Rapid Communication

J. Biochem. **125,** 658-661 (1999)

Inhibitory Effects of Catecholamines and Anti-Oxidants on the Fluorescence Reaction of 4,5-Diaminofluorescein, DAF-2, a Novel Indicator of Nitric Oxide[1]

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Received January 13, 1999; accepted February 8, 1999

4,5-Diaminofluorescein (DAF-2) is a newly developed indicator of nitric oxide (NO). Two amino groups of DAF-2 are oxidized by NO. We investigated the effects of reducers on the NO-induced oxidation of DAF-2. NOC-5 (0.1-10 μM), a NO-donor, concentration-dependently elicited fluorescence with 10 μM DAF-2. The rate of the fluorescence reaction was dependent on the width of the excitation band path. The presence of catecholamines (1 μM), but not tyrosine or phenylephrine, attenuated the fluorescence induced by NOC-5. Ascorbate and other reducers like dithiothreitol, 2-mercaptoethanol, or glutathione (all 1 mM) abolished the fluorescence. These results suggest that reducers attenuate the NO-induced fluorescence of DAF-2 mainly through an anti-oxidative action.

Key words: catecholamines, 4,5-diaminofluorescein, fluorescence, nitric oxide, reducers.

Nitric oxide (NO) exerts a variety of biological actions. Recently, the effects of NO have been proposed to be dependent on the localized concentration initially formed by NO synthases: lower concentrations than 1 μM regulate the cardiovascular function, middle range concentrations of around 1-10 μM affect neurotransmission, and higher concentrations elicit immunological responses (*1*). Thus, various methods for determining NO involving measurement of the ESR spectrum, luminescence intensity, current through the electrode and so on have been developed (*2*). Very recently, 4,5-diaminofluorescein (DAF-2) was synthesized as a specific NO indicator (*3*). DAF-2 selectively traps NO between two amino groups in its molecule, and yields triazolofluorescein, which emits green fluorescence when excited at 490-495 nm. The fluorescence intensity is dependent on the amount of NO trapped by DAF-2. Thus, we applied DAF-2 to the detection of NO liberated from vascular vessels. However, when catecholamine, which causes contraction of vascular smooth muscle, was added to the medium, the fluorescence signal of DAF-2 was significantly inhibited. Since catecholamines are known to be reducers (*4*), we investigated the effects of other reducers on the fluorescence signal produced on the reaction of DAF-2 with NO.

DAF-2 (purchased from Daiichi Pure Chemicals, Tokyo) was dissolved in 1 mg/0.55 ml dimethyl sulfoxide, and then diluted to 10 μM with a physiological salt solution composed of NaH_2PO_4 1.3, KCl 5.4, glucose 5.6, $NaHCO_3$ 24, NaCl 120, $MgCl_2$ 1, and $CaCl_2$ 2 (mM). The solution was bubbled with 20% O_2, 75% N_2, 5% CO_2 (37°C, pH 7.4). The fluorescence signal caused by the reaction of DAF-2 with NO was measured using a fluorescence spectrometer (F-4010, Xe lamp 150 W; Hitachi, Tokyo). NOC-5, 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (Dojindo, Kumamoto), was used as a NO-releasing agent. L-Ascorbic acid, DTT, GSH, 2-mercaptoethanol, L-phenylephrine hydrochloride, (−)-epinephrine bitartrate salt, (−)-norepinephrine bitartrate salt, dopamine hydrochloride, L-dopa, and L-tyrosine were purchased from Sigma, MO, USA. All the results given were obtained through at least duplicated experiments.

When NOC-5 (0.1, 1, and 10 μM) was added to the DAF-2 containing solution, the fluorescence intensity increased in a concentration-dependent manner (Fig. 1). Using a narrow excitation band path (5 nm), the fluorescence intensity increased linearly in 30 min after the addition of NOC-5 at less than 10 μM (Fig. 1A). Such elevation of the fluorescence intensity was not detected after the addition of 0.01 μM NOC-5. The rate of increase in the fluorescence reaction shown in Fig. 1A did not seem to be exactly proportional to the added concentration of NOC-5. Presumably, NO yielded with a low concentration of NOC-5 is readily converted to other nitric oxides in the presence of oxygen, so that 0.1 μM NOC-5 gives a much smaller rate of the fluorescence reaction than predicted, in addition to the experimental variance due to the solubilization of NOC-5. On the other hand, when the excitation band path was 10 or 20 nm, *i.e.* wider than 5 nm, the rate of the fluorescence reaction after the addition of 1 μM NOC-5 was

[1] This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: DAF-2, 4,5-diaminofluorescein; NO, nitric oxide; NOC-5, 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene.

faster, and the plateau level was attained earlier with a wider band path (Fig. 1B). The attained plateau level did not significantly decay in 30 min. After the plateau level was attained, shutting-off of the excitation light abolished the fluorescence intensity and then re-excitation with the light by re-opening of the light path promptly raised the fluorescence intensity to the original plateau level. Figure 2C shows the rapid elevation followed by the plateau phase of the fluorescence reaction with the 20 nm excitation band path after the successive addition of NOC-5 at concentrations from 0.1 and 1 μM. With the 20 nm excitation band path, the increase in fluorescence intensity was minimum after the addition of 0.01 μM NOC-5 and the upper detection limit of the spectrometer was overshot after that of 10 μM NOC-5 (Fig. 1C). Practically, with the use of a fluorescence spectrometer, DAF-2 is capable of detecting NO released from NOC-5 at concentrations of more than 0.1 μM. The rate of the fluorescence reaction appears to be dependent on the excitation band path, as a wider band path seems to be better for the stable detection of NO.

Figure 2 shows the effects of catecholamines and ascorbate on the DAF-2-fluorescence (excitation band path, 20 nm) induced by the cumulative addition of NOC-5 (1, 3, and 10 μM). The presence of catecholamines, dopa, dopamine, norepinephrine, or epinephrine (all 1 μM), attenuated the fluorescence induced by NOC-5 at concentrations of 1 and 3 μM, but inhibition was not apparent for the 10 μM NOC-5-induced fluorescence. Dopamine at the high concentration of 10 μM markedly attenuated the 10 μM NOC-5-induced fluorescence (Fig. 2F). Other tested catecholamines at 10 μM caused comparable inhibition of 10 μM NOC-5-induced fluorescence to that caused by dopamine (data not shown). In contrast, phenylephrine, a synthetic derivative of epinephrine lacking a hydroxyl group on the benzene ring, even at the high concentration of 10 μM did not affect the DAF-2 fluorescence responses to 1-10 μM NOC-5 (data not shown). L-Tyrosine, which does not possess a catechol moiety and is a precursor of catecholamines, at 10 μM did not inhibit the fluorescence responses to NOC-5 at all (data not shown).

We further investigated the effects of various reducers on the NOC-5-induced DAF-2 fluorescence. The presence of 1 μM ascorbate attenuated the DAF-2 fluorescence induced by NOC-5 at concentrations of 1 and 3 μM (Fig. 2G). This inhibitory effect was similar to those of the tested catecholamines described above. Ascorbate at the high concentration (1 mM) abolished the fluorescence reaction to NOC-5 at the tested concentrations of 1-10 μM (Fig. 2H). Also, the presence of DTT or 2-mercaptoethanol (both 1 mM) abolished the increase in fluorescence (data not shown). In the presence of 1 mM GSH, a thiol compound present in living organisms at concentrations of 1-10 mM (*5*), 1 and 3 μM NOC-5 did not elicit fluorescence and 10 μM NOC-5 elicited a marginal increase in the fluorescence (data not shown), indicating that GSH inhibits the DAF-2 fluorescence response to NOC-5.

The present experiments suggest that NO concentration-dependently reacts with DAF-2, resulting in concentration-dependent fluorescence upon excitation. Once DAF-2 is fully excited, the fluorescence intensity is very stable for more than 30 min and the fluorescence reaction promptly occurs after excitation, indicating that triazolofluorescein formed through the reaction of DAF-2 with NO is very stable, not being decomposed by the excitation light. While the rate of the fluorescence reaction appears to be dependent on the width of the excitation band path. Thus, some step of triazolofluorescein formation seems to be accelerated proportionally by the amount of excitation light, not by the intensity of the light. When measured using a NO-sensitive electrode (*6*), the NO liberation from NOC-5 was found to be fast, being well comparable with the fluorescence reaction with the 20 nm excitation band path, suggesting that the liberation of NO from NOC-5 is not affected by the excitation light. In a bioassay system involving isolated rat aorta, 0.1 μM NOC-5 elicited rapid relaxation in the presence of 1 μM phenylephrine, but after the preincubation of NOC-5 plus 10 μM DAF-2 under dark conditions the relaxation activity of NOC-5 was reduced (unpublished data). The reduction in the relaxation activity was dependent on the duration of preincubation and the half time of reduction was 2-3 min. Thus, the association of NO with DAF-2 seems to be relatively rapid, *i.e.* of a minute

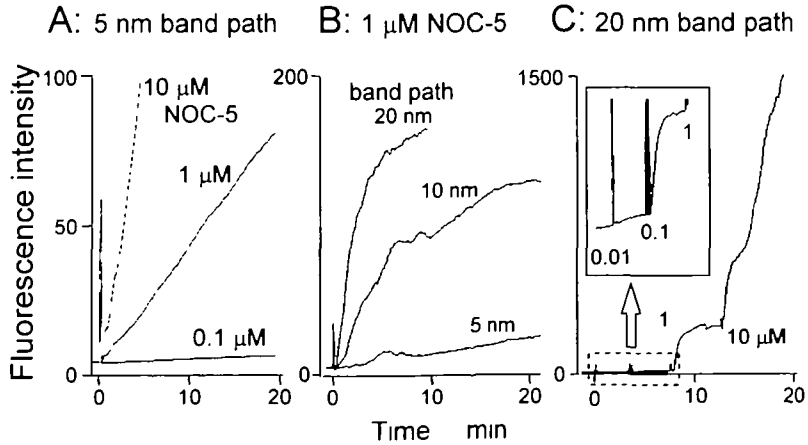

Fig. 1. **Fluorescence reaction of 4,5-diaminofluorescein (DAF-2, 10 μM) with NOC-5 with excitation band paths of various width at 37°C.** In A, the fluorescence changes after the addition of NOC-5 at 0.1, 1, and 10 μM were monitored through a 5 nm excitation band path and a 5 nm emission band path. Three different traces are superimposed. In B, fluorescence elicited by 1 μM NOC-5 was monitored with a 5 nm excitation path (5 nm emission path), 10 nm excitation path (2.5 nm emission path), or 20 nm excitation path (1.25 nm emission path). In C, 0.01, 0.1, 1, and 10 μM NOC-5 were successively added and the DAF-2 fluorescence reaction was monitored with a 20 nm excitation path. Inset in C: the fluorescence trace enclosed by the dotted line is expanded 75-fold on the ordinate. NOC-5 at each concentration was dissolved immediately before addition. The transient increases in the fluorescence intensity at 0 time were artifacts due to the opening of the lid of the dark chamber to add NOC-5. On the ordinate, the fluorescence intensity is expressed in arbitrary units, and the presented intensities are proportional. Note: Experimental variation of the slope and the maximum level of the fluorescence reaction can be observed in A, B, and C, since representative data for A, B, and C were obtained through different sets of experiments. A narrower emission band path with a wider excitation band path is necessary to reduce the background light intensity with a wider excitation band path.

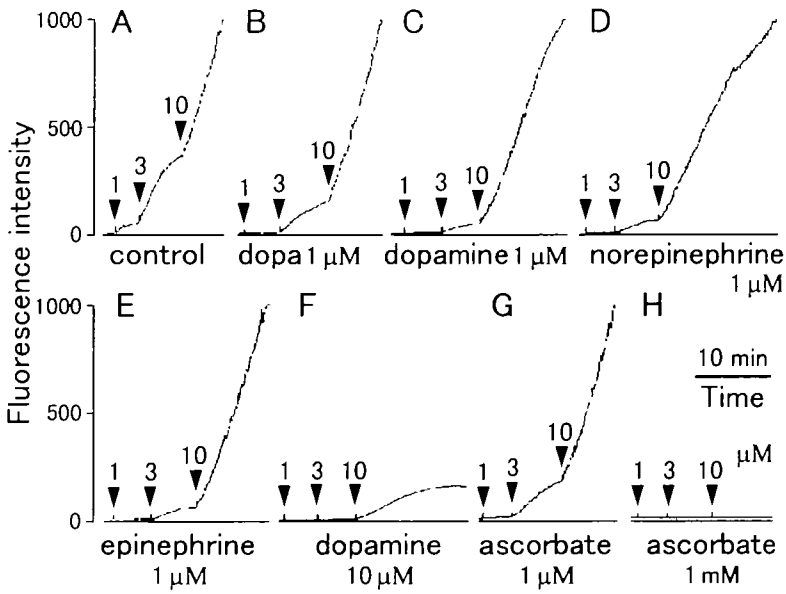

Fig. 2. **Inhibitory effects of catecholamines (dopa, dopamine, norepinephrine, and epinephrine) and sodium ascorbate on the DAF-2 fluorescence response to the successive addition of 1, 3, and 10 μM NOC-5.** Fluorescence was measured with a 20 nm excitation band path. A shows a control. B, C, D, E, F, G, and H show the effects of treatment with 1 μM dopa, 1 μM dopamine, 1 μM norepinephrine, 1 μM epinephrine, 10 μM dopamine, 1 μM ascorbate, and 1 mM ascorbate, respectively. The tested agents were added 5 min before the addition of 1 μM NOC-5. The fluorescence reactions after the addition of 10 μM NOC-5 overshoot the upper detection limit of fluorescence except in F and H.

order. On the other hand, with the narrower excitation band path (5 nm), the DAF-2 fluorescence reaction gradually increased over 30 min. The probability of the formation of triazolofluorescein through the activation of a small area of the solution by light in a unit time may be low under the presently employed experimental conditions, so the rate of DAF-2 fluorescence reaction may be low on excitation with the narrow excitation band path.

The present experiments further demonstrated that all tested reducers, including catecholamines, inhibit the DAF-2 fluorescence reaction to NOC-5. One possible mechanism for this inhibition is the direct interaction of reducers with DAF-2. Since the fluorescence of triazolofluorescein was reported to be almost completely quenched when the phenolic OH group in the molecule is protonated (*7*), the tested reducers may protonate the phenolic OH groups of DAF-2 to interfere with the fluorescence reaction. An alternative mechanism for the inhibition of the fluorescence reaction could be considered with respect to the interaction of NO with reducers. Since an SH group is known to trap NO under basic conditions (*8*), reducers like glutathione, dithiothreitol, 2-mercaptoethanol, *etc.* may be capable of forming nitrosothiol to reduce the effective concentration of NO for the reaction to DAF-2. Catecholamines have also been reported to react with NO, presumably through the formation of N_2O_3, resulting in the production of 6-nitrosocatecholamines at neutral pH (*9-11*), and 6-nitrosonorepinephrine was found in the brain, although in a low amount compared with that of norepinephrine (*12*). Thus, it is feasible that catecholamines inhibit the DAF-2 fluorescence reaction by trapping NO at the 6th position of the catechol moiety. Peroxynitrite formed from NO specifically also nitrosates tyrosine (*13*) and thiols (*14*). However, this peroxynitrite formation may not occur under our experimental conditions, since the presence of tyrosine or phenylephrine did not affect the fluorescence reaction of DAF-2 with NOC-5 at all. Although both mechanisms described above are very feasible, the exact mechanism responsible for the reducer-induced inhibition of the DAF-2 fluorescence reaction with NOC-5 can not be delineated at present and thus awaits future investigations. At present, it is notable that reducers ordinarily used in laboratories significantly inhibit the fluorescence reaction of DAF-2 with NO. Also, catecholamines at 1 μM, the concentration usually used in laboratories to investigate catecholaminergic function, inhibit the DAF-2 fluorescence reaction. Further, the recommended addition of ascorbate to diluted solution of catecholamines (*15*) is not suitable for DAF-2 fluorescence measurement. On the other hand, synthetic reagents acting on catecholamine-receptors such as phenylephrine did not inhibit the DAF-2 fluorescence reaction. Therefore, to measure DAF-2 fluorescence, we recommend against the use of reducers and catecholamines in the experimental system. In addition, measurement of the DAF-2 fluorescence reaction is better performed with a wide excitation band path, but one should avoid the damage to intact cells caused by the strong light when applied to living biological preparations.

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