Development of a Fluorescent Indicator for the Bioimaging of Nitric Oxide

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Nitric oxide (NO) has been reported to play various roles as a signal transmitter. However, detailed functions of NO have yet to be clarified. We have developed a fluorescent indicator for NO imaging in living cells. The N-nitrosation of newly designed and synthesized 4-((3-amino-2-naphthyl)aminomethyl)benzoic acid (DAN-1) by NO yielded the highly fluorescent triazole-form. The membrane permeable ester derivative of DAN-1 (DAN-1 EE) was applied to the imaging of NO produced in activated rat aortic smooth muscle cells. After DAN-1 EE has been loaded into cells, the ester bond is hydrolyzed by intracellular esterase, yielding original DAN-1 with less permeability. The fluorescence intensity of the cells loaded with DAN-1 EE increased according to NO production. The imaging method with fluorescent indicators will be significant for the functional clarification of NO *in vivo*.

Key words nitric oxide; imaging; fluorescent indicator; diaminonaphthalene; vascular smooth muscle cell

Nitric oxide (NO), which is biologically synthesized from L-arginine (L-Arg), has been reported to play a role as a signal transmitter not only in the vascular endothelium¹⁾ but also in the central nervous system²⁾ and in phagocytic cells.³⁾ However, the cellular function of NO is not yet completely clear, especially in the central nervous system. This is partially due to the difficulty of direct detection of NO at functioning time, which hinders the discussion based on the quantity and the distribution. In order to determine the level of NO, which is unstable and produced at a low concentration,⁴⁾ in biological systems, a new detecting method with high sensitivity and specificity is required. Chemiluminescence detection^{1,5,6)} has the highest sensitivity of all existing methods; however, the selectivity is not high. The imaging of NO based on fluorescence microscopy with a high resolution of space and time is more effective in the functional clarification of NO in living cells, in consideration of experimental feasibility. Fluorescent indicators for cytosolic free Ca²⁺, such as fura-2,⁷⁾ have contributed to the clarification of biological responses concerning Ca²⁺.

First, the reaction of NO with amines was examined in detail⁸⁾ to establish the key reaction of selective NO trapping. As a result, we found that aromatic vicinal diamines react with NO to yield corresponding triazole-rings in the presence of dioxygen under a neutral condition. One of the possible reaction species is nitrous anhydride (N₂O₃), which is formed as an intermediate in the rapid autoxidation of NO to produce NO₂⁻ and NO₃⁻.^{9,10)} Under these reaction conditions, NO₂⁻ and NO₃⁻ cannot convert the vicinal diamines into the corresponding triazole compounds. Therefore, the selectivity for NO of this principle is high, and it is therefore possible to detect NO without the inhibition of its function because diamines do not react directly with NO. Thus, we started to establish a method of detecting NO with 2,3-diaminonaphthalene (DAN). The reaction of NO and DAN (excitation (Ex.): 335 nm, emission (Em.): 385 nm) yields naphthotriazole (Ex.: 375 nm, Em.: 425 nm). In the

mean time, a similar method was reported.¹⁰⁾ However, it was impossible to use DAN as a probe for the imaging of NO because DAN leaked through cell membranes easily after the loading. Ester derivatization is known as a method for loading chemicals into cells (Fig. 1).¹¹⁾ Therefore, an ester group was introduced into DAN to yield DAN-1 EE (Chart 1). DAN-1 EE is transformed into DAN-1 with less membrane permeability by esterase in cytosol after the dye permeates the cell membrane. Since we speculated that the modification of a primary amine to a secondary amine does not hinder the reaction of NO, the modification was carried out at the amino group. A benzyl group was introduced between the amine and ester in order to avoid intramolecular cyclization by the reaction of ester and amine.

NONOates, which are the NO adducts of the general

Fig. 1. Schematic Representation of DAN-1 and Its Derivatives (DAN-1 EE, DAN-1 T)

Chart 1

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chemical structure $R[N(O)NO]^-$, spontaneously release NO at predictable rates.¹²⁾ NOC13, a NONOate, was used to confirm the reaction of DAN-1 with NO.

The administration of cytokines causes an increase in the inducible type of NO synthase (NOS) in vascular smooth muscle cells.¹³⁾ We tested the usefulness of DAN-1 EE with the activated cells.

MATERIALS AND METHODS

General Experimental Procedure ¹H-NMR spectra were recorded on a JEOL JNM-GSX 400 instrument at 400 MHz or on a JNM-LA 300 instrument at 300 MHz with tetramethylsilane (TMS) as an internal standard. MS were measured with a JEOL JMS-DX 300 mass spectrometer. IR spectra were measured on a JASCO FT/IR-5300 instrument. UV spectra were taken on a Shimadzu UV-265 UV-VIS spectrophotometer (Kyoto, Japan). Fluorescence spectra were obtained with a Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan).

Synthesis 4-((3-Amino-2-naphthyl)aminomethyl)-benzoic acid (DAN-1): *N,N*-Dimethylformamide (DMF) solution (20 ml) of 4-(bromomethyl)benzoic acid (8.39 g, 39.0 mmol) was added dropwise to a solution of 2,3-diaminonaphthalene (9.26 g, 58.5 mmol) in DMF (20 ml) at 100 °C. After the addition, the solution was stirred at 145 °C for 2 h. After cooling, the resulting dark-colored solution was poured into ice-cold water (800 ml), and the precipitate was collected, then dissolved in 2 M NaOH. After being washed with dichloromethane the solution was acidified with HCl and the precipitate was collected and dried (7.0 g, 61%). Moreover, the crude product was purified by silica gel column chromatography eluted with methanol-dichloromethane (3:97 v/v), then recrystallized from ethanol (mp 223–224 °C). ¹H-NMR (DMSO-*d*₆, 400 MHz) δ : 4.50 (d, 2H, $J=3.9$ Hz, $ArCH_2NH$), 5.13 (s, 2H, NH_2), 5.80 (t, 1H, $J=3.9$ Hz, CH_2NH), 6.54 (s, 1H, C1'-H), 6.86 (s, 1H, C4'-H), 6.97 (m, 2H, C6'-H, C7'-H), 7.31 (d, 1H, $J=7.2$ Hz, C8'-H), 7.36 (d, 1H, $J=7.2$ Hz, C5'-H), 7.52 (d, 2H, $J=8.3$ Hz, C3-H, C5-H), 7.90 (d, 2H, $J=8.3$ Hz, C2-H, C6-H), 12.7 (br, 1H, COOH). IR (KBr) cm^{-1} : 1698 (C=O). MS m/z : 292 (M^+), 248. *Anal.* Calcd for $C_{18}H_{16}N_2O_2$: C, 73.95; H, 5.52; N, 9.59. Found: C, 74.01; H, 5.39; N, 9.30.

4-((3-Amino-2-naphthyl)aminomethyl)benzoic acid ethyl ester (DAN-1 EE): Concentrated H_2SO_4 (0.1 ml) was added to an ethanol solution (1 ml) of DAN-1 (60 mg, 0.21 mmol) and the solution was refluxed for 3.5 h. After cooling, ethanol was removed *in vacuo* and the residue was poured into ice-cold water to collect the resulting crystal (1/2 H_2SO_4 -salt, 82 mg, 95%). The crystal was suspended to an aqueous solution of sodium hydrogen carbonate, extracted with dichloromethane, and purified by silica gel column chromatography eluted with dichloromethane (obtained as a free base). Concentrated HCl was added to an ether solution of the free DAN-1 EE, and the precipitate was collected and recrystallized from ether-ethanol to give the HCl-salt (mp 143 °C (dec)). ¹H-NMR (free base, $CDCl_3$, 400 MHz) δ : 1.39 (t, 3H, $J=7.2$ Hz, CH_2CH_3), 3.7 (br, 2H, NH_2), 4.0 (br, 1H, CH_2NH), 4.38 (q, 2H, $J=7.2$ Hz, CH_2CH_3), 4.51

(s, 2H, $ArCH_2NH$), 6.85 (s, 1H, C1'-H), 7.10 (s, 1H, C4'-H), 7.20 (m, 2H, C6'-H, C7'-H), 7.51 (d, 2H, $J=8.8$ Hz, C3-H, C5-H), 7.55 (m, 2H, C5'-H, C8'-H), 8.04 (d, 2H, $J=8.8$ Hz, C2-H, C6-H). IR (free base, KBr) cm^{-1} : 1714 (C=O). MS (free base) m/z : 320 (M^+). *Anal.* (HCl-salt) Calcd for $C_{20}H_{21}ClN_2O_2$: C, 67.31; H, 5.93; N, 7.85. Found: C, 66.89; H, 6.02; N, 7.61.

4-((1-Naphtho[2',3':4,5]triazolyl)methyl)benzoic acid (DAN-1 T): NO gas was bubbled through the methanol solution of DAN-1 in air. After removing the solvent, the residue was recrystallized from ethanol (mp >300 °C). ¹H-NMR (DMSO-*d*₆, 300 MHz) δ : 6.19 (s, 2H, $ArCH_2N$), 7.42 (d, 2H, $J=8.2$ Hz, C3-H, C5-H), 7.48 (t, 1H, $J=8.3$ Hz, C6'-H), 7.54 (t, 1H, $J=8.3$ Hz, C7'-H), 7.90 (d, 2H, $J=8.2$ Hz, C2-H, C6-H), 8.04 (d, 1H, $J=8.3$ Hz, C8'-H), 8.16 (d, 1H, $J=8.3$ Hz, C5'-H), 8.36 (s, 1H, C1'-H), 8.79 (s, 1H, C4'-H), 13.0 (br, 1H, COOH). IR (free base, KBr) cm^{-1} : 1688 (C=O). MS (free base) m/z : 303 (M^+). *Anal.* Calcd for $C_{18}H_{13}N_3O_2$: C, 71.27; H, 4.32; N, 13.86. Found: C, 71.01; H, 4.21; N, 13.86.

Reagents NONOate was also synthesized from NO gas (purchased from Sumitomo-Seika Chemicals, Osaka, Japan) and *N*-methyl-1,3-propanediamine as described by Hrabie *et al.*¹²⁾ The structure of NOC13 is $MeN[N(O)NO]^- (CH_2)_3NH_3^+$. Dimethylsulfoxide (DMSO, grade for fluorometric analysis) was purchased from Dojin Chemical Research Laboratory (Kumamoto, Japan). Fetal bovine serum (FBS) was purchased from Bioserum (Victoria, Australia). Recombinant murine interleukin-1 β (IL-1 β) were purchased from R&D Systems (Minneapolis, MN, U.S.A.). DMSO for biochemistry, L-Arg, and L-NMMA was purchased from Wako Pure Chemical Industries (Osaka, Japan). 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).

Preparation of Cells Vascular smooth muscle cells were isolated from the thoracic aorta of male Wistar rats by the standard explant method of Ross *et al.*,¹⁴⁾ and were then grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. Cells were incubated at 37 °C in a humid atmosphere of 95% air/5% CO_2 on glass-bottomed culture dishes (P35G-101: MatTek Corp., Ashland, MA, U.S.A.). Confluent cells between the 10th and 20th passage were used for these experiments. Next, IL-1 β (final concentration of 25 U/ml) was added to the medium. After another 12 h of incubation, the activated cells were subjected to the experiment.

Imaging Cells were incubated for 1 h at 37 °C in KRP containing 10 mM DAN-1 EE (0.2% (v/v) DMSO was used as cosolvent) to load DAN-1 EE, and were then washed with KRP and filled with KRP containing L-Arg or L-NMMA. They were mounted on an inverted fluorescence microscope (IX70: Olympus, Tokyo, Japan) equipped with an objective lens ($\times 20$), a excitation filter (330–385 nm), a dichroic mirror (400 nm), and a long-pass emission filter (420 nm). The temperature of this stage was maintained at 37 °C with a microwarm plate (DC-MP10DM: Kitazato, Shizuoka, Japan). The light exposure time was 264 ms. Optical signals were recorded

Fig. 2. Excitation and Emission Spectra of DAN-1 before and after the Reaction with NO

NO gas was bubbled into DAN-1 (100 μ M) in sodium phosphate buffer (0.1 M, pH 7.4) for several seconds. A: The interval of contour lines is 2 units of fluorescence intensity. B: The interval of contour lines is 20 units of fluorescence intensity. The slit widths of excitation and emission were both 2.5 nm. The photomultiplier voltage was 700 V.

Fig. 3. Time-course of Fluorescence Intensity of DAN-1 Depending on NO Generated from NONOates

NOC13 was incubated in 2 ml reaction volumes containing 0.1 M sodium phosphate buffer (pH 7.4) and 100 mM DAN-1 at 37 °C. The fluorescence intensity was determined at 440 nm with excitation at 365 nm. a, NOC 13 (final concentration 5 μ M) was added immediately after it had been dissolved in 0.1 M sodium phosphate buffer (pH 7.4). b, NOC 13 (50 μ M) was added. The slit widths of excitation and emission were both 2.5 nm. The photomultiplier voltage was 950 V.

by a MCID imaging system (Imaging Research, Inc., St. Catharines, Ontario, Canada) with a charge-coupled device (CCD) camera. Images were taken at intervals of 10 s.

RESULTS AND DISCUSSION

Although DAN-1 (Ex.: 340 nm, Em.: 385 nm) had little fluorescence, unlike DAN, DAN-1 reacted with NO to form a corresponding highly fluorescent triazole-form (DAN-1 T; Ex.: 360 nm, Em.: 447 nm), as expected (Fig. 2). Extinction coefficients of DAN-1 and DAN-1 T were both $6.0 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 340 and 360 nm, respectively. Quantum efficiencies of the fluorescence of DAN-1 and DAN-1 T were also obtained: 0.002 and 0.63, respectively. The calibration was done by comparison of the integral of the corrected emission spectrum of the test sample with that of a solution of *p*-terphenyl in cyclohexane (quantum efficiency of 0.87) and also a solution of quinine in 0.1 M H_2SO_4 (quantum efficiency of 0.55), respectively, according to the literature.¹⁵⁾ Figure 3 shows that the fluorescence intensity increased according to the amount of NO released from one of the NONOates, NOC13. Not one proton in the triazole of DAN-1 T caused no change in fluorescence intensity around a neutral pH, while the

Fig. 4. Fluorescence Images of IL-1 β Activated Rat Aortic Smooth Muscle Cells Loaded with DAN-1 EE in the Presence of L-Arg (1 mM) or L-NMMA (1 mM)

These images are shown in pseudocolor and correspond to the fluorescence intensity data in Fig. 5.

Fig. 5. Fluorescence Response of DAN-1 EE-loaded Rat Aortic Smooth Muscle Cells in NO Production

A: Fluorescence intensity of activated cells obtained when L-Arg (1 mM) was present in the medium. B: The response in the presence of NOS inhibitor, L-NMMA (1 mM). The medium was changed to KRP containing L-Arg or L-NMMA 10 min before 0 time. Lines show the average fluorescence intensity in arbitrary areas containing cells.

fluorescence of naphthotriazole was higher in an alkaline solution by the deprotonation of triazole (data not shown). This property of DAN-1 T is advantageous to bioimaging.

We tested this new probe with activated rat aortic smooth muscle cells. Figure 4 shows the fluorescence images of activated rat aortic smooth muscle cells cor-

Fig. 6. NO Production Response of DAN-1 EE-loaded Rat Aortic Smooth Muscle Cells by Changing Medium

A: After cells were incubated solely with 0.5 mM L-Arg from 13 min before 0 time, 10 mM L-NMMA was added at 100 s. B: After cells were incubated solely with 1 mM L-NMMA from 13 min before 0 time, 10 mM L-Arg was added at 100 s. Lines show the average fluorescence intensity in arbitrary areas containing cells.

responding to the intensity profile of Fig. 5. Figure 5A shows that the fluorescence intensity in activated cells increased with NO production. Although intracellular L-Arg exists, L-Arg was added to the medium in order to quickly produce NO. The increase was depressed by an inhibitor of NOS, N-monomethyl-L-arginine (L-NMMA) (Fig. 5B), which competes with L-Arg. The results were reproducible among the different cell plates ($n=4$). We had determined that L-NMMA suppressed NO production from these cells with DAN (data not shown). Photobleaching of the autofluorescence in cells, or a slight leaking of DAN-1 T, might cause a small reduction of fluorescence intensity when L-NMMA is added. The increase of fluorescence intensity was stopped when L-NMMA twenty times as high as L-Arg concentration was added to the medium (Fig. 6A). Inversely, the fluorescence intensity increased when L-Arg ten times as high as L-NMMA was added to the medium (Fig. 6B). Similar data were obtained between the different cell plates ($n=2$).

The damage caused by loading the dye was not apparently observed. These results show that DAN-1 EE enables the bioimaging of NO. The accurate quantification of NO concentration in cells, however, is quite difficult because unreacted DAN-1 had too little fluorescence to calibrate the concentration of loaded dye, and high auto-fluorescence was created by UV excitation.

The imaging method based on fluorescent indicators will be useful for the revelation of the dynamic biological action of NO at a molecular level with high resolution of space and time.

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