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Detection and Imaging of Nitric Oxide with Novel Fluorescent Indicators: Diaminofluoresceins

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Nitric oxide is a gaseous, free radical which plays a role as an intracellular second messenger and a diffusable intercellular messenger. To obtain direct evidence for NO functions in vivo, we have designed and synthesized diaminofluoresceins (DAFs) as novel fluorescent indicators for NO. The fluorescent chemical transformation of DAFs is based on the reactivity of the aromatic vicinal diamines with NO in the presence of dioxygen. The N-nitrosation of DAFs, yielding the highly green-fluorescent triazole form, offers the advantages of specificity, sensitivity, and a simple protocol for the direct detection of NO (detection limit 5 nM). The fluorescence quantum efficiencies are increased more than 100 times after the transformation of DAFs by NO. Fluorescence detection with visible light excitation and high sensitivity enabled the practical assay of NO production in living cells. Membrane-permeable DAF-2 diacetate (DAF-2 DA) can be used for real-time bioimaging of NO with fine temporal and spatial resolution. The dye was loaded into activated rat aortic smooth muscle cells, where the ester bonds are hydrolyzed by intracellular esterase, generating DAF-2. The fluorescence in the cells increased in a NO concentration-dependent manner.

Nitric oxide is a messenger molecule involved in the regulation of diverse physiological and pathophysiological mechanisms in the cardiovascular system, the central and peripheral nervous systems, and the immune system.[1−3] However, many proposed physiological roles of NO were not proved directly by measuring NO. One of the reasons for this is the difficulty of direct, real-time detection of this gaseous, free-radical species. Although several methods of detecting NO, which is unstable and produced at low concentration, have been developed,[4−7] a new method is required which is satisfactory for studies in living cells in terms of selectivity, and experimental feasibility. Experimental studies on Ca^{2+}-dependent signal transduction in cells were greatly facilitated by the development of fluorescent indicators for cytosolic Ca^{2+}.[8,9] Although bioimaging of NO has already been reported by chemiluminescence[10] and ESR,[11] they have disadvantages to the functional analysis of NO, such as the use of cytotoxic H_2O_2 or low spatial resolution. The use of NO-reactive fluorescent indicators, in conjunction with confocal laser microscopy, should make possible the bioimaging of NO, which is suitable for real-time analysis of intracellular NO. The fluorescence detection of intracellular NO based on the conversion of 2,7-dichlorofluorescin was reported; however, the system is unable to distinguish between NO and reactive oxygen species, and the limit of the detection of NO is 16 μM.[12] We therefore newly designed and synthesized fluorescent NO indicators to detect NO in living cells as a means to examine the physiological functions of NO. First, the reactivity of NO was examined in order to find a suitable reaction for selective NO trapping. Wink et al. reported that NO deaminates deoxynucleosides, deoxynucleotide, and intact DNA at physiological pH.[13] We found that aromatic amines react with NO in the presence of dioxygen to produce the corresponding triazenes,[14] and the corresponding triazole ring compounds are generated from aromatic vicinal diamines under neutral conditions. The reaction mechanism is proposed to involve nitrous anhydride, which is generated according to the following scheme:[15]

$$2NO + O_2 \rightarrow 2NO_2 \qquad (1)$$

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

We started to investigate the design and synthesis of fluorescent compounds based originally on 2,3-diaminonaphthalene as a vicinal diamine. During this work, a similar method was reported.[16] We developed a new fluorescent indicator (DAN-1 EE) for bioimaging of NO[17] and found some problems, such as cytotoxcity and strong autofluorescence owing to the requirement for excitation for UV light, small extinction coefficient, and poor solubility in neutral buffer.

Fluorescein is widely used in biology as a fluorophore because of its convenient wavelengths for biological measurement, high extinction coefficient, and high fluorescence quantum yield in water. Fluoresceinamine was reported to show quenched fluorescence, but the conversion of amine to amide resulted in fluorescent properties.[18] This result implies that the fluorescence was quenched because of the electron-donating group attached to the phthalic ring of fluorescein. However, when the electron-donating group was transformed into a less electron-donating group, the fluorescence recovered. Based on these findings, we designed diaminofluoresceins (DAFs, Figure 1) as indicators for NO. In other words, we considered that the electron donation of the functional groups attached to fluorescein would be reduced by reaction with NO via triazole ring formation, leading to an NO concentration-dependent enhancement of fluorescence.

In order to load the cells efficiently with the probe, it was necessary to prepare the diacetate (DAF-2 DA, Figure 1). It can permeate readily into the cells, where it is hydrolyzed by intracellular esterases existing in many cell types, to generate DAF-2.[19] *Escherichia coli* lipopolysaccharide (LPS) and certain kinds of cytokines cause an increase in an inducible type of NO synthase in vascular smooth muscle cells.[20] We evaluated this new probe for real-time biological imaging of NO in cultured rat aortic smooth muscle cells.

EXPERIMENTAL SECTION

Synthesis of DAFs. ^{1}H NMR spectra were recorded on a JEOL JNM-LA300 instrument at 300 MHz; δ values in ppm relative to tetramethylsilane are given. Mass spectrometry (MS) spectra were measured with JEOL JMS-DX 300 mass spectrometer. IR spectra were measured on a Jasco FT-IR-5300 instrument. The synthetic pathways employed to obtain the new dyes are outlined in Figure 1.

I. Acetic anhydride (15 mL, 0.16 mol) was added to a solution of 2,3-dimethyl-6-nitroaniline (25.3 g, 0.152 mol) in hot glacial acetic acid (290 mL). The mixture was refluxed for 1 h, acetic acid was removed by evaporation, and the residue was recrystallized from ethanol to give a quantitative yield of 2,3-dimethyl-6-nitroacetanilide (**I**): ^{1}H NMR ($CDCl_3$) δ 2.18 (s, 3H, CH_3), 2.24 (s, 3H, $COCH_3$), 2.39 (s, 3H, CH_3), 7.18 (d, 1H, ArH, J = 8.3 Hz), 7.80 (d, 1H, ArH, J = 8.3 Hz), 8.46 (s, 1H, NH).

II. A suspension of potassium permanganate (133 g, 841 mmol) in water (1 L) was added in several portions, as dissolution of the solid proceeded, to a suspension of **I** (29.2 g, 140 mmol) in hot water (1 L) with magnesium sulfate (100 g, 831 mmol). The resulting brown suspension was filtered hot, and the filtrate was saturated with NaCl. After cooling to room temperature, the yellow filtrate was acidified with HCl and the cream-colored precipitate was collected. In addition, the filtrate was extracted with ethyl acetate and the extract was worked up in the usual way (19.6 g, 52% yield): ^{1}H NMR (dimethyl sulfoxide (DMSO)-d_6) δ 1.97 (s, 3H, $COCH_3$), 7.90 (d, 1H, ArH, J = 8.4 Hz), 8.04 (d, 1H, ArH, J = 8.4 Hz), 10.13 (s, 1H, NH), 13.5 (br, 2H, COOH).

III. Acetyl chloride (30 mL) was added to **II** (19.0 g, 70.8 mmol) in acetic anhydride (180 mL) at 80 °C, and the mixture was refluxed for 2 h. The solvent was removed in vacuo, a small amount of anhydrous dichloromethane was added, and the cream-colored precipitate was collected (9.87 g, 56%): ^{1}H NMR ($CDCl_3$) δ 2.35 (s, 3H, $COCH_3$), 7.89 (d, 1H, ArH, J = 8.2 Hz), 8.45 (d, 1H, ArH, J = 8.2 Hz), 9.01 (s, 1H, NH).

III → IV, V, VI, and VII. A mixture of 3-acetamido-4-nitrophthalic anhydride (**III**) and resorcinol (or 4-chlororesorcinol) (2 equiv) was stirred at 180 °C for 2 h. After the addition of $ZnCl_2$ (0.25 equiv), the temperature was raised to 210 °C and stirring was continued until a dry residue was obtained. After cooling, the black powder was refluxed in 0.6 M HCl (containing 1 equiv of HCl) for 1 h.[21] The black solid was collected by filtration and dried. The products were purified by silica gel chromatography eluted with methanol/dichloromethane (**IV**, **V**, the concentration of methanol was gradually raised from 7 to 15% v/v; **IV**, **V**, the concentration of methanol was gradually raised from 3 to 10% v/v), to afford **IV** (5%) and **V** (2%) (or **VI** (6%) and **VII** (1%)): ^{1}H NMR (DMSO-d_6) (**IV**) δ 6.38 (d, 1H, ArH, J = 8.6 Hz), 6.56 (dd, 2H, ArH, J = 8.6, 2.4 Hz), 6.66 (d, 2H, ArH, J = 2.4 Hz), 6.80 (d, 2H, ArH, J = 8.6 Hz), 7.96 (s, 2H, NH_2), 8.35 (d, 1H, ArH, J = 8.6 Hz), 10.18 (s, 2H, OH); (**V**) δ 6.40 (s, 2H, NH_2), 6.56 (dd, 2H, ArH, J = 8.6, 2.4 Hz), 6.71 (d, 2H, ArH, J = 2.4 Hz), 6.72 (d, 2H, ArH, J = 8.6 Hz), 7.22 (d, 1H, ArH, J = 8.6 Hz), 8.38 (d, 1H, ArH, J = 8.6 Hz), 10.18 (s, 2H, OH); (**VI**) δ 6.41 (d, 1H, ArH, J = 8.6 Hz), 6.87 (s, 2H, ArH), 7.03 (s, 2H, ArH), 7.83 (s, 2H, NH_2), 8.36 (d, 1H, ArH, J = 8.6 Hz), 11.23 (s, 2H, OH). VII: δ 6.60 (s, 2H, NH_2), 6.93 (s, 2H, ArH), 6.93 (s, 2H, ArH), 7.22 (d, 1H, ArH, J = 8.6 Hz), 8.40 (d, 1H, ArH, J = 8.6 Hz), 11.17 (s, 2H, OH).

IV → DAF-1, V → DAF-3, VI → DAF-4, and VII → DAF-6. Aminonitrofluoresceins were reduced with Na_2S and NaSH in water, as described by McKinney et al.,[22] to give diaminofluoresceins (DAF-1, 76%; DAF-3, 30%; DAF-4, 52%; DAF-6, 16%). DAFs were purified by silica gel chromatography eluted with methanol/dichloromethane (DAF-1, 1:9; DAF-3, 7:93; DAF-4, 5:95; DAF-6, 5:95 v/v) and recrystallized from isopropyl alcohol. (DAF-1) mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 5.01 (s, 2H, NH_2), 5.88 (s, 2H, NH_2), 6.05 (d, 1H, ArH, J = 7.5 Hz), 6.52 (dd, 2H, ArH, J = 8.6, 2.4 Hz), 6.60 (d, 2H, ArH, J = 2.4 Hz), 6.64 (d, 2H, ArH, J = 8.6 Hz), 6.78 (d, 1H, ArH, J = 7.5 Hz), 9.97 (s, 2H, OH); MS (EI),

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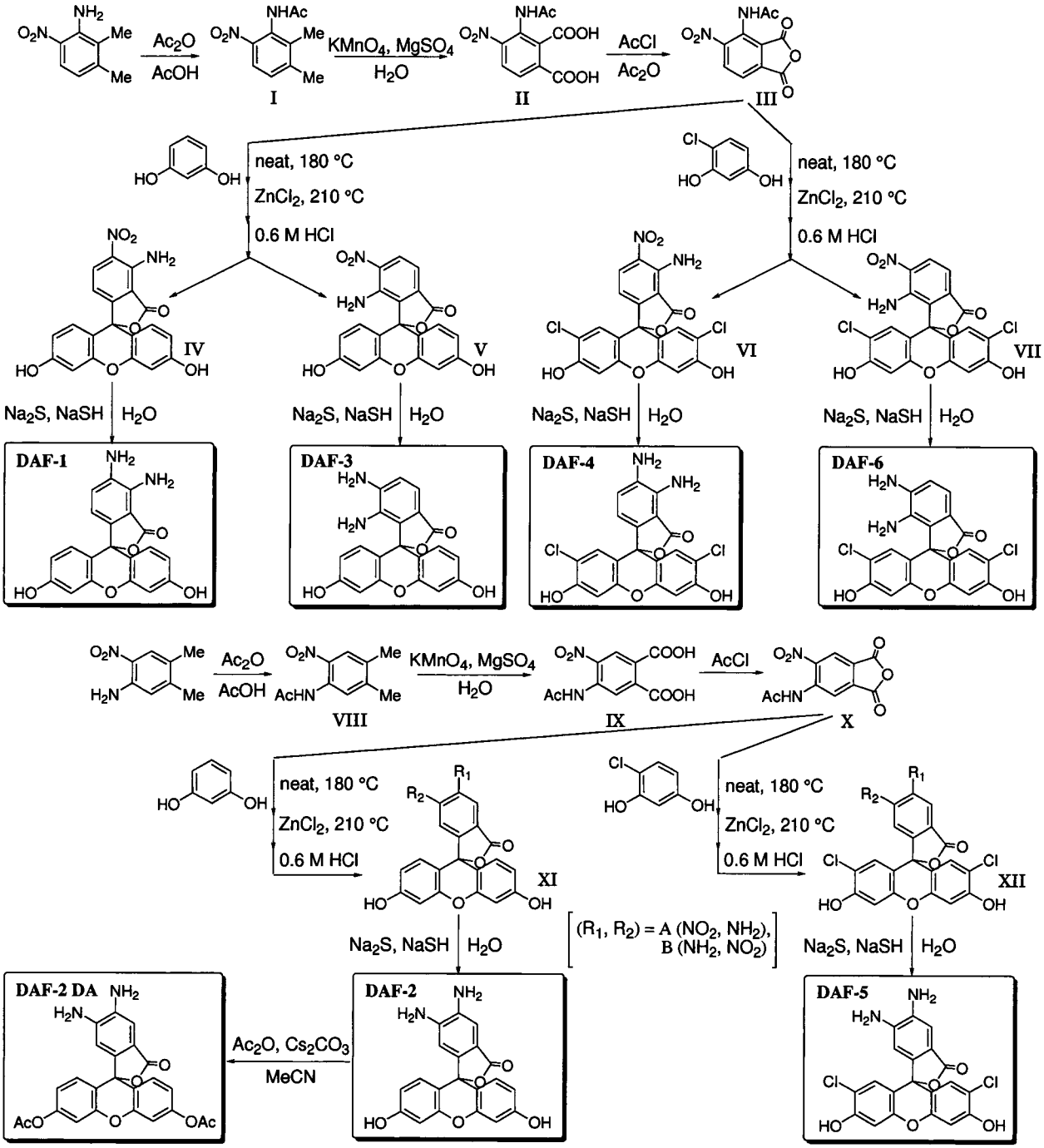

Figure 1. Synthesis of DAFs. Roman numerals correspond to the synthetic details given under Experimental Section. **XI** and **XII** represent mixtures of isomers.

m/*z* (M^+) 362; IR (KBr) 1730 (C═O), 1615 (aromatic), 1453 (aromatic) cm^{-1}. Elemental Anal., Calcd for $C_{20}H_{14}N_2O_5 \cdot 2C_3H_8O$: C, 64.71; H, 6.27; N, 5.81. Found: C, 64.62; H, 6.21; N, 5.48. (DAF-3) mp 220−230 °C; ^{1}H NMR (DMSO-d_6) δ 3.80 (s, 2H, NH_2), 5.58 (s, 2H, NH_2), 6.53 (dd, 2H, ArH, J = 8.6, 2.4 Hz), 6.62 (d, 2H, ArH, J = 8.6 Hz), 6.65 (d, 2H, ArH, J = 2.4 Hz), 6.79 (d, 1H, ArH, J = 7.9 Hz), 7.07 (d, 1H, ArH, J = 7.9 Hz), 10.01 (s, 2H, OH); MS (EI), *m*/*z* (M^+) 362; IR (KBr) 1730 (C═O), 1611 (aromatic), 1453 (aromatic), 1383 (aromatic) cm^{-1}. Elemental Anal., Calcd for $C_{20}H_{14}N_2O_5 \cdot 1.4H_2O$: C, 61.98; H, 4.37; N, 7.23. Found: C, 61.70; H, 4.09; N, 7.18. (DAF-4) mp >300 °C (sublimed at 240 °C); ^{1}H NMR (DMSO-d_6) δ 5.14 (s, 2H, NH_2), 5.97 (s, 2H, NH_2), 6.12 (d, 1H, ArH, J = 7.7 Hz), 6.68 (s, 2H, ArH), 6.81 (d, 1H, ArH, J = 7.7 Hz), 6.85 (s, 2H, ArH), 10.97 (s, 2H, OH); MS (EI), *m*/*z* (M^+) 430; IR (KBr) 1705 (C═O), 1610 (aromatic), 1381 (aromatic) cm^{-1}. Elemental Anal., Calcd for $C_{20}H_{12}Cl_2N_2O_5 \cdot C_3H_8O$: C, 56.22; H, 4.10; N, 5.70. Found: C, 55.82; H, 4.15; N, 5.79. (DAF-6) mp >300 °C (sublimed at 230 °C); ^{1}H NMR (DMSO-d_6) δ 4.06 (s, 2H, NH_2), 5.74 (s, 2H, NH_2), 6.61 (s, 2H, ArH), 6.84 (d, 1H, ArH, J = 7.9 Hz), 6.89 (s, 2H, ArH), 7.10 (d, 1H, ArH, J = 7.9 Hz), 10.97 (s, 2H, OH); MS (EI), *m*/*z* (M^+) 430; IR (KBr) 1719 (C═O), 1456 (aromatic), 1385 (aromatic) cm^{-1}. Elemental Anal., Calcd for $C_{20}H_{12}Cl_2N_2O_5 \cdot C_3H_8O$: C, 56.22; H, 4.10; N, 5.70. Found: C, 55.97; H, 4.02; N, 6.00.

VIII. Acetic anhydride (10 mL, 0.11 mol) was added to a solution of 4,5-dimethyl-2-nitroaniline (17.1 g, 103 mmol, Aldrich) in hot acetic acid (200 mL). The mixture was refluxed for 1 h, acetic acid was removed by evaporation, and the residue was recrystallized from ethanol to afford a quantitative yield of 4-acetamido-5-nitroxylene (**VIII**): ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 3H, $COCH_3$), 2.28 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 7.98 (s, 1H, ArH), 8.54 (s, 1H, ArH), 10.30 (s, 1H, NH).

IX. A suspension of potassium permanganate (42.4 g, 268 mmol) in water (300 mL) was added dropwise to a suspension of **VIII** (11.8 g, 56.7 mmol) in hot water (700 mL) with magnesium sulfate (42.4 g, 352 mmol). The resulting brown suspension was filtered hot, and the yellow filtrate was acidified with HCl and extracted with ethyl acetate. 4-Acetamido-5-nitrophthalic acid (**IX**) was obtained after evaporation (8.9 g, 59%): ^{1}H NMR (DMSO-d_6) δ 2.10 (s, 3H, $COCH_3$), 7.91 (s, 1H, ArH), 8.26 (s, 1H, ArH), 10.53 (s, 1H, NH), 13.0 (br, 2H, COOH).

X. **IX** (6.0 g, 22 mmol) was dehydrated in acetyl chloride (60 mL) under reflux.[23] Acetyl chloride was removed, and the recrystallization of the residue from benzene gave 4-acetamido-5-nitrophthalic anhydride (**X**) (5.0 g, 90%): ^{1}H NMR ($CDCl_3$) δ 2.40 (s, 3H, $COCH_3$), 8.85 (s, 1H, ArH), 9.54 (s, 1H, ArH), 10.64 (s, 1H, NH).

X → XI and XII. **XI** and **XII** were synthesized from **X** by the same method as used to obtain **IV**−**VII** (**XI**, A 34%, B 19%. **XII**, A + B 30% mixture after solidification was used for the next reaction): ^{1}H NMR (DMSO-d_6) (**XI**-A) δ 6.58 (dd, 2H, ArH, J = 8.6, 2.2 Hz), 6.63 (s, 1H, ArH), 6.65 (d, 2H, ArH, J = 2.2 Hz), 6.75 (d, 2H, ArH, J = 8.6 Hz), 7.92 (s, 2H, NH_2), 8.49 (s, 1H, ArH), 10.18 (s, 2H, OH). (**XI**−B) δ 6.55 (dd, 2H, ArH, J = 8.6, 2.2 Hz), 6.65 (d, 2H, ArH, J = 2.2 Hz), 6.74 (d, 2H, ArH, J = 8.6 Hz), 7.59 (s, 1H, ArH), 7.73 (s, 2H, NH_2), 7.74 (s, 1H, ArH), 10.12 (s, 2H, OH). (**XII**-A) δ 6.66 (s, 1H, ArH), 6.89 (s, 2H, ArH), 6.91 (s, 2H, ArH), 7.96 (s, 2H, NH_2), 8.48 (s, 1H, ArH), 11.11 (s, 2H, OH). XII−B: δ 6.9 (br, 2H, ArH), 6.91 (s, 2H, ArH), 7.63 (s, 1H, ArH), 7.77 (s, 2H, NH_2), 7.86 (s, 1H, ArH), 11.07 (s, 2H, OH).

XI → DAF-2 and XII → DAF-5. These compounds were synthesized from **XI** or **XII** by a method similar to that used for DAF-1, 3, 4, and 6 (DAF-2, from **XI**-A 97%, **XI**-B 30%. DAF-5, from **XII**-A + B (mixture) 53%). (DAF-2) mp 240−250 °C; ^{1}H NMR (DMSO-d_6) δ 5.00 (s, 2H, NH_2), 5.58 (s, 2H, NH_2), 6.07 (s, 1H, ArH), 6.52 (dd, 2H, ArH, J = 8.6, 2.2 Hz), 6.60 (d, 2H, ArH, J = 2.2 Hz), 6.60 (d, 2H, ArH, J = 8.6 Hz), 6.89 (s, 1H, ArH), 9.99 (s, 2H, OH); MS (EI), m/z (M^+) 362; IR (KBr) 1732 (C═O), 1618 (aromatic), 1458 (aromatic), 1383 (aromatic) cm^{-1}. Elemental Anal., Calcd for $C_{20}H_{14}N_2O_5 \cdot 2C_3H_8O$: C, 64.71; H, 6.27; N, 5.81. Found: C, 64.67; H, 6.15; N, 6.08. (DAF-5) mp 215−220 °C; ^{1}H NMR (DMSO-d_6) δ 5.12 (s, 2H, NH_2), 5.69 (s, 2H, NH_2), 6.12 (s, 1H, ArH), 6.63 (s, 2H, ArH), 6.85 (s, 2H, ArH), 6.93 (s, 1H, ArH), 10.96 (s, 2H, OH); MS (EI), m/z (M^+) 430; IR (KBr) 1717 (C═O) cm^{-1}. Elemental Anal., Calcd for $C_{20}H_{12}Cl_2N_2O_5 \cdot C_3H_8O$: C, 56.22; H, 4.10; N, 5.70. Found: C, 56.22; H, 3.98; N, 5.84.

DAF-2 → DAF-2 DA. Cesium carbonate (166 mg, 0.509 mmol) was added to an acetonitrile solution (100 mL) of DAF-2 (124 mg, 0.341 mmol) and then acetic anhydride (105 μL, 1.12 mmol) was added dropwise. The mixture was stirred for 1 h, the solvent was removed by evaporation, and the residue was subjected to silica gel chromatography eluted with methanol/dichloromethane (3:97 v/v) to afford 4,5-diaminofluorescein diacetate (DAF-2 DA) (137 mg, 90%): mp 110−120 °C; ^{1}H NMR (acetone-d_6) δ 2.27 (s, 6H, $COCH_3$), 4.68 (s, 2H, NH_2), 5.16 (s, 2H, NH_2), 6.34 (s, 1H, ArH), 6.92 (dd, 2H, ArH, J = 8.6, 2.2 Hz), 7.00 (d, 2H, ArH, J = 8.6 Hz), 7.12 (d, 2H, ArH, J = 2.2 Hz), 7.15, (s, 1H, ArH); MS (EI), m/z 446 (M^+). IR (KBr) 1750 (lactone), 1606 (aromatic) cm^{-1}. Elemental Anal., Calcd for $C_{24}H_{18}N_2O_7$: C, 64.57; H, 4.06; N, 6.28. Found: C, 64.27; H, 4.16; N, 6.18.

Triazole Forms of DAFs. NO gas was introduced into a methanol solution of each DAF to yield the corresponding triazolofluorescein (DAF-1 T-DAF-6 T). Each triazolofluorescein was purified by silica gel chromatography eluted with methanol/dichloromethane (1:9 v/v, a small amount of acetic acid (0.02% v/v) was added only in the case of purification of DAF-4 T), and the dried residue was dissolved in 2 M NaOH. Then the solution was adjusted to pH 3−4 with HCl, and the precipitate was collected and dried. (DAF-1 T) mp >300 °C; ^{1}H NMR (acetone-d_6) δ 6.60 (dd, 2H, ArH, J = 8.6, 2.4 Hz), 6.77 (d, 2H, ArH, J = 2.4 Hz), 6.77 (d, 2H, ArH, J = 8.6 Hz), 7.23 (d, 1H, ArH, J = 7.7 Hz), 8.43 (d, 1H, ArH, J = 7.7 Hz), 9.05 (s, 2H, OH); MS (EI), m/z (M^+) 373; IR (KBr) 1734 (C═O), 1615 (aromatic), 1453 (aromatic) cm^{-1}. (DAF-2 T) mp >300 °C; ^{1}H NMR (acetone-d_6) δ 6.59 (dd, 2H, ArH, J = 8.8, 2.4 Hz), 6.72 (d, 2H, ArH, J = 8.8 Hz), 6.75 (d, 2H, ArH, J = 2.4 Hz), 7.68 (s, 1H, ArH), 8.56 (s, 1H, ArH); MS (EI), m/z (M^+) 373; IR (KBr) 1725 (C═O), 1616 (aromatic), 1474 (aromatic), 1383 (aromatic) cm^{-1}. (DAF-3 T) mp >300 °C; ^{1}H NMR (acetone-d_6) δ 6.54 (dd, 2H, ArH, J = 8.6, 2.4 Hz), 6.67 (d, 2H, ArH, J = 8.6 Hz), 6.79 (d, 2H, ArH, J = 2.4 Hz), 8.00 (d, 1H, ArH, J = 8.0 Hz), 8.24 (d, 1H, ArH, J = 8.0 Hz), 9.00 (s, 2H, OH); MS (EI), m/z (M^+) 373; IR (KBr) 1734 (C═O), 1611 (aromatic), 1474 (aromatic), 1383 (aromatic) cm^{-1}. (DAF-4 T) mp >300 °C; ^{1}H NMR (acetone-d_6) δ 6.97 (s, 2H, ArH), 7.00 (s, 2H, ArH), 7.31 (d, 1H, ArH, J = 9.0 Hz), 8.46 (d, 1H, ArH, J = 9.0 Hz), 9.70 (s, 2H, OH); MS (EI), m/z (M^+) 441; IR (KBr) 1748 (C═O), 1582 (aromatic), 1491 (aromatic), 1385 (aromatic) cm^{-1}. (DAF-5 T) mp >300 °C; ^{1}H NMR (acetone-d_6) δ 6.86 (s, 2H, ArH), 6.93 (s, 2H, ArH), 7.8 (br, 1H, ArH), 8.6 (br, 1H, ArH), 9.62 (s, 2H, OH); MS (EI), m/z (M^+) 441; IR (KBr) 1724 (C═O), 1385 (aromatic) cm^{-1}. (DAF-6 T) mp >300 °C; ^{1}H NMR (acetone-d_6) δ 6.88 (s, 2H, ArH), 7.00 (s, 2H, ArH), 8.04 (d, 1H, ArH, J = 8.0 Hz), 8.23 (d, 1H, ArH, J = 8.0 Hz), 9.68 (s, 2H, OH); MS (EI), m/z (M^+) 441; IR (KBr) 1717 (C═O), 1636 (aromatic), 1458 (aromatic), 1385 (aromatic) cm^{-1}.

Preparation of NO Donor and NO Solution. The NONOates were prepared according to the methods of Hrabie and his associates.[24] The structures of NOC12 and NOC13 are $EtN[N(O)NO]^-(CH_2)_2NH_2^+Et$ and $MeN[N(O)NO]^-(CH_2)_3NH_3^+$, respectively. NO solution was prepared by bubbling NO through sodium phosphate buffer for about 30 s. The buffer was deoxidized by bubbling argon through it for 1 h before NO introduction, and the concentration was determined by horseradish peroxidase assay.[25]

Fluorometric Analysis. A fluorescence spectrophotometer (F4500, Hitachi, Tokyo, Japan) was used. The slit width was 2.5

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nm for both excitation and emission. The photomultiplier voltage was 950 V. DAFs were dissolved in DMSO to obtain 10 mM stock solutions. Relative quantum efficiencies of fluorescence of DAFs and triazolofluoresceins were obtained by comparing the area under the corrected emission spectrum of the test sample at 492-nm excitation with that of a solution of fluorescein in 0.1 M NaOH, which has a quantum efficiency of 0.85 according to the literature.[26]

HPLC Analysis. The HPLC unit consisted of a pump (PU980, Jasco), a detector (UV-970 or FU-920, Jasco), and a column (Finepak SIL C18-5, 6.0 × 250 mm, Jasco). The eluent for analysis was pH 7.4 sodium phosphate buffer/10 mM acetonitrile (94:6 v/v). The flow rate was 1.0 mL/min. Absorbance was monitored at 495 nm for colorimetric assay. Fluorescence was monitored at ex/em 495 nm/515 nm. Aliquots of 10 μL were injected for analysis. Macrophages (RAW 264.7) were cultured on 24-well plates, and DAF was added to the culture medium. The macrophages were stimulated 16 h before measurement with LPS (10 ng/mL) and recombinant murine interferon-γ (IFN-γ) (10 units/mL), when necessary. After 2 h, an aliquot (10 μL) of each cell supernatant was taken and analyzed by HPLC.

Preparation of Activated Rat Aortic Smooth Muscle Cells. Fetal bovine serum (FBS) was purchased from Bioserum (Victoria, Australia). Recombinant murine interleukin-1β (IL-1β) and recombinant murine tumor necrosis factor-α (TNF-α) were purchased from R&D Systems (Minneapolis, MN). IFN-γ was purchased from Genzyme (Cambridge, MA). LPS was purchased from Difco Laboratories (Detroit, MI). L-Arginine (L-Arg), and *N*-monomethyl-L-arginine (L-NMMA) were 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).

Vascular smooth muscle cells were isolated from thoracic aorta of male Wistar rats by a standard explant method[25] and grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% FBS 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). Confluent cells between the 10th and 20th passages were used for these experiments. Prior to each experiment, the medium was switched to serum-free DME and the cells were incubated for 24 h. Then, a cytokine cocktail containing final concentrations of 25 units/mL IL-1β, 30 ng/mL TNF-α, 150 units/mL IFN-γ, and 12.5 μg/mL LPS was added to the medium. After another 12-h incubation, the activated cells were used for the experiment.

Imaging Procedures. The cells were incubated for 1 h at 37 °C in KRP containing 10 μM DAF-2 DA (0.2% DMSO was used as a cosolvent) for loading, washed with KRP, and placed in KRP containing L-Arg or L-NMMA. They were mounted on an inverted fluorescence microscope (IX70; Olympus, Tokyo, Japan) equipped with an objective lens (×20), an excitation filter (490 nm), a dichroic mirror (505 nm), and a long-pass emission filter (515 nm). The temperature of the stage was maintained at 37 °C with a microwarm plate (DC-MP10DM; Kitazato, Shizuoka, Japan). Light exposure time was 132 ms. Optical signals were recorded with an MCID (Imaging Research Inc., St. Catharines, ON, Canada), which is an imaging system including a charge-coupled device (CCD) camera. Images were taken at intervals of 30 s. A confocal laser scanning microscope (Noran Instruments Inc., Middleton, WI) equipped with an objective lens (×40) and a long-pass emission filter (515 nm) was used for scanning cells in 2-μm sections from the bottom.

Table 1. Absorbance and Fluorescence Properties of Dyes[a]

DAF	extinctn coeff and abs max,[b] × 10^4 M^{-1} cm^{-1} diamine	extinctn coeff and abs max,[b] × 10^4 M^{-1} cm^{-1} triazole	fluorescence max of triazole forms, nm	rel quantum effic diamine	rel quantum effic triazole
1	8.1 (489)	5.6 (493)	521	0.004	0.78
2	7.9 (486)	7.3 (491)	513	0.005	0.92
3	8.1 (493)	6.6 (493)	521	0.005	0.85
4	9.7 (501)	9.2 (505)	530	0.002	0.75
5	9.8 (499)	7.9 (503)	523	0.007	0.70
6	11 (505)	4.3 (506)	528	0.003	0.53

[a] All data were obtained at 20 °C in 0.1 M sodium phosphate buffer, pH 7.4. Relative quantum efficiencies were measured as described under Experimental Section. [b] Values in parentheses are nm.

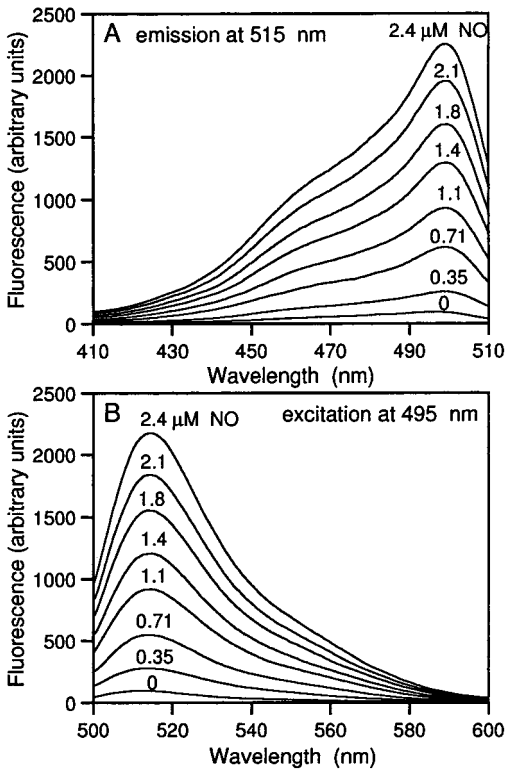

Figure 2. Excitation (A) and emission (B) spectra for DAF-2 at 37 °C in 0.1 M sodium phosphate buffer (pH 7.4) with NO values ranging from 0 to 2.4 μM. NO solution was added to 10 μM DAF-2 solution under aerobic conditions. The spectra were obtained from an average of seven accumulations 15 min after the addition of NO solution.

RESULTS AND DISCUSSION

Chemical Properties of DAFs. Diaminofluoresceins (Figure 1) were obtained according to the reaction scheme shown. Reduction of aminonitrofluoresceins by catalytic hydrogenation or with acid and metal combinations proved unsatisfactory, so we employed an alkaline reduction system with sulfide and hydrosulfide ion.[22]

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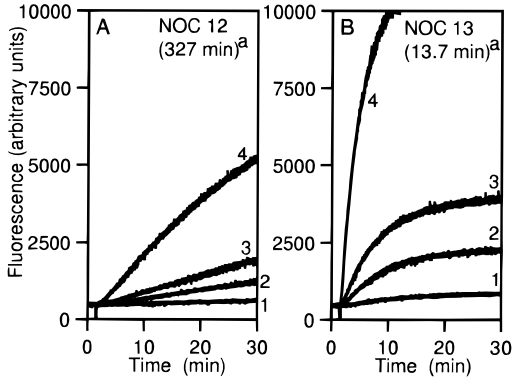

Figure 3. Time course of fluorescence intensity of DAF-2 depending on NO generated from NONOates. Varying concentrations of two NONOates with different half-lives were incubated in 2-mL reaction volumes containing 0.1 M sodium phosphate buffer (pH 7.4) and 10 μM DAF-2 at 37 °C. The fluorescence intensity was determined at 515 nm with excitation at 495 nm. (A) NOC 12 was added immediately after it had been dissolved in 0.1 M sodium phosphate buffer (pH 7.4). (B) NOC 13 was added. Final concentrations: (1) 5 (2) 50, (3) 100, and (4) 500 μM. [a]Half-lives at 22 °C are taken from the literature.[24]

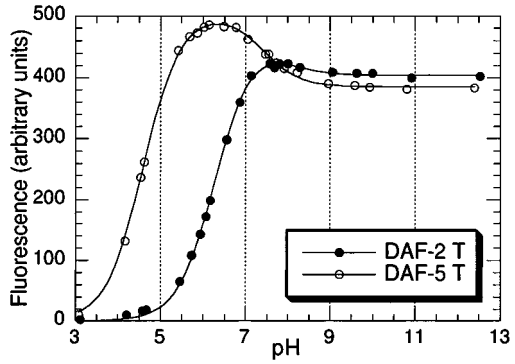

Figure 4. Effect of pH on the fluorescence intensity of DAF-2 T and DAF-5 T (1 μM) at 20 °C. Triazole forms of DAF-2 and DAF-5 (final 1 μM) were added to sodium phosphate solution (78 mM) adjusted to various pH values. The pH was measured after mixing. The fluorescence intensities of DAF-2 T and DAF-5 T were determined at 515 and 520 nm with excitation at 495 and 505 nm, respectively. The curves were fitted with the following equations: (DAF-2 T) intensity = $460/(1 + 10^{6.27-pH}) - 55.6/(1 + 10^{7.94-pH})$, $R = 0.9997$; (DAF-5 T) intensity = $505/(1 + 10^{4.59-pH}) - 119/(1 + 10^{7.41-pH})$, $R = 0.9996$.

The absorbance and fluorescence properties for DAF-1−DAF-6 are summarized in Table 1. Conversion of DAFs to the triazole form by reaction with NO caused little change of the absorbance maximums, but greatly increased the quantum efficiency. The excitation and emission wavelengths of DAF-4−DAF-6, with a chlorine atom adjacent to phenolic OH, were shifted to longer wavelengths than those of DAF-1−DAF-3. DAFs possess favorable properties for cellular imaging applications; namely, DAF has a visible excitation wavelength, which is less damaging to cells, and is not subject to interference from the autofluorescence of biological samples. For example, Figure 2 shows the excitation and emission spectra of DAF-2. The increase of fluorescence intensity was dependent on the concentration of NO. Figure 3 shows the time course of fluorescence intensity of DAF-2 after the addition of NO-releasing compounds (NOCs).[24] The intensity increased according to the half-life and concentration of added NOC.

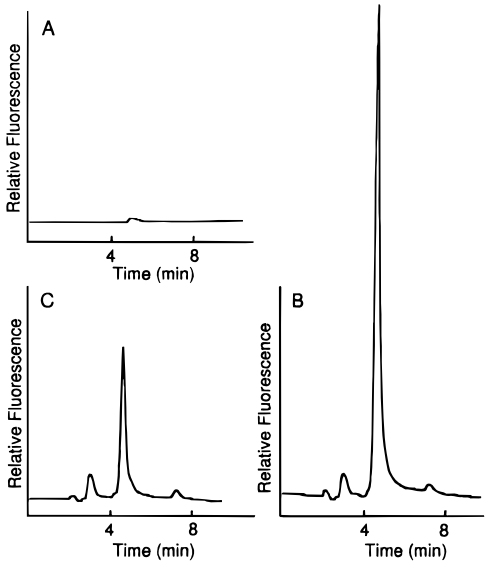

Figure 5. Detection of triazole formation by endotoxin-stimulated macrophages using reversed-phase HPLC. Fluorescence was monitored at an excitation wavelength of 495 nm and an emission wavelength of 515 nm. HPLC conditions are given the Experimental Section. (A) Standard DAF-2. (B) Endotoxin-stimulated RAW 264.7 cells were incubated with DAF-2 for 2 h. Endotoxins were LPS (10 ng/mL) and IFN-γ (10 units/mL). (C) Nonstimulated RAW 264.7 cells were incubated with DAF-2 for 2 h.

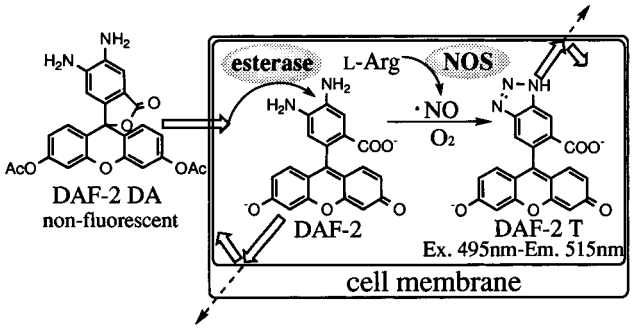

Figure 6. Schematic representation of dye behavior in the cell. DAF-2 DA permeates through the cell membrane and is hydrolyzed to yield DAF-2, which is retained in the cell owing to its relatively poor permeating ability. Loaded DAF-2 reacts with NO to form fluorescent DAF-2 T.

Although the mechanism of fluorescence of the triazole form compounds is not entirely clear, one possibility is as follows. Minta et al. proposed for fluorescent xanthene derivatives that electron donation from the amino nitrogen to the phthalic ring and then to the xanthene would increase the double-bond character between the phthalic ring and the xanthene, resulting in hindered planarity of the xanthene and radiation deactivation of fluorescence.[9] Although the structure in the excited state rather than in the ground state must be taken into consideration because the absorption spectra of the diamino forms and triazole forms are similar, it can be qualitatively assumed that fluorescence disappears when the functional groups donate electrons to the phthalic ring by resonance. Thus, the fluorescence intensity of fluorescein derivatives can be modified by functional group substitution with electron-donating or -withdrawing groups.

Judging from NO standard curves calibrated with 10 μM DAFs, DAF-2 and DAF-4 showed a little higher sensitivity than the others. The detection limit of NO by DAF-2 was 5 nM.

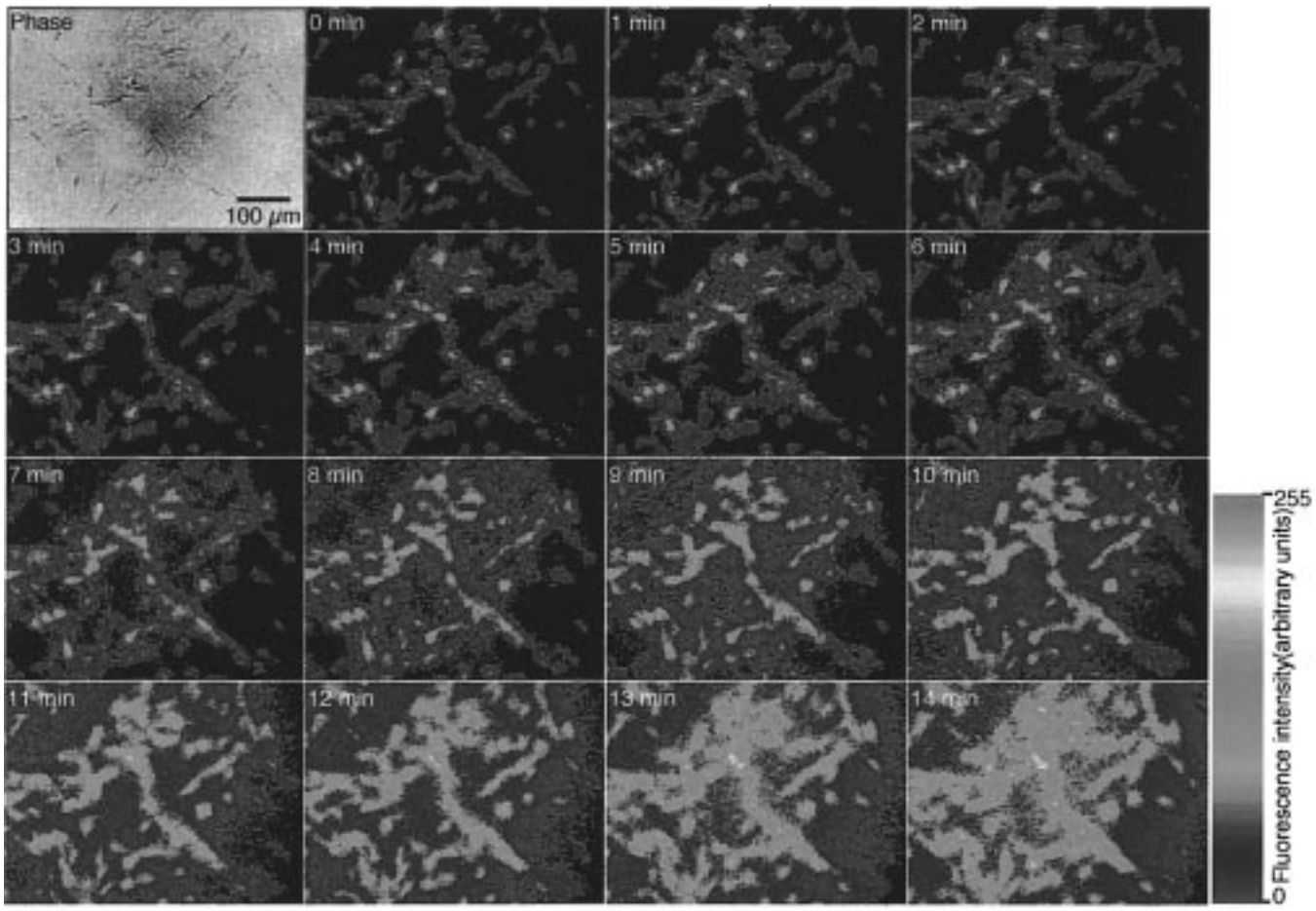

Figure 7. Bright-field and fluorescence images of the same group of activated rat aortic smooth muscle cells loaded with DAF-2 DA in the presence of L-Arg (1 mM). These images are shown in pseudocolor and correspond to the fluorescence intensity data in Figure 8A. Instrument settings were similar to those described in Figure 8.

The fluorescence intensity of fluorescein with two phenolic OH groups is almost completely quenched when the phenolic OH group is protonated. DAFs exhibit similar properties. Figure 4 shows the pH profile of fluorescence intensity of the triazole forms of DAF-2 and DAF-5 (DAF-2 T, DAF-5 T). The fluorescence of DAF-2 T strikingly decreased below pH 7. The titration curve fitted a pK_a of 6.27 ± 0.02. Conversely, it was found that DAF-1−DAF-3 were useful in media above pH 7. In fact, we had designed and synthesized DAF-4−DAF-6 with the electron-withdrawing chlorine substituent to lower the pK_a value with the aim of obtaining a stable fluorescence intensity at physiological pH. Although the electron-withdrawing effect of the chlorine atom did lower the pK_a values of DAFs, as shown in Figure 4, DAF-5 T (pK_a, 4.59 ± 0.01), there was a reduction of their fluorescence intensity from pH 7 to 9, probably due to deprotonation of the triazole moiety (pK_a, 7.94 ± 0.17 for DAF-2 T; pK_a, 7.41 ± 0.04 for DAF-5 T). It was reported that the pK_a of benzotriazole was 8.2 and that of 5-chlorobenzotriazole was 7.7.[28,29] The protonation of phenolic OH significantly reduced the fluorescence intensity because of the blue shift of the absorption wavelength owing to hindrance of electron delocalization, whereas deprotonation of the triazole ring slightly decreased the intensity because of the small electron donation to the conjugated double bonds. The chlorine substitution, lowering the pK_a value of phenolic OH, resulted in unstable fluorescence intensity around physiological pH. Taking these effects into consideration, we concluded that DAF-2 would be most appropriate for detecting NO in neutral pH buffers.

The reaction mechanisms of NO and DAF are complex because DAFs react not with NO itself but with an active intermediate formed in the course of the oxygen oxidation of NO to NO_2^-. N_2O_3 is formed as shown in eq 2 and is a potent nitrosating reagent. The rate constant for eq 2 (1.1×10^9 M^{-1} s^{-1})[30] indicates that N_2O_3 is immediately formed when NO is oxidized to $^{\bullet}NO_2$. The oxidizing reaction of N_2O_3 is competitive with hydrolysis to NO_2^-. We found that the yield of triazolofluorescein based on NO was 18% in neutral solution in the absence of biological samples. Since it was reported that the rate constant for nitrosation of morpholine by N_2O_3 was 6.4×10^7 M^{-1} s^{-1},[31] this step cannot be rate-determining. The rate-determining step is probably the formation of $^{\bullet}NO_2$ from NO (eq 1). The third-order rate constant for reaction of two NO and one oxygen

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molecule was reported by Kharitonov et al. to be 6.3 ± 10^6 M^{-2} s^{-1}.[32] Due to the slow reaction speed of NO oxidation, the chemical yield of the product was low. This result has two implications. The first is that the detection limit could be higher if the reaction speed were faster; so in other words, the rate at which NO is oxidized imposes an intrinsic limitation on detection by this method. The other point is that this detection reagent does not react directly with NO, but reacts with the oxidized form of NO. So, the measurement should not interfere too much with the signal transduction by NO. Nevertheless, the specificity for NO is high, because DAFs did not react in neutral solution with mobile and stable oxidized forms of NO, such as NO_2^- and NO_3^-, and other reactive oxygen species, such as $O_2^{\bullet -}$, H_2O_2, and $ONOO^-$, to yield any fluorescent product. Under physiological conditions, triazolofluorecein is not formed in the absence of NO.

Detection of NO Production by Macrophages. Isocratic elution with 10 mM sodium phosphate buffer pH 7.4/acetonitrile (94:6 v/v) on a reversed-phase column separated DAF-2 and DAF-2 T. Judging from the determination by HPLC with UV−visible detector, the retention times (r_t) of DAF-2 and DAF-2 T were 4.81 and 4.23 min, respectively. Figure 5 shows the triazole formation evaluated by HPLC with a fluorescence detector. Standard DAF-2 shows little fluorescence at r_t 4.81 min (Figure 5A). The supernatant of cultured macrophages (RAW 264.7) showed marked fluorescence at r_t 4.23 min, and this was augmented after endotoxin stimulation (Figure 5B). The non-stimulated cells produced a smaller amount of the triazole form (Figure 5C). This result reflects substantial NO production by macrophages even without LPS and IFN-γ stimulation.

NO Imaging. We designed and synthesized DAF-2 DA (Figures 1 and 6) for imaging of NO and confirmed that it permeated well into the cells, where it was quickly transformed into DAF-2 by esterase in the cytosol. The hydrolysis was confirmed by HPLC detection. DAF-2 DA (10 μM) was hydrolyzed quickly within 10 min by the cytosolic solution of cell lysate (data not shown). During the acetylation of the phenolic OH of DAF-2, amino groups might also be acetylated. Therefore, phenolic OH was deprotonated over cesium carbonate and acetylation was carried out carefully with acetic anhydride (see Figure 1). This procedure avoided the acetylation of the amines.

The dye was applied to the imaging of NO in cultured rat aortic smooth muscle cells by using a fluorescence microscope equipped with fluorescence filters for fluorescein chromophores. The cells were incubated with 10 μM DAF-2 DA for 1 h at 37 °C for dye loading. Fluorescein is easily photobleached by strong excitation light. Photobleaching was observed with DAF-2, but reduction of the light exposure time and the lower excitation frequency reduced this effect to a manageable level. Confocal laser scanning microscopy indicated that fluorescence was emitted from the whole cell body, including the nucleus (data not shown). This implies that DAF-2 regenerated intracellularly is distributed throughout the whole cell. There was no indication of any cell damage caused by loading the dye. Figure 7 shows the bright-field and fluorescence images, corresponding to the fluorescence intensity data of Figure 8A. Figures 7 and 8A show that the fluorescence intensity in endotoxin- and cytokine-activated cells

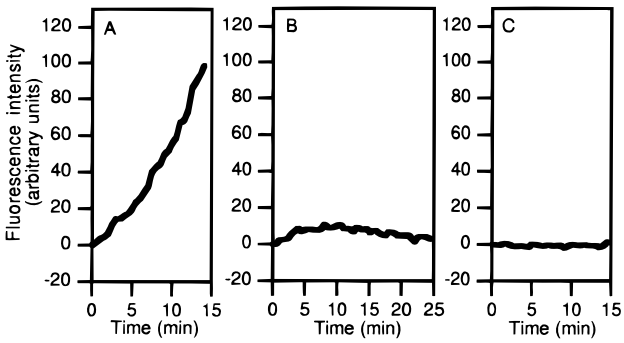

Figure 8. Fluorescence response of DAF-2 DA-loaded rat aortic smooth muscle cells, reflecting NO production. Smooth muscle cells, 12 h after LPS and cytokine activation, were incubated with 10 μM DAF-2 DA for 1 h at 37 °C for dye loading. Then, the cells were washed with KRP, and the medium was changed to KRP containing L-arginine (L-Arg) or *N*-monomethyl-L-arginine (L-NMMA) 10 min before time 0. Fluorescence excited at 490 nm (515-nm emission) was measured at 30-s intervals. (A) Fluorescence intensity of activated cells obtained when L-Arg (1 mM) was present in the medium. (B) Fluorescence intensity of activated cells in the presence of L-Arg (1 mM) and L-NMMA (10 mM). (C) Fluorescence intensity of inactivated cells in the presence of L-Arg (1 mM). Lines show mean values of the average fluorescence intensity in areas containing a cell. The zero point fluorescence intensity was taken as the initial intensity at the start of measurement.

increased owing to DAF-2 T production from DAF-2 by reaction with NO. The increase was suppressed by the NO synthase inhibitor L-NMMA (Figure 8B). The initial increase of fluorescence resulted from NO generated before the inhibition of NO synthase. Figure 8C shows that the fluorescence did not increase in inactivated cells, which did not produce NO. When an NO donor (NONOate, 1 mM NOC13) was added to the medium of inactivated cells loaded with DAF-2 DA, the fluoresence intensity in the cells was suddenly increased (data not shown).

We determined NO evolved in the supernatant by the addition of the nonesterified indicator to the medium and by the measurement of the triazole formation with a fluorescence spectrophotometer. As a result, it was estimated that NO was produced at the rate of 2 fmol/h by a single cell under these conditions. It is highly likely that the concentration of NO in the cell before diffusion is considerably higher than that in the medium thus examined. Real-time visualization of the production and diffusion of NO with our new fluorescent indicators is useful for direct analysis of the cellular functions of NO.

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