Distinction between Nitrosating Mechanisms within Human Cells and Aqueous Solution*

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The quintessential nitrosating species produced during NO autoxidation is N2O3. Nitrosation of amine, thiol, and hydroxyl residues can modulate critical cell functions. The biological mechanisms that control reactivity of nitrogen oxide species formed during autoxidation of nano- to micromolar levels of NO were examined using the synthetic donor NaEt₂NN(O)NO (DEA/NO), human tumor cells, and 4,5-diaminofluorescein (DAF). Both the disappearance of NO and formation of nitrosated product from DAF in aerobic aqueous buffer followed second order processes; however, consumption of NO and nitrosation within intact cells were exponential. An optimal ratio of DEA/NO and 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (PTIO) was used to form N2O3, through the intermediary of NO2. This route was found to be most reflective of the nitrosative mechanism within intact cells and was distinct from the process that occurred during autoxidation of NO in aqueous media. Manipulation of the endogenous scavengers ascorbate and glutathione indicated that the location, affinity, and concentration of these substances were key determinants in dictating nitrosative susceptibility of molecular targets. Taken together, these findings suggest that the functional effects of nitrosation may be organized to occur within discrete domains or compartments. Nitrosative stress may develop when scavengers are depleted and this architecture becomes compromised. Although NO2 was not a component of aqueous NO autoxidation, the results suggest that the intermediary of this species may be a significant factor in the advent of either nitrosation or oxidation chemistry in biological systems.

An early discovery in the nitrogen oxide field was that macrophages utilize nitric oxide (NO) derived from l-arginine in their tumoricidal armature (1). Although NO directly reacts primarily with metalloproteins, reactive nitrogen oxide species (RNOS)1 formed during the autoxidation of NO can engage an alternate and broader range of molecular targets (2–5). The salient nitrosating species produced during NO autoxidation is N2O3, which can react with amines, thiols, or hydroxyl groups to form NO adducts (6). Nitrosative mechanisms have been implicated in ion conductance (7–10), signal transduction (11), glycogenolysis (12), apoptosis (13), and DNA repair (14, 15), underscoring the pivotal role these modifications may play in many facets of cellular function. Excessive formation of N2O3 can exert toxicological changes that culminate as nitrosative stress if critical systems become adversely affected (5, 16, 17). Therefore, the chemistry leading to N2O3 formation may figure prominently in both functional and cytotoxic aspects of NO in vivo.

Several mechanisms have been proposed for autoxidation of NO (17–30). However, few studies have examined this process in the context of the intracellular milieu (29, 30). We tested the hypothesis that the autoxidation process within the architecture of intact cells is distinct from that which occurs in the aqueous extracellular medium. The results suggest that NO2 is not an intermediate species during NO autoxidation in aqueous phase. Cellular hydrophobic domains in conjunction with scavenger composition and location may serve to focus nitrosation chemistry to discrete sites during NO autoxidation in biological systems.

EXPERIMENTAL PROCEDURES

Compounds Used to Generate RNOS—NaEt₂NN(O)NO (DEA/NO) was a generous gift from Dr. Joseph Saavedra (NCI, National Institutes of Health, Frederick, MD). DEA/NO decomposes to release free NO into solution with a half-life of 2.5 min at neutral pH and 37 °C (Refs. 31 and 32; see also Fig. 2A). Stock solutions (~1 mM) were prepared in 10 mM NaOH and were stored at –20 °C, and concentrations were determined from the absorbance values at 250 nm in 10 mM NaOH (ε = 8000 M⁻¹ cm⁻¹, Ref. 31) directly prior to use. The rate of DEA/NO decomposition was unaltered by the presence of either DAF or 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (PTIO).

Ascorbic acid, 2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), buthionine sulfoximine (BSO), diethylenetriaminepentaacetic acid (DTPA), and glutathione (GSH) were purchased from Sigma. PTIO, 4,5-diaminofluorescein-2 (DAF), and DAF-diacetate were purchased from Calbiochem (San Diego, CA).

Initial experiments indicated that, during aerobic decomposition of 0.5 μM DEA/NO in a PBS-buffered solution, the fluorescence signal from the DAF reaction product triazolofluorescein was linearly dependent on DAF, with concentrations ranging from 1 nM to 1 μM. At higher DAF concentrations, nonlinearity in fluorescence response was observed indicative of an interference effect; therefore, solution data were accumulated in PBS solutions containing 1 μM DAF and the metal chelator DTPA (50 μM) unless otherwise indicated. The level of ambient light was kept to a minimum during all steps involving DAF. For anaerobic analysis, the assay buffer was deoxygenated by bubbling with argon through a septum-sealed cuvette.

Cell Conditions—The human cancer cell lines MCF-7 (breast), A-549 (lung), and HT-29 (colon) were obtained from American Type Tissue Collection (Manassas, VA) and were cultured as attached cells to 80% confluence in either T-75 flasks (Falcon) or 96-well, black-walled plates (Corning) containing RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT), 4.5 g/liter glucose, 2 mM t-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified incubator with 5% CO2 and 95% air. As indicated, cells were treated

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1 The abbreviations used are: RNOS, reactive nitrogen oxide species; DEA/NO, NaEt₂NN(O)NO; DAF, 4,5-diaminofluorescein-2; PBS, phosphate-buffered saline; DTPA, diethylenetriaminepentaacetic acid; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide; BSO, buthionine sulfoximine; ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid).
with either 50 µM BSO or 200 µM ascorbate for 16 h prior to testing. Cells subsequently were gently rinsed with PBS and incubated at 37 °C for 30 min with a PBS solution containing 5 µM DAF-diacetate and DTPA (50 µM). After replacement of the DAF solution with fresh PBS, the cells were dislodged and were either washed three times by a cycle of suspension and centrifugation or, in the case of the 96-well plate format, were rinsed by cycles of aspiration and PBS addition.

Differences in uptake efficiency of DAF between tumor cell lines were determined by reacting lysates (two pulses of 10 s with an ultrasonicator; Cole-Palmer, Vernon Hills, IL) of each sample preparation with a large excess of DEA/NO (500 µM) to give maximal fluorescent signal. The total DAF uptake by MCP-7 and A-549 cells was equivalent, whereas that by HT-29 cells was ~65–75% lower. This approach also confirmed that the total level of intracellular DAF for each cell line was in excess relative to the concentration of introduced nitrogen oxides in subsequent experiments. Ultrasonication of intact cells following complete decomposition of DEA/NO did not affect the intensity of fluorescence, indicating that interference quenching within the enclosure of the cell did not occur. Treatment with BSO augmented the absolute level of intracellular DAF roughly 3–4-fold in MCP-7 and A-549 cells, but had little effect on DAF uptake in HT-29 cells. Pre-incubation of MCP-7, A-549, and HT-29 cells with ascorbate did not significantly alter DAF uptake. The diacetate derivative of DAF was designed to aid in retention of DAF within intact cells. The level of DAF leakage from cells following the addition of fresh PBS-buffered solution was determined from the signals obtained in supernatant relative to the lysed cell pellet following exposure to DEA/NO (500 µM). Leakage of the dye back into solution or incidental cellular lysis resulted in a signal that was 2–3% of the total amount of fluorophore present in 1 × 10^6 cells. To avoid misinterpretation of this solution artifact as intracellular signal, DAF-loaded cells were washed and suspended into fresh buffer immediately prior to testing. Trypan blue dye exclusion indicated that viability of intact cells at the end of all treatment conditions was in the range of 92–100%.

**Instrumentation and Data Analysis**—UV-visible spectroscopy was performed with a Hewlett-Packard 8452A diode array spectrophotometer. Fluorescence measurements were obtained on a PerkinElmer Life Sciences LS50B fluorometer with excitation at 495 nm and emission at 515 nm with either 2.5- or 5.0-mm slit widths as indicated. The reaction solution (2 ml) was stirred and maintained at 37 °C with a water-jacketed cuvette holder. Additional fluorescence measurements were obtained on a PerkinElmer Life Sciences HTS 7100 fluorescent plate reader (200-µl volume, 37 °C). Kinetic analyses (KaleidaGraph software; Synergy, Reading, PA) of fluorescence signal increases were performed on data sets excluding the first 2 min to ignore the contribution from DEA/NO decay prior to the NO maximum. Kinetic simulations were obtained using Stella II software (High Performance Systems). NO measurements were made using a NO-specific electrochemical probe (World Precision Instruments, Sarasota, FL) suspended into a fluorometer cuvette controlled by a DUO18 amplifier and software. Signals were calibrated using argon-purged PBS solutions of saturated NO gas (Matheson, Montgomeryville, PA) following determination of concentration with ABTS (660 nm, ε = 12,000 M⁻¹ cm⁻¹; Ref. 32). The background signals were assessed using nitrre or DEA/NO pre-incubated for 4 h in buffer. The microscopic characteristics of DAF-loaded cells were viewed using a Zeiss LSM 210 microscope equipped with a mercury lamp and a fluorescein filter set.

**RESULTS**

*N-Nitrosation of the aromatic vicinal amines of DAF results in formation of a triazolofluorescein product that exhibits fluorescence with a high extinction coefficient and quantum yield (34). Decomposition of DEA/NO in a PBS-buffered solution containing DAF generated fluorescent product (Fig. 1A). Since NO was introduced by decay of DEA/NO (k = 4.6 × 10^3 s⁻¹; Ref. 30) as opposed to bolus addition, the temporal increase in signal intensity is reflective initially of NO release from this donor compound (Reaction 1).*  

\[ \text{NO} + \text{PTIO} \rightarrow \text{NO}_2 + \text{PTI} \]  
**REACTION 3**

As NO levels decreased with decay of DEA/NO, the rate-limiting step for fluorescent product formation becomes autoxidation, which is second order in NO (Reaction 2; k = 8 × 10^6 M⁻² s⁻¹; Refs. 18 and 19).

\[ 4\text{NO} + \text{O}_2 \rightarrow 2\text{N}_2\text{O}_3 \rightarrow 4\text{H}^+ + 4\text{NO}_2^- - d[\text{NO}]dt = k[\text{NO}]^{2}[\text{O}_2] \]  
**REACTION 2**

Kinetic analysis of the data, excluding the first 2 min, confirmed that the signal increase conformed to an apparent second order process (Fig. 1A, k = 4.8 ± 0.4 × 10^3 M⁻¹ s⁻¹). Electrochemical detection of the NO concentration in solution corroborated this interpretation as the free NO level rose to a maximum, indicative of production exceeding the rate of consumption, then declined by a second order process (Fig. 2A, k = 1.6 ± 0.1 × 10^3 M⁻¹ s⁻¹, buffer). At the detection limits of the fluorometer, the process by which low nanomolar levels of NO elicited increases in fluorescent product remained second order (data not shown). Inclusion of the metal chelator DTPA did not affect the reaction. Addition of nitrre, diethylamine, or previously decomposed DEA/NO did not generate fluorescent product when DAF was present in either solution or within cells. DAF nitrosation during decomposition of DEA/NO was dependent on O₂ and did not develop upon re-oxygenation of solutions previously reacted in the absence of O₂. These results indicated that NO does not nitrosate DAF directly and were consistent with formation of N₂O₃ consequent to autoxidation of NO.

To test the hypothesis that the intracellular milieu alters nitrosation, tumor cells were loaded with DAF and remained either intact or were ruptured by ultrasonication immediately prior to exposure to equal concentrations of DEA/NO. The fluorescent signal generated from N₂O₃ reaction with DAF inside intact MCP-7 cells was 54 ± 12% lower than the signal observed with corresponding lysate (Fig. 1B). Comparable results were obtained with A-549 cells (data not shown). Similar to cell-free solution, the rate of fluorescence increase following cellular disruption was second order (Fig. 1A, lysed, k = 5.2 ± 0.8 × 10^3 M⁻¹ s⁻¹). In contrast, the appearance of fluorescent product from DAF localized within intact cells was exponential with an apparent first order rate constant of 4.0 ± 3.3 × 10⁻¹ s⁻¹. Given the similarity between this value and the rate for DEA/NO decomposition (4.6 × 10⁻¹ s⁻¹; Ref. 32), we tested the hypothesis that the enhanced solubility of NO and O₂ within the hydrophobic phase of cellular membranes may accelerate the rate of N₂O₃ formation (29) beyond the rate of DEA/NO decomposition at all time points. Simulations predicted that the maximum NO concentration would decrease 4-fold under these conditions (Fig. 2B); however, this model was incongruous with experimental data. The presence of MCF-7 tumor cells (1 × 10⁶, 2-ml volume, stirring, 37 °C) resulted in only a small reduction (~20%) in the electrochemical signal peak height during decomposition of 1 µM DEA/NO (Fig. 2A). If cells consume NO (30), then nitrosation during autoxidation would be decreased. An increased first order rate of NO disappearance (k = 2.5 ± 0.4 × 10⁻³ s⁻¹) was evident from solution containing cells exemplified by a faster return of the electrochemical signal to baseline (Fig. 2A). Consistent with this, inhibition of fluorescence generated with DEA/NO and free DAF in aerobic solution was observed upon addition of intact tumor cells (Fig. 3A). Notably, a reciprocal plot of these data revealed that inhibition occurred by two distinct processes (Fig. 3B).
We used PTIO as a tool to investigate the relationship between cells and NO₃ formation during NO autoxidation. Electrochemical measurements verified that the level of free NO present in solution during DEA/NO decomposition was decreased by PTIO in a dose-dependent fashion (data not shown). PTIO at 500-fold or greater excess relative to DEA/NO resulted in nearly complete inhibition of fluorescent product formation from DAF (Fig. 4A). Under these conditions, conversion of NO to NO₂ by PTIO approached 100%, indicating that NO₂ alone cannot nitrosate DAF. In contrast, the rate and yield of product formation in both cells (Fig. 1C) and solution (Fig. 4A) were markedly augmented with lower concentrations of PTIO, where some portions of NO remained available to react with NO₂, forming N₂O₃ (Reaction 4; \( k = 1.1 \times 10^9 \) s⁻¹, Refs. 22 and 26).

\[
\text{NO} + \text{NO}_2 \rightarrow \text{N}_2\text{O}_3
\]

**Reaction 4**

The optimal ratio to achieve maximal nitrosation of DAF was ~10–15-fold excess PTIO to DEA/NO (Fig. 4A). The fluorescent product yield under these conditions was 10-fold higher relative to the signal observed with autoxidation alone (Fig. 1C). The absence of a headspace in the reaction vessel had a negligible effect (±5%; data not shown) on fluorescent product formation during DEA/NO decomposition in aerobic solution. Therefore, the marked increase in nitrosated product yield observed with NO/PTIO was not the result of NO capture prior to escape into room air. A double-reciprocal plot of fluorescence as a function of DAF concentration indicated there was a vast difference between the affinities of the nitrosating species produced during DEA/NO decomposition in either the presence (\(-1/X_{max} = 0.44 \pm 0.04 \) μM) or absence (\(-1/X_{int} = 5.5 \pm 0.6 \) μM) of PTIO (Fig. 4B). At infinite concentrations of DAF, the maximal fluorescence values were similar (\(1/Y_{max} = 2900\), DEA/NO; 4000, DEA/NO + PTIO), indicating that the absolute levels of nitrosating species formed were comparable.

Although PTIO is cell-impermeable, the NO/PTIO combination resulted in an augmented level of intracellular DAF nitrosation relative to that achieved solely with NO autoxidation (Fig. 1D). Fluorescent production formation was further enhanced in lysate relative to the signal obtained with DAF inside intact cells. Experiments with PTIO, DEA/NO, and free DAF in solution showed that addition of intact cells progressively inhibited formation of nitrosated product (Fig. 3C). A reciprocal plot of these data indicated a single inhibitory process (Fig. 3D). The slope (\( m = 0.0031 \)) of this line was similar to the slower process (\( m = 0.0018 \)) observed with DEA/NO in the absence of PTIO (Fig. 3B).

The role of endogenous quenchers of nitrosation was evaluated. Nitrosation of DAF (1 μM) in aqueous buffer was more susceptible to ascorbate (IC₅₀ = 2 μM, \( r^2 = 0.996 \)) than GSH (IC₅₀ = 90 μM, \( r^2 = 0.990 \)) during DEA/NO decomposition (1 μM). Extracellular GSH at a ratio of ≥200:1 completely inhibited intracellular nitrosation of DAF during DEA/NO decomposition. In contrast, much lower levels of GSH (≤6:1) quenched DAF product formation within cells when nitrosation

PTIO (5 μM) was added to DAF in solution as in A. The reaction was first order in the presence of PTIO (\( k = 5.5 \times 10^{-3} \) s⁻¹, line overlay) and remained second order in its absence (\( k = 4.4 \times 10^{-3} \) M⁻¹ s⁻¹, line overlay). D. DEA/NO (25 μM) was added to buffer containing PTIO (25 μM) and either intact or lysed cells as in B. Fluorescent product formation was first order under both conditions (\( k = 9.3 \times 10^{-3} \) s⁻¹ and \( k = 8.2 \times 10^{-3} \) s⁻¹, respectively, line overlay). Fluorescence changes were monitored at λ_{max} of 495/515 nm with either 5-mm (A and B) or 2.5-mm (C and D) slit widths.

**Fig. 1.** Fluorescence changes elicited by DEA/NO decomposition with DAF present free in solution and within either intact or lysed MCF-7 tumor cells. A, DEA/NO (1 μM) was added to a PBS-buffered solution (2 ml, stirring, 37 °C) containing DAF (1 μM) and the metal chelator DTPA (50 μM). The rate of increase in fluorescent product was second order (\( k = 4.8 \times 10^{-3} \) M⁻¹ s⁻¹, line overlay). B, DAF-diacectate was incorporated into cells during a 30-min incubation period followed by centrifugation and wash steps to remove extracellular DAF. Cells (2 × 10⁵) were resuspended in buffer as in A and remained either intact or were lysed by a brief sonication pulse. Increase in fluorescence after addition of DEA/NO (10 μM) followed a single exponential with intact cells (\( k = 3.8 \times 10^{-3} \) s⁻¹, line overlay), while the rate law in lysate was second order (\( k = 5.2 \times 10^{-3} \) M⁻¹ s⁻¹, line overlay). C, DEA/NO (0.5 μM) in either the presence or absence of DEA/NO + PTIO (25 μM) was added to buffer containing PTIO (25 μM) and either intact or lysed cells as in B.
was elicited in the presence of PTIO (Fig. 5). Cells were incubated with either ascorbate or BSO, the competitive inhibitor of γ-glutamylcysteine synthetase (37, 38), to determine the degree to which ascorbate or GSH may protect intracellular constituents from nitrosation. The level of fluorescence generated by exposure to DEA/NO was increased roughly 2-fold in MCF-7 and A-549 cells depleted of GSH with BSO (Fig. 6). Nitrosation in GSH-depleted HT-29 cells was relatively unchanged (data not shown). The rate constants for fluorescent product formation in MCF-7 cells treated with BSO were first order (4.0 ± 0.8 × 10⁻³ s⁻¹), similar to those observed in non-BSO-treated cells (data not shown). Fluorescence maxima produced by DEA/NO were reduced ~30–60% in each tumor cell type previously supplemented with ascorbate (Fig. 5).

A heterogeneous pattern of intracellular fluorescence was evident when tumor cells loaded with DAF were viewed under a microscopic following exposure to DEA/NO (data not shown). Qualitatively, fluorescence was often organized into discrete regions resulting in punctate appearance. A diffuse pattern that filled the entire cell, however, was evident in many cases. The fluorescence per individual cell ranged from none (background) to greater than 95%.

**DISCUSSION**

We tested the hypothesis that the chemistry of NO autoxidation in aqueous solution was not synonymous with the reaction within intact cells. Nitrosation of free DAF in aerobic solution occurred by an apparent second order process consist-

![Fig. 2. Comparison of NO disappearance in the presence and absence of MCF-7 tumor cells.](http://www.jbc.org/)

![Fig. 3. Characteristics of extracellular DAF nitrosation inhibition by MCF-7 tumor cells.](http://www.jbc.org/)
Mechanisms of Intracellular Nitrosation via NO Autoxidation

Dynamic modulation of both the rates of NO formation by NOS isoforms (2, 16, 39, 40) and of NO consumption by different cell types (30) may serve to regulate nitrosation chemistry within a particular region of tissue. NO levels generated from constitutive NOS isoforms are generally limited by feedback inhibition from nitrosyl complex formation at the catalytic heme site (39, 40). Several studies have noted nitrosation generated within neurons and endothelia using DAF (41–43), while we observed second order dependence for fluorescent product formation in solution at submicromolar concentrations of NO from synthetic donors (e.g. Fig. 1C). These data are consistent with the viewpoint that physiologic NO formation can lead to subtle levels of nitrosation, which may function to modulate residues critical for channel gating, subunit dimerization, and enzyme activity. Measurements in the current in vitro studies were conducted in the absence of glucose. We have

![Image](338x203 to 525x346)

**Fig. 4.** Relationship between PTIO and DEA/NO concentrations on formation of fluorescence from DAF. A, DEA/NO (1 μM) was decomposed in aerobic PBS-buffered solution containing DAF (1 μM), DTPA (50 μM), and PTIO as indicated. The level of fluorescence was measured (λex/em 490/525 nm) after a 45-min incubation period at 37 °C (200-μl wells, unstirred). B, double-reciprocal plots of fluorescence (collected at λex/em: 495/515 nm, slit widths 2.5 mm) versus DAF concentration. The relative affinities of the nitrosating species formed by DEA/NO (1 μM) decomposition in aerobic PBS-buffered solution containing DTPA (50 μM) and DAF (1 nM to 1 μM) either without (A) or with (B) PTIO (25 μM) are indicated. Increases in fluorescence after addition of DEA/NO (5 μM) were monitored at λex/em of 495/515 nm with 2.5-mm slit widths.

![Image](338x440 to 525x730)

**Fig. 5.** Effect of GSH on fluorescence changes elicited by DEA/NO ± PTIO with DAF within MCF-7 tumor cells. DAF-diacetate was incorporated into cells during a 30-min incubation period. Following centrifugation and wash steps to remove extracellular DAF, cells (2 × 10⁶) were resuspended in PBS-buffered solution (2 ml, stirring, 37 °C) containing DTPA (50 μM) either without (A) or with (B) PTIO (25 μM) and GSH (30 μM) as indicated. Increases in fluorescence after addition of DEA/NO (5 μM) were monitored at λex/em of 495/515 nm with 2.5-mm slit widths.

![Image](80x430 to 267x730)

**Fig. 6.** Influence of intracellular ascorbate and GSH levels on nitrogen oxide-induced fluorescence in tumor cells. The human tumor cell lines MCF-7 or A-549 were plated into a 96-well culture plate at a density of 5000 cells/well. Cells were treated for 16 h with either ascorbate (Asc, 200 μM) or BSO (5 mM). Following a 1-h incubation period in PBS-buffered solution containing 5 μM DAF-diacetate, cells were gently rinsed twice and reacted with DEA/NO (20 μM) in buffer for 1 h at 37 °C. Representative data are shown as the mean ± S.E. of quadruplicate wells (n = 3).
observed that manipulation of mitochondrial status in MCF-7 cells (± glucose, rotenone, actinomycin D) had little effect on both NO consumption and extracellular DAF nitrosation. However, intracellular DAF nitrosation levels were influenced by respiration, albeit only modestly (10–15%; data not shown). Localization of DAF within mitochondria has been examined (44), and intraorganellate nitrosation mechanisms remain an active area of inquiry.

Although cells can affect the rate of nitrosation, nucleophilic substances such as GSH and ascorbate can limit the yields of nitrosation by competitive scavenging of N₂O₃ (16, 19, 45–48). MCF-7 breast carcinoma cells contain an estimated 5–10 mM intracellular GSH (38), a concentration that completely depletes these defenses, salubrious nitrosative reactions (3 and 4; Refs. 35 and 36). In addition to circumventing the rate-limiting step in autoxidation, the optimal balance of PTIO scavenger substances for RNOS can lead to either nitrosative or oxidative stress. These results provide a novel framework for the mechanisms of nitrosation at physiological NO concentrations in biological systems.

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