Interfering with Nitric Oxide Measurements

4,5-DIAMINOFLUORESCIN REACTS WITH DEHYDROASCORBIC ACID AND ASCORBIC ACID*

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4,5-Diaminofluorescein (DAF-2) is widely used for detection and imaging of NO based on its sensitivity, noncytotoxicity, and specificity. In the presence of oxygen, NO and NO-related reactive nitrogen species nitrosate 4,5-diaminofluorescein to yield the highly fluorescent DAF-2 triazole (DAF-2T). However, as reported here, the DAF-2 reaction to form a fluorescent product is not specific to NO because it reacts with dehydroascorbic acid (DHA) and ascorbic acid (AA) to generate new compounds that have fluorescence emission profiles similar to that of DAF-2T. When DHA is present, the formation of DAF-2T is attenuated because the DHA competes for DAF-2, whereas AA decreases the nitrosation of DAF-2 to a larger extent, possibly because of additional reducing activity that affects the amount of available N2O3 from the NO. The reaction products of DAF-2 with DHA and AA have been characterized using capillary electrophoresis with laser-induced fluorescence detection and electrospray mass spectrometry. The reactions of DAF-2 with DHA and AA are particularly significant because DHA and AA often colocalize with nitric-oxide synthase in the central nervous, cardiovascular, and immune systems, indicating the importance of understanding this chemistry.

NO is involved in a variety of important biological functions in the cardiovascular system, the central and peripheral nervous system, the reproductive system, and the immune system (1–6). NO is normally generated via an enzymatically regulated pathway with the conversion of L-arginine to citrulline by a family of at least three distinct nitric-oxide synthase (NOS) enzymes, the neuronal, inducible, and endothelial NOS forms (or NOS-I, -II, and -III) (7–9). Endothelial and neuronal NOS, also named constitutive NOS, are calcium-dependent. Although cells expressing constitutive NOS generally produce small amounts of NO (submicromolar at the cellular level), inducible NOS, which is synthesized in immune-competent cells when stimulated by cytokines, endotoxins, and other biologically active compounds, is calcium-independent and produces NO at higher levels (1–10 μM in microphages) (10). Although in situ and immunohistochemical techniques allow one to determine whether NOS is present in a particular tissue, whether the NOS is actively producing NO under specific conditions and the amount of NO produced are important questions to answer to understand the physiological roles of NOS.

Direct detection (or imaging) of NO production in a biological system was unsatisfactory until the development of a series of fluorescent indicators for NO, the diaminofluoresceins (DAFs) in 1998 by the Nagano group (11). In the presence of oxygen, NO nitrosates DAFs to produce the highly fluorescent triazofluoresceins. DAFs provide the advantages of sensitivity (detection limits of 5 nM), simple protocols, and noncytotoxicity, and they are also believed to offer high specificity to NO. Since then, an increasing number of researchers have used them for NO detection and NO imaging (10, 12).

The specificity of 4,5-diaminofluorescein (DAF-2), a commonly used form of DAF, has been examined. Broillet et al. (13) reported that Ca2⁺, Mg2⁺, or incident light promoted the production of DAF-2T, the reaction product of DAF-2 and NO, by using NO donors in Ringer solution. It was later reported (14) that the enhancement of fluorescence intensity was not caused by increasing the reaction rate between DAF-2 and NO but by the potentiation of the release rate of NO from NO donors, and it was concluded that DAF-2 could provide reliable information on NO production in a biological system. Recently, Roychowdhury et al. (15) evaluated the efficiency and specificity of DAF-2 for the detection of endogenously produced NO in mouse glial cell culture and reported that DAF-2 was more sensitive for peroxynitrite. Jourd’heul (16) described that DAF-2 may be oxidized by either peroxynitrite or a peroxidase to form an intermediate that directly combines with NO to bypass NO/O2 reaction and suggested that the results from DAF-2 fluorometric assays are quantitatively difficult to interpret in cells when oxidants and NO are cogenerated. In addition, Nagata et al. (17) investigated the effects of reducers on the NO-induced nitrosation of DAF-2 and reported that catecholamines attenuated the fluorescence induced by NO donor, whereas ascorbic acid (AA, ascorbate, or vitamin C) and other reducers abolished the fluorescence. The authors suggested that the attenuation was mainly through anti-oxidative action of the reducers and oxidized NO and N2O3 or possibly through a direct interaction of reducers with DAF-2. We demonstrate here that DAF-2 directly reacts with dehydroascorbic acid (DHA) and AA to form fluorescent compounds with emission spectra similar to that of DAF-2T.

AA is the primary water-soluble antioxidant in biological systems, donating reducing hydrogen when being oxidized to...
DHA (18, 19). Uptake of AA into cells has been suggested to be via DHA, which is preferentially transported through the cell membrane (20). Once inside the cell, DHA is rapidly reduced to AA through enzymatic processes that involve GSH, NADH, or NADPH reductants (21). Tissues vary widely in AA content with a range of 1–10 mM (22–25), but the highest concentrations occur in the pituitary and adrenal glands and in the brain (22). For example, catecholamines require ascorbic acid for synthesis and protection from oxidation. Large quantities of AA are required for collagen synthesis (26). Leukocytes, particularly neutrophils, possess high concentrations of AA and may be the primary vehicle for its distribution throughout the body. It has been suggested that AA accumulates in activated human neutrophils to 14 mM concentrations (23).

This study reports a cross-reaction of DHA/AA with DAF-2, confounding detection and measurements of NO levels in biological systems. We investigated the effect of this newly described reaction using capillary electrophoresis (CE) with wavelength-resolved fluorescence detection and chemically characterized the new products using electrospray ionization-mass spectrometry (ESI-MS). Because of the extremely high physiological concentrations of AA and DHA colocalized with nanomolar levels of NO, even a poor reactivity of DAF-2 to DHA/AA can generate significant amounts of DAF-2-DHA/AA products. This is a potentially serious complication for direct NO measurements using DAF-2 because the same tissues that contain NO synthases have high levels of AA and DHA.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—All of the chemicals used were of the highest available purity and were obtained from Sigma unless otherwise noted. Three different buffers were used in this work. The 0.1 m phosphate buffer (pH 7.4 ± 0.1) was prepared using 0.54 g of monobasic sodium phosphate (NaH2PO4·H2O; Fisher) and 1.64 g of dibasic sodium phosphate (Na2HPO4·7H2O; Fisher) in 100 ml of ultrapure water (Millipore, Bedford, MA). The 0.1 m acetate buffer (pH 5.0 ± 0.1) was prepared using 0.96 g of sodium acetate (CH3COONa·3H2O) and 0.17 ml of glacial acetic acid dissolved in a final volume of 100 ml of ultrapure water. The borate buffer (pH 8.5 ± 0.1) was prepared using 3 g of boric acid (H3BO3) and 9.2 g of sodium borate (Na2B4O7·10H2O) in 1.00 liters of milli-Q water. DAF-2 was obtained either in solid form or as a 1.9 mM NO solution in pH 7.4 phosphate buffer (pH 7.4) was added to the vial. The cerebral ganglia were treated with 1% protease IX (Sigma, P-6141) and incubated at room temperature as described below. For the purpose of evaluation and calibration, a series of known concentrations of NO donor solution was added to and mixed with DAF-2. If the effects of other chemicals (such as DHA, AA, or GSH) were monitored, NONOate solution was combined with the compound just prior to the addition of DAF-2 solution. Unless otherwise stated, all of the reactions were performed at room temperature for 30 or 40 min (30-min reaction for CE assays and 40-min reaction for fluorometric measurements). The reaction products were subjected to subsequent CE or fluorometric analysis or transferred to dry ice for storage if immediate analysis was not available. Because of the instability of DHA, AA, and GSH, all of the solutions were freshly prepared before the analysis.

RESULTS

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Reaction of DAF-2 with DHA and AA

DAF-2 cross-reacted with DHA to produce fluorescent compounds, which are called the DAF-2-DHAs. Combining DHA and DAF-2 in solution (pH 7.4) for 30 min produced two new major compounds detected with CE-LIF, DAF-2-DHA1 and DAF-2-DHA2 (Fig. 1B). DAF-2 in the presence of both DHA and NO generated DAF-2-DHAs as well as DAF-2T (Fig. 1C). Comparison of these three reaction protocols (Fig. 1D) demonstrated that more DAF-2 was used by 1 mM DHA than 30 μM NO donor (Fig. 1D, left panel). Moreover, the signal of DAF-2-DHAs was almost unchanged with the presence of NO (Fig. 1D, middle panel), whereas the amount of DAF-2T generated (from 30 μM NO donor) decreased by about 45% with the inclusion of 1 mM DHA (Fig. 1D, right panel).

DAF-2 reacted with AA to generate the same fluorescent compounds as DAF-2 with DHA. Although stronger DAF-2-DHA peak signals were observed for samples with longer incubation times of 7–24 h (data not shown), only a small amount was detected after an incubation period of 30 min using CE-LIF (Fig. 2). Concomitantly, AA attenuated the formation of DAF-2T to a greater extent (~98%) than DHA (45%). Besides using an NO donor, authentic NO bubbled in buffer solutions (both pH 5 and pH 7.4) were tested. In all cases, similar results were obtained.

Time course (5 min to 24 h) examinations of the reaction of DAF-2 with DHA/AA at both pH 5 and 7.4 demonstrated ever-increasing DAF-2-DHA2 signals as well as complex patterns of a series of unresolved peaks between DAF-2-DHA1 and DAF-2-DHA2 (data not shown). The major reaction products were those observed at pH 7.4 with a 30-min incubation time, DAF-2-DHA1 and DAF-2-DHA2 (Figs. 1 and 2). A reaction time longer than 24 h produced only DAF-2-DHA2 for DAF-2 + DHA/AA at both pHs.

Fluorometry—The reaction of DAF-2 + NO, DAF-2 + DHA/AA and the effect of DHA and AA on the fluorescence of DAF-2 in response to NO were monitored using a fluorimeter with 495-nm excitation. This scheme measured the total fluorescence from all fluorescent compounds in the solution. Fluorescence spectra of DAF-2 with NONOate at different concentrations are shown in Fig. 3. Fluorescence intensity was linear with the NONOate concentration (r² = 0.9974) within the tested range of 10 nM to 10 μM.

Appreciable fluorescence signal was observed from DAF-2 + DHA mixtures. As shown in Fig. 3, the fluorescence intensity of the reaction mixture of DAF-2 with 1 mM DHA was in between the fluorescence produced by 100 nM and 1 μM NONOate. A semi-quantitative calculation based on the calibration curve of DAF-2T fluorescence versus NO donor concentration revealed that 1 mM DHA produces a fluorescence signal indistinguishable from ~300 nM NONOate, 10 μM DHA + 10 μM NONOate, and 1 mM AA + 20 nM NONOate after an incubation period of 40 min with DAF-2. Furthermore, the fluorescence emission profiles of DAF-2-DHAs and DAF-2T showed a high degree of similarity (both had emission maxima at 515 nm). The excitation maxima differed slightly between DAF-2-DHAs (485 nm) and DAF-2T (495 nm).

Consistent with the CE-LIF assays, similar attenuation was observed on the total fluorescence of DAF-2 + NO when DHA or AA was present (Fig. 4). The total fluorescence from DAF-2T generated from 1 μM NONOate was attenuated by about 57 and 75% with 1 mM DHA and AA, respectively.

ESI-MS—The DAF-2-DHA products were characterized with ESI-MS. The solvent dependence of the reaction products aided in this analysis. In an ammonium acetate media (pH 4) where CE-LIF mainly showed DAF-2-DHA2, a molecular ion with m/z 519.1 was observed from the DAF-2 + DHA reaction mixture. An additional weak peak at m/z 501.1 was detected...
from a reaction mixture prepared in water, which has a weak DAF-2-DHA1 peak and a strong DAF-DHA2 signal in the CE-LIF analysis. These two ions with \( m/z \) 519.1 and 501.1 (M/H^+) were therefore assigned to DAF-2-DHA2 and DAF-2-DHA1, respectively. We name these compounds DAF-2-DHA-518 and DAF-2-DHA-500. Analysis of the MS-MS fragmentation pattern and the proposed structures of the two compounds are summarized in Table I.

**Cellular Analysis**—The reaction of DAF-2 with DHA/AA was also observed in the cellular samples using CE-LIF. Fig. 5 demonstrates typical wavelength-resolved electropherograms of molluscan MCCs and a few small adjacent cells homogenated and incubated with DAF-2 solution. Besides the expected cellular compounds (such as the neurotransmitter, serotonin; amino acids, tryptophan and tyrosine; NOS cofactor, tetrahydrobiopterin) that are natively fluorescent with 257-nm excitation, we also detected DAF-2-DHAs generated from DAF-2 in reaction with...
Table I

| MS-MS fragments | Δ Mass | Ion | Structure for DAF-2-DHA-518* |
|----------------|--------|-----|-----------------------------|
| from m/z 519.1 (m/z) | (from m/z) | | |
| 501.1 | -18 | (m/z 519.1) | H₂O |
| 459.1 | -60 | (m/z 519.1) | C₂H₂O₂ |
| 429.1 | -30 | (m/z 459.1) | CHOH |
| 401.1 | -28 | (m/z 429.1) | CO |
| 373.1 | -28 | (m/z 401.1) | CO |

| MS-MS fragments | Structure for DAF-2-DHA-500** |
|----------------|-------------------------------|
| from m/z 501.1 (m/z) | |
| 441.1 | -60 | (m/z 501.1) | C₂H₂O₂ |
| 413.1 | -28 | (m/z 441.1) | CO |
| 385.1 | -56 | (m/z 441.1) | 2CO |

Note: Key moieties
- 1H-Quinoxaline-2-one
- Quinoxaline

Fig. 5. Wavelength-resolved electropherograms of DAF-2-treated cell homogenates. A. MCCs from P. californica, 2-h incubation. B. MCCs and surrounding cells from P. californica, 45-min incubation. Peak identities are serotonin (arrow a), tryptophan (arrow b), tyrosine (arrow c), and tetrahydrobiopterin (arrows d, d', and d**). Note that protein in the cellular samples prolonged the migration time, because of the coating of the capillary wall. All of the labeled peaks were identified by comparison with standards run immediately before or after the CE assay and confirmed by spiking with standards.

endogenous DHA/AA. In the control samples without DAF-2 incubation, no DAF-2-DHAs were observed. Unresolved peaks eluting between DAF-2-DHA1 and DAF-2-DHA2, as in the time course of DAF-2 + DHA/AA reaction, were also observed.

DISCUSSION

Reaction Scheme of DAF-2 with DHA/AA—CE-LIF results indicate that AA and DHA react with DAF-2 under physiologically relevant conditions to form a unique series of fluorescent products, from which we identified two major compounds, DAF-2-DHA-518 and DAF-2-DHA-500. We propose a reaction mechanism where the two primary amines from DAF-2, acting as electron-donating groups, react with the 1,2-carbonyl groups from DHA/AA to form a conjugated system containing -C=N moieties. Reactions between ascorbate and diaminobenzene, the end functional group of DAF-2, have been documented (32–34). The reaction products between these two reactants have been previously suggested to contain either a quinoxaline structure (see Table I footnote) with the five-member ring of ascorbate remaining closed (32) or a 1H-quinoxaline-2-one structure (see Table I footnote) with the ascorbate ring opened (33, 34). From these reports, we expect a similar reaction scheme resulting in the two predicted structures in Table I. These structures have been partially confirmed by ESI-MS and MS-MS analysis, although complete MS-MS fragmentation was not obtained. The proposed reaction and reaction products are further supported by an earlier study that reported the use of 2,3-diaminonaphthalene to detect AA (35). Yang et al. (35) proposed a similar reaction scheme in which the two primary amine groups from 2,3-diaminonaphthalene reacted with the carbonyl moiety on ascorbate to form a quinoxaline structure. Interestingly, 2,3-diaminonaphthalene, a previously defined fluorescent indicator for NO, reacts with NO in the presence of oxygen to generate a triazole structure; this is the reaction upon which the development of DAFs and diaminorhodamines as fluorescent NO indicators was based (12). It is reasonable to predict that other structurally similar members of the DAF family such as DAF-FM and diaminorhodamine 4M-AM also react with DHA/AA to form fluorescent products similar to those we have described here. The reactivity of DAF-FM and diaminorhodamine 4M-AM with DHA/AA and the fluorescence properties of the products (if any) will be the focus of future investigations.

Here, we report the formation of fluorescent products of both DHA and AA combined with DAF-2. AA autoxidizes to form DHA (36). Thus it is likely that DAF-2 + AA fluorescent products may be acting through DHA, given that only small amount of same products, DAF-2-DHA-500 and DAF-2-DHA-518, were observed for the DAF-2 + AA mixture (Fig. 2). To ensure the presence of AA alone in solution, it is necessary to carefully control the reaction conditions. To suppress the AA autooxidation, we could eliminate oxygen from the solution, but the reaction of DAF-2 with NO requires the presence of oxygen. A second alternative would be the use of GSH at pH 7.4 to prevent AA oxidation (37); however, we found no significant difference on the reaction products of DAF-2 + AA with or without GSH. Rather, GSH itself was found to attenuate the DAF-2T signal, consistent with the observation by Nagata et al. (17). Thus this method is not practical in our study for the preservation of AA in solution. It is unclear from our data whether or not AA reacts directly with DAF-2. The unresolved peaks between DAF-2-DHA1 and DAF-2-DHA2 observed in the cellular analysis (Fig. 5) and in the time course studies may be attributed to the ascorbate cascade resulting from AA autooxidation to DHA and the subsequent DHA degradation (38, 39).

DAH/AA Inhibits NO-induced Nitrosation of DAF-2 and Obscures DAF-2T Emission—Both DHA and AA attenuate the formation of DAF-2T. The effect by DHA is mainly due to the competition for DAF-2. Physiologically relevant levels of NO may be low enough so that the reaction of DHA with DAF-2 interferes with NO measurements. AA seems to inhibit the DAF-2 + NO reaction to a greater extent. This can be attributed to additional redox activity between AA and N₂O₃ to form NO, which decreases the available amount of N₂O₃ to react with DAF-2 to form DAF-2T (17).
As we have shown, DAF-2 reacts with DHA/AA to form fluorescent compounds, DAF-2-DHAs. Unfortunately, standard fluorescence spectroscopy is not able to distinguish DAF-2-DHAs from DAF-2T because of the similarity in the emission profiles. Although the fluorescence properties (extinction coefficient and fluorescence quantum yield) of the DAF-2-DHAs are much lower than those of DAF-2T, the concentration of ascorbate in biological systems (0.1 to 10 mM) is often orders of magnitude higher than the expected NO level (10 nM to 10 μM).

As an example, the measured AA and DHA concentrations in the Aplysia MCC neuron are ~1 mM and ~300 μM respectively.\(^2\) Our observation of DAF-2-DHA peaks in the MCC neurons incubated with DAF-2 clearly indicates that this reaction occurs under endogenous physiological conditions. Thus we suggest that the fluorescence observed with spectrofluorometry upon DAF-2 application may contain a significant DAF-2-DHAs signal, in addition to the expected DAF-2T fluorescence.

Biological Implications—NO research has expanded of the last 5 years. There have been more than 100 studies published using variants of DAF fluorescence indicators to detect NO in biological systems. However we demonstrate here novel reactions of DAF-2 with DHAA. Without chromatographic or electrophoretic separation, fluorescence spectroscopy detection lacks the ability to differentiate compounds with similar emission profiles. Several studies have used high performance liquid chromatography to separate weakly fluorescent DAF-2 and highly fluorescent DAF-2T but have not reported other fluorescent compounds from the direct reaction with DAF-2 (10, 41, 42). Our work appears to be the first report that DAF-2 reacts with physiologically relevant compounds other than NO-related compounds.

NO is an important messenger molecule involved in the regulation of diverse physiological and pathophysiological mechanisms in biological systems. AA plays an antioxidant role in many of the same biological activities, including endothelial function, infection and inflammation, and fertilization (26, 43–46). It has also been suggested that AA potentiates NO synthesis in endothelial cells and preserves the endothelial NO activity (47, 48). Based on these findings, our experimental results demonstrate a significant potential bias in the methodology of NO assays using DAF-2.

It has been suggested that the major advantage of DAF-2 fluorescence spectroscopy over other NO sensitive imaging/ detection techniques (such as electron paramagnetic resonance or EPR with NO spin trapping) is the ability to detect the basal NO production from endothelial and neuronal cells, with micromolar NO concentrations (41, 49, 50). The intracellular level of ascorbate is as high as 9 mM in human endothelial cells (24) and is in the range of 1–10 mM in neurons (25). Our data indicate that these high concentrations of AA can attenuate the DAF-2T level to below its detection limits or even prevent the formation of DAF-2T. Concomitantly, fluorescence of DAF-2-DHAs generated from the DAF-2 + AA/DHA reactions is expected that is indistinguishable from DAF-2T fluorescence. To exclude this possibility, further work using specific constitutive NO inhibitors should be performed.

We have observed a constant increase of DAF-2-DHAs signal with longer DAF incubation periods that may be misinterpreted as inducible NO production. As one example, DAF-2 has been used together with NOS-II in situ hybridization to simultaneously monitor NO production and inducible NOS mRNA expression in an experimental model of central nervous system inflammation (51). NO production estimated from DAF fluorescence was observed in a much broader area than inducible NOS mRNA expression and remained at a high level for more than 24 h even though the inducible NOS mRNA signal returned to base line. The authors hypothesized that the interleukin-1β (used to induce inflammation) also activated remote cells that, together with other immune-related molecules, penetrated the blood-brain barrier and progressively increased NO production.

Our results suggest the possibility that significant amounts of DHA/AA in these tissues are in fact producing the fluorescence signal because AA is one of the major cytoprotective agents to biotic and abiotic stress. A growing body of evidence suggests that animals and plants increase their capacity to scavenge reactive oxygen species and reactive nitrogen species in response to such stresses generated in inflammation (52, 53). It has even been reported that extracellular DHA levels increase in the disease state (23).

Although our results indicate the potential interference of measuring NO production using DAF-2, many of the prior reports have used elegantly designed controls such as NO inhibitors and NO scavengers (40–42, 46, 49, 50, 54), so that the results are, at least qualitatively, unchanged with this unwanted reaction pathway. Furthermore, the work reported here also indicates the possibility of using DAF-2 to image ascorbate distributions in the central nervous system and other tissues. The use of DAfS and other related compounds as fluorescent probes for ascorbate is certainly an intriguing possibility.

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