). To confirm that the increase of fluorescence intensity was dependent on the triazole formation of DAF-2 by the reaction of NO released from the cells, the supernatant of the cells was subjected to HPLC analysis. Standard DAF-2 showed weak fluorescence at a retention time (r.t.) of 4.81 min

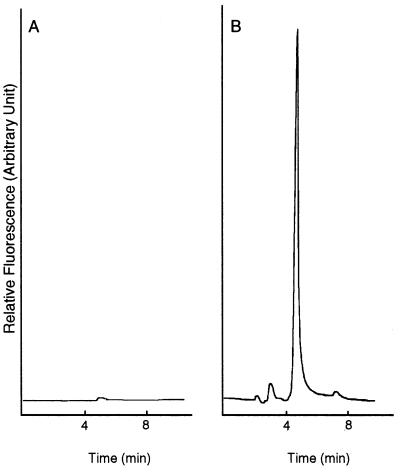

Fig. 3. Detection of triazole formation by endotoxin-induced mouse macrophage cell line RAW 264.7 using reversed-phase HPLC. RAW 264.7 cells were stimulated with 10 ng/ml LPS and 10 U/ml IFN-γ. A: Standard DAF-2. B: Endotoxin-induced RAW 264.7 cells were incubated with DAF-2 for 2 h. The excitation wavelength was 495 nm and the emission wavelength was 515 nm.

(Fig. 3A). The supernatant of the LPS-treated cells showed a marked fluorescent peak at r.t. 4.23 min due to triazolofluorescein (DAF-2 T), as shown in Fig. 3B.

We next tried to apply this fluorometric assay to ECs. As shown in Fig. 4, the fluorescence intensity of the supernatant of 1 μM bradykinin-treated cells after incubation with DAF-4, increased time dependently. An increase of fluorescence intensity was also observed without bradykinin. This reflects the basal NO production. Furthermore, L-NAME, a known inhibitor of NOS, blocked the increase of fluorescence intensity in a dose-dependent manner, while D-NAME did not block it (Fig. 5).

Thus, DAFs could be used for detection of NO from not only macrophages, but also bovine aortic ECs.

4. Discussion

The direct measurement of NOS activity and NO production in biological systems of activated macrophages, endothelial cells, and nerve cells has become increasingly important, and several methods for assaying NO in biological systems have been reported [3–11]. The DAN assay for fluorometric determination of NO [11–14] is more sensitive than the Griess assay, but even the DAN assay is unsatisfactory for ECs in terms of sensitivity.

In order to obtain higher sensitivity, we designed and synthesized DAFs as novel fluorescent NO indicators [15]. The reaction of NO and DAFs produces highly fluorescent triazolofluoresceins. The detection limits of DAF-2 and DAF-4 were about the same. Therefore, we applied both DAF-2 and DAF-4 to biological systems. NO could be detected by DAF-2 instead of DAF-4 and vice versa.

We found that DAFs can readily detect the high concentration of NO produced by activated macrophages. The requisite incubation time for DAFs assay was less than 2 h. In contrast, the incubation time of Griess assay is about 9–40 h [5,6]. Moreover, the lower concentration of NO released from ECs was also detected by DAFs. That is, under the basal conditions, a small increase of fluorescence intensity was observed, and this reflects the basal release of NO by cNOS in ECs. In both macrophages and ECs, known inhibitors of NOS blocked the increase of fluorescence intensity. Thus we conclude that DAFs are useful for detecting the generation of NO from cultured cells.

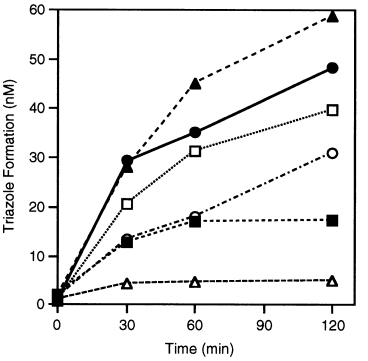

Fig. 4. Time course of fluorescence intensity augmentation of DAF-4 by NO released from bovine aortic endothelial cells with 100 μM L-arginine. Basal conditions (open circles), in the presence of 1 μM bradykinin with 0 μM (closed circles), 10 μM (open squares), 100 μM (closed squares), 1000 μM (open triangles) L-NAME and 1000 μM (closed triangles) D-NAME. All data have no deviation ($n = 3$).

NO trapping efficiency by DAF-2 was calculated around 9.6%. The NO which did not react with DAF-2 may react with thiols and amines in the cells.

We proposed the reaction mechanism of DAFs with NO to involve nitrous anhydride (N_2O_3) (Fig. 6). N_2O_3 is generated according to the following scheme [17]:

$$2NO + O_2 \rightarrow 2NO_2, \quad (1)$$

$$2NO_2 + 2NO \leftrightarrow 2N_2O_3. \quad (2)$$

According to this reaction scheme, the formation of the N-nitrosation species, N_2O_3, requires two molecules of NO. Therefore, the stoichiometry of the reaction between DAFs and NO is 1:2. It also means that DAF does not react directly with NO, but reacts with the oxidized form of NO.

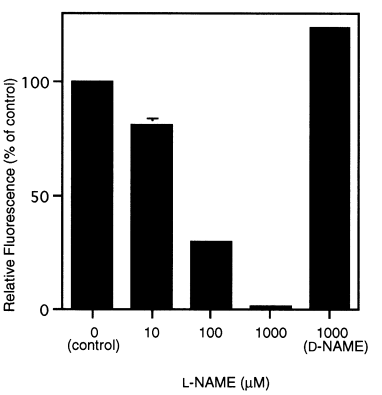

Fig. 5. Effect of various concentrations of L-NAME and D-NAME on fluorescence generation. The fluorescence intensity due to NO release from bovine aortic endothelial cells incubated with DAF-4 was measured in the presence of arginine analogues. Fluorescence intensity was measured after 120 min incubation with 1 μM DAF-4. Relative fluorescence is expressed as a percentage of control values in the absence of L-NAME (0 μM). All data are mean ± S.D. ($n = 3$).

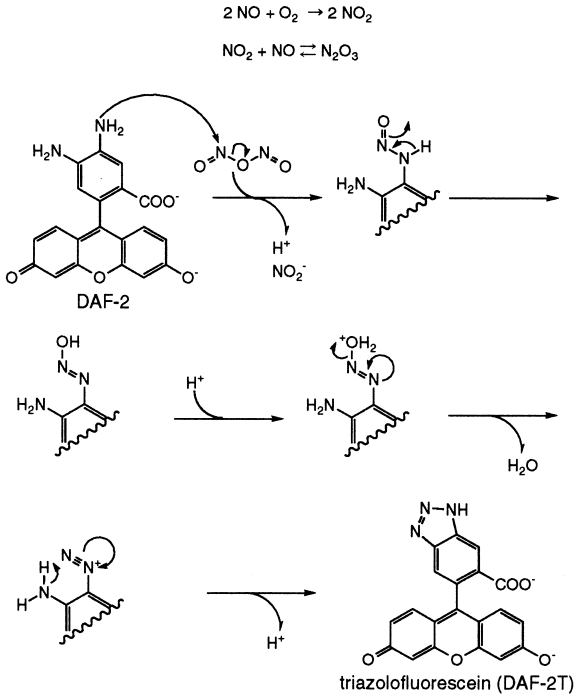

Fig. 6. Proposed mechanism in the reaction of DAF-2 with NO in the presence of dioxygen.

The maximum concentration of NO generated by ECs (1.17 mg total protein/well) was estimated to be around 300 nM after 2 h of incubation. The basal NO formation was about 100 nM after 2 h.

In conclusion, this fluorometric determination using new fluorescence indicators, DAFs, provides a rapid, easy, and sensitive assay for detecting NO from not only activated macrophages, but also ECs, which produce only a small amount of NO. Moreover, the assay with these fluorescence indicators can be done in conjunction with a 96-well microplate reader so that large numbers of samples can be conveniently handled.

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