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Analytical methods

Calibration of nitric oxide flux generation from diazeniumdiolate ·NO donors

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ABSTRACT

The 1-(secondary amino) diazen-1-ium-1,2-diolates (NONOates) are the most commonly utilized nitric oxide (·NO, nitrogen monoxide) donor because of the ability of different NONOates to spontaneously break down liberating ·NO at different rates, which can be utilized to control ·NO fluxes. However, the parameters that determine these fluxes of ·NO generation, half-lives and stoichiometry of ·NO per donor, can vary significantly with specific experimental conditions in addition to the donor chosen. Here we report straightforward methods that can be used to determine these parameters. For donors of intermediate half-life (10–80 min) a real-time oxymyoglobin (oxyMb) assay can be analyzed to simultaneously determine both the half-life and the total amount of ·NO liberated, from which the ·NO flux can be obtained for any given donor concentration. The half-lives obtained by oxyMb assay are very similar to those obtained by following NONOate decomposition kinetics spectrophotometrically, and a survey of several NONOates from different commercial sources show consistent results. These data provide validation for the methodologies employed. In addition, procedures are described for calibration of donors with shorter (<10 min) and longer (>80 min) half-lives. These procedures can be used to reproducibly and routinely calibrate ·NO fluxes for a variety of donors under any specific condition.

Introduction

Nitric oxide (·NO, nitrogen monoxide), a small diatomic molecule, is involved in essentially every aspect of physiology and pathophysiology [1]. In order to study its novel biological functions, several classes of ·NO donors have been developed and used as exogenous sources of ·NO [2,3]. Under biological conditions, ·NO is produced as a flux of varying rates, making it essential to have donors that also generate ·NO at varying (and controllable) rates. The 1-(secondary amino) diazen-1-ium-1,2-diolates (diazeniumdiolates, NONOates)[1] are without doubt the ·NO donor class of choice because these compounds spontaneously liberate ·NO with first-order kinetics [4]. In addition, NONOates have half-lives of ·NO generation ranging from seconds to hours depending on the secondary amino group, and therefore can provide a wide range of ·NO exposure time to simulate various biological environments.

The two characteristics for a given NONOate that determine the ·NO flux are the rate constant (or half-life, $t_{1/2}$) of ·NO liberation and the total amount of ·NO liberated after complete decomposition (which we will refer to as "·NO content"). The ·NO content is given by the NONOate concentration ($[D]_0$, concentration at zero time) multiplied by the stoichiometry of ·NO per donor (which we refer to as "α"), thus equal to $\alpha[D]_0$. Several physicochemical properties for a variety of NONOates have been summarized previously, including $t_{1/2}$, α and spectrophotometric properties [5]. Theoretically, by utilizing published values for $t_{1/2}$, α, and spectrophotometric measurement of $[D]_0$, the ·NO flux generated from a given concentration of NONOate can be calculated. However, as has been indicated previously [5,6], specific experimental conditions significantly affect the rate and extent of ·NO formation (e.g., temperature, pH, ionic strength, and also presence of membranes [7]). In this context, it is important to standardize and calibrate NONOates from batch to batch under individual laboratory conditions, ideally utilizing a convenient method [6,8,9].

The oxyhemoglobin/oxymyoglobin (oxyHb/oxyMb) assay has been extensively used for measuring ·NO release *in vitro* and *in vivo* [10,11]. We describe here an application of this method and kinetic analysis which is a straightforward procedure that can be applied routinely under individual experimental conditions and allows simultaneous determination of both the rate constant and the ·NO content of a NONOate. To validate this method, we examine NONOates from different commercial sources, and also compare the results to those obtained by another method (NONOate decomposition kinetics).

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[1] The 1-(secondary amine) NONOates are primarily donors of ·NO, while primary amine NONOates release nitroxyl as well [25].

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Experimental procedures

Materials

Myoglobin (Mb) from equine skeletal muscle, diethylenetriamine pentaacetic acid (DTPA) and sodium hydrosulfite ($Na_2S_2O_4$) were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Sodium hydroxide (NaOH) and sodium phosphate were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Disodium 1-[2-(carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate-methanol (PROLI/NO) was from Alexis Biochemicals (San Diego, CA, USA). (Z)-1-[*N*,*N*-diethylamino]diazen-1-ium-1,2-diolate-diethylammonium salt (DEA/NO), (Z)-1-[*N*-(3-aminopropyl)-*N*-(*n*-propyl)amino]diazen-1-ium-1,2-diolate (PAPA/NO) and (Z)-1-[*N*-(3-aminopropyl)-*N*-(i-propyl)amino]diazen-1-ium-1,2-diolate (NOC-5) were supplied by Alexis Biochemicals (San Diego, CA, USA), Dojindo Laboratories (Kumamoto, Japan), Cayman Chemical (Ann Arbor, MI, USA) or Sigma–Aldrich, Inc. (St. Louis, MO, USA) as indicated. Aliquots of NONOates in 10 mM NaOH were used as stock solutions and were stable for months at −80 °C.

Preparation of oxyMb

The preparation of oxyMb from Mb has been described in detail [10]. Briefly, excess sodium hydrosulfite was added to Mb solution in 100 mM sodium phosphate buffer containing 100 μM DTPA, pH 7.4 (working buffer). The color change of the solution from dark brown to wine-red indicates the reduction of metmyoglobin (metMb) and the formation of deoxymyoglobin (deoxyMb). After two sequential desalting steps by PD10 column containing Sephadex™ G-25 Medium from GE Healthcare (Uppsala, Sweden), deoxyMb was oxygenated and the concentration of oxyMb was determined spectrophotometrically by averaging the values from three wavelengths (ε_{418nm} = 128 $mM^{-1}cm^{-1}$, ε_{543nm} = 13.6 $mM^{-1}cm^{-1}$ and ε_{581nm} = 14.6 $mM^{-1}cm^{-1}$) [10,12].

Determination of working wavelengths and conversion extinction coefficients

The working wavelengths and the molar extinction coefficients for the conversion of oxyMb to metMb ($\Delta\varepsilon = \varepsilon_{oxyMb} - \varepsilon_{metMb}$) were measured as described previously for hemoglobin [10]. Known concentrations of oxyMb were oxidized completely by excess ·NO generated from PROLI/NO (half-life as short as 1.8 s at pH 7.4, and 37 °C [13]). In the visible region, the absorbance changes at 582 and 545 nm were found highest, therefore, these two wavelengths were chosen as the working wavelengths. Other wavelengths with high differences such as the Soret (405 nm) can also be applied [10,11]. The absorbance changes at working wavelengths were plotted vs. oxyMb concentrations, and the conversion extinction coefficients were derived from the slope of the straight line obtained by linear regression fit. $\Delta\varepsilon$ = 11.50 ± 0.03 and 8.60 ± 0.03 $mM^{-1}\,cm^{-1}$ (mean ± SD, n = 3) were determined for 582 and 545 nm, respectively in working buffer.

Kinetic measurements

All kinetic measurements were performed at 25 °C in working buffer using a Shimadzu UV-2501PC Spectrophotometer with temperature control (Kyoto, Japan). For the oxyMb assay, 995 μl of 50–60 μM oxyMb solution was placed in both reference and sample cuvettes. After the baseline scan, 5 μl of 0.5–1 mg/ml NONOate in 10 mM NaOH was added into sample cuvette, while 5 μl of 10 mM NaOH was added into reference cuvette. The absorbance changes at both 582 and 545 nm were followed immediately after quick addition and mixing. For the kinetics of NONOate decomposition, the absorbance decrease of 12.5–25 μg/ml NONOate at 250 nm was followed. Data were analyzed by Microcal (TM) Origin (Version 6.0 from Microcal Software, Inc., Northampton, MA, USA) using non-linear regression function BoxLucas1 as described in the Results Section [14]. At least five half-lives of data were used for analysis.

Statistics

Data in Table 1 are presented as mean ± SD from at least three independent measurements. Values from this work in Table 2 were obtained from all measurements listed in Table 1 without distinguishing different vendors. SD for α was calculated by $SD(\alpha)^2/Mean(\alpha)^2 = SD(\alpha[D]_0)^2/Mean(\alpha[D]_0)^2 + SD([D]_0)^2/Mean([D]_0)^2$, where $SD(\alpha)$, $SD(\alpha[D]_0)$ and $SD([D]_0)$ are the SD for α, $\alpha[D]_0$ and $[D]_0$, respectively; $Mean(\alpha)$, $Mean(\alpha[D]_0)$ and $Mean([D]_0)$ are the mean values for α, $\alpha[D]_0$ and $[D]_0$, respectively.

Results

Standardization and calibration of ·NO content and half-life by oxyMb assay

The oxyHb assay utilizes the facile ·NO reaction with oxyHb yielding methemoglobin (metHb) and nitrate (rate constant k_2 $3.4 \times 10^7\ M^{-1}\,s^{-1}$) [15] and is commonly used to determine ·NO by measuring oxyHb conversion to metHb spectrophotometrically [10]. With proper data analysis this method can be used to simultaneously determine both $t_{1/2}$ and $\alpha[D]_0$ for a NONOate. The method involves following oxyMb[2] conversion to metMb after addition of NONOate. Under these conditions, the reactions taking place are given by Eqs. (1) and (2):

$$D \xrightarrow{k_1} \alpha NO + P \tag{1}$$

$$NO + oxyMb \xrightarrow{k_2} metMb + NO_3^- \tag{2}$$

where D is the ·NO donor (NONOate here), P is the product of NONOate decomposition, α is the stoichiometry of ·NO per donor, and k_1 and k_2 are the rate constants of D decomposition and ·NO/oxyMb reaction, respectively. The concentration of donor at any time is given by Eq. (3):

$$[D] = [D]_0 - \frac{[oxyMb]_0 - [oxyMb]}{\alpha} \tag{3}$$

where $[D]_0$ and $[oxyMb]_0$ are the initial concentrations of D and oxyMb, respectively. The rate of change of oxyMb is given by Eq. (4):

$$\frac{d[oxyMb]}{dt} = -k_2[NO][oxyMb] \tag{4}$$

For the derivation we make the steady-state assumption[3] [16,17]:

$$\frac{d[NO]}{dt} = \alpha k_1[D] - k_2[NO][oxyMb] = 0 \tag{5}$$

and combining with Eq. (4),

[2] We utilize Mb because, unlike hemoglobin, it does not possess cysteine, and thus avoids the potential complication of additional chemistry such as nitrosothiol formation [26].

[3] The steady-state assumption is based on the phenomenon that very soon after initiation of the reaction the rate of consumption will increase due to increasing [·NO]. Within a short period of time this consumption rate will reach a point where it will equal the rate of production. After this point the [·NO] will not change but will be very low until the completion of either reaction (Eqs. (1) and (2)), because oxyMb scavenging is much faster than the ·NO generation, that is, ·NO is trapped by oxyMb once it is produced from donor.