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A New and Simple Method for Delivering Clamped Nitric Oxide Concentrations in the Physiological Range: Application to Activation of Guanylyl Cyclase-Coupled Nitric Oxide Receptors

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ABSTRACT

The signaling molecule nitric oxide (NO) could engage multiple pathways to influence cellular function. Unraveling their relative biological importance has been difficult because it has not been possible to administer NO under the steady-state conditions that are normally axiomatic for analyzing ligand-receptor interactions and downstream signal transduction. To address this problem, we devised a chemical method for generating constant NO concentrations, derived from balancing NO release from a NONOate donor with NO consumption by a sink. On theoretical grounds, 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) was selected as the sink. The mixture additionally contained urate to convert an unwanted product of the reaction (NO_2) into nitrite ions. The method enabled NO concentrations covering the physiological range (0–100 nM) to be formed within approximately 1 s. Moreover, the concentrations were sufficiently stable over at least several minutes to be useful for biological purposes. When applied to the activation of guanylyl cyclase-coupled NO receptors, the method gave an EC_{50} of 1.7 nM NO for the protein purified from bovine lung, which is lower than estimated previously using a biological NO sink (red blood cells). The corresponding values for the $\alpha1\beta1$ and $\alpha2\beta1$ isoforms were 0.9 nM and 0.5 nM, respectively. The slopes of the concentration-response curves were more shallow than before (Hill coefficient of 1 rather than 2), questioning the need to consider the binding of more than one NO molecule for receptor activation. The discrepancies are ascribable to limitations of the earlier method. Other biological problems can readily be addressed by adaptations of the new method.

Nitric oxide (NO), synthesized from the amino acid L-arginine, functions as a signaling molecule throughout the body, in which it elicits diverse effects such as smooth muscle relaxation, platelet disaggregation, and synaptic plasticity. Physiological NO signal transduction occurs through the activation of intracellular guanylyl cyclase (GC)-coupled receptors, leading to cGMP formation (Denninger and Marletta, 1999; Koesling and Friebe, 2000). Depending on its concentration and/or other experimental conditions, however, biological effects can be exerted through other pathways, including the binding of NO to cytochrome *c* oxidase, resulting in the inhibition of mitochondrial respiration (Brown, 1999), reaction with other radicals (e.g., with superoxide ions to give reactive peroxynitrite anions), and the production of nitrosating species after the reaction of NO with oxygen (Beckman and Koppenol, 1996; Augusto et al., 2002). The extent to which these other reactions contribute to the biology of NO in vivo remains uncertain. Nevertheless, the spectrum of possible effects of NO in vitro can make the interpretation of experimental findings difficult, particularly when NO is applied in constantly changing concentrations, as happens with the current methods of delivery.

NO can be dispensed from concentrated anaerobic solutions, but on dilution into aerobic solutions used in the laboratory, it is consumed by reaction with oxygen (autoxidation) at a rate proportional to the square of its concentration (Ford et al., 1993). Alternatively, NO can be provided by donors, of which the so-called NONOates are preferred because they degrade to release NO with predictable kinetics (Morley and Keefer, 1993), and different NONOates with widely differing half-lives (1.8 s to 20 h) are commercially available. When added to biological media, however, the NO concentration increases (at a rate governed by the half-life)

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ABBREVIATIONS: NO, nitric oxide; CPTIO, 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DEA/NO, diethylamine/nitric oxide adduct; DETA/NO, diethylenetriamine/nitric oxide adduct; GC guanylyl cyclase; SPER/NO, spermine/nitric oxide adduct; DTT, dithiothreitol; NOC, nitric oxide-amine complex.

and then decreases as the autoxidation rate exceeds the NO release rate (Schmidt et al., 1997). Moreover, autoxidation itself creates problems because it leads to the generation of reactive nitrosating agents (e.g., NO_2 and N_2O_3), and the rate of autoxidation at a given NO concentration will be approximately 10-fold higher in the hyperoxic environment in which cells are maintained in vitro than it would be in vivo (Ford et al., 1993; Augusto et al., 2002).

Clearly, methods are needed for applying NO in the controlled manner that would be axiomatic for meaningful studies of the biology of other signaling molecules. To try to address this problem, an apparatus for maintaining "clamped" NO concentrations has been designed (Zhelyaskov and Godwin, 1999), but it is complex, expensive to construct, and unsuited to most biological applications. In principle, steady NO concentrations can be achieved by marrying a constant rate of NO production with an appropriate rate of inactivation. We recently exploited this concept by using red blood cells as biological NO sinks, which allowed for the determination of the absolute and relative sensitivities of the purified lung GC-coupled receptor and mitochondrial respiration to NO (Bellamy et al., 2002). Nevertheless, the method has several limitations, including the following: 1) having to prepare a washed red blood cell suspension for each experiment; 2) a slow increase of the NO level to plateau concentrations (60 s) so that, for rapid kinetics experiments, additions need to be made to a pre-equilibrated mixture in a small enough volume not to disturb the equilibrium, which limits the scope of the technique; 3) even a small leakage of free hemoglobin could compromise the experiment, because free hemoglobin inactivates NO at a much higher rate than when the protein is packaged in red blood cells (Liu et al., 1998); and 4) possible interference from bioactive substances taken up into, or released from, the red blood cells.

To address these and other limitations we sought to devise a cell-free method for generating steady NO concentrations covering the presumed physiological range (0–100 nM). This was achieved satisfactorily using the combination of a NONOate donor and the chemical NO scavenger CPTIO. When used to address one key issue in NO biology, namely the sensitivity of the GC-coupled receptors to NO, the new method gave results that differed in important ways from those obtained previously using red blood cells to provide the clamped NO concentration.

Materials and Methods

Materials. SPER/NO, DEA/NO, DETA/NO and CPTIO were all obtained from Alexis Corporation (Bingham, Nottingham, UK). All other reagents were obtained from Sigma Chemical (Poole, Dorset, UK). NO donor stock solutions were made in 10 mM NaOH and kept on ice until use. Uric acid (30 mM) was dissolved in 60 mM NaOH and kept at room temperature. Stocks of CPTIO (20 mM) and superoxide dismutase (100,000 U/ml) were prepared in water and stored on ice until use. Ferrous oxyhemoglobin was prepared as described previously (Martin et al., 1985).

Measurement of NO. NO concentrations were recorded in buffer (1 ml) incubated in a sealed, stirred vessel (at 37°C) equipped with an NO electrode (Iso-NO; World Precision Instruments, Stevenage, Hertfordshire, UK). The rate of NO release from SPER/NO was measured by the addition of 10 μM of the donor to 50 mM Tris-HCl buffer containing 1000 U/ml superoxide dismutase (pH 7.4 at 37°C). After the 15 to 30 s required for the electrode response to settle (see *Results*), the measured NO concentration increased linearly for approximately another 60 s until autoxidation became significant as the NO concentration exceeded 250 nM. The NO release rate was obtained by measuring the gradient between 30 and 60 s after the addition of the donor.

Measurement of NO-Evoked GC Activity. Experiments were carried out in 50 mM Tris-HCl buffer supplemented with 1000 U/ml superoxide dismutase, 300 μM uric acid, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.01 mM DTT, 0.05% bovine serum albumin, and 1 mM GTP, pH 7.4 at 37°C, and, except when DEA/NO was used, CPTIO (200 μM unless specified otherwise). When cell extracts were assayed, the buffer also contained 5 mM creatine phosphate and 200 μg/ml of creatine kinase. Receptor protein purified from bovine lung (soluble guanylyl cyclase; Alexis) was diluted in a buffer, pH 7.4, containing 50 mM Tris-HCl, 1 mM DTT, and 0.5% bovine serum albumin to give a stock concentration of 5 μg/ml, which was stored on ice and subsequently diluted 1:100 into assay buffer maintained at 37°C. NO donor was added, and aliquots of the reaction mix were removed at intervals and inactivated in boiling buffer (50 mM Tris, 4 mM EDTA). To examine individual receptor isoforms, COS-7 cells were transfected with combinations of either the α1 and β1 subunits or the α2 and β1 subunits (both rat) as described previously (Gibb et al., 2003) and maintained for 48 h before harvesting by trypsinization. The cells were pooled, pelleted at 1500*g* for 5 min, and resuspended at 3 mg protein/ml in ice-cold lysis buffer, pH 7.4, containing 50 mM Tris-HCl, 0.1 mM DTT, and a protease inhibitor cocktail (complete mini EDTA-free; Roche Diagnostics, East Sussex, UK). After the addition of glycerol (to give 5%), the homogenate was frozen until use. The NO-evoked GC activity of the two isoforms was compared directly. The homogenates were thawed, stored on ice, and diluted 1:10 into assay buffer pre-equilibrated at 37°C. SPER/NO was added to achieve varying steady-state NO concentrations, and 2 min later, aliquots of the reaction mixture were removed and inactivated as described above. cGMP levels were quantified by radioimmunoassay. Data are given as means ± S.E.M., and results were analyzed using an unpaired Student's *t* test (two-tailed).

Mathematical Modeling. The chemical reactions on which the NO delivery method depends were incorporated into a mathematical model using the rate constants listed in Table 1. The model consisted of the following differential equations, where x is the number of moles of NO released per mole of SPER/NO:

TABLE 1
Reactions considered in the model
For the autoxidation reaction (reaction 5), the concentration of O_2 was assumed to be 185 μM (that of an air-equilibrated solution).

Reaction No.	Reaction	Rate Constant	Reference
1	SPER/NO $\rightarrow$ SPER + $NO^{\bullet}$	$k_1 = 2.96 \times 10^{-4} s^{-1}$	Keefer et al., 1996
2	$NO^{\bullet}$ + CPTIO $\rightarrow$ CPTI + $NO_2^{\cdot}$	$k_2 = 1.6 \times 10^{4} M^{-1}s^{-1}$	Present study
3	$NO_2^{\cdot}$ + $NO^{\bullet}$ $\rightarrow$ N_2O_3	$k_3 = 1.1 \times 10^{9} M^{-1}s^{-1}$	Hogg et al., 1995; Ford et al. 2002
4	$NO_2^{\cdot}$ + $NO_2^{\cdot}$ $\rightarrow$ N_2O_4	$k_4 = 4.5 \times 10^{8} M^{-1}s^{-1}$	Hogg et al., 1995; Augusto et al., 2002
5	$2NO^{\bullet}$ + O_2 $\rightarrow$ $2NO_2^{\cdot}$	$k_5 = 13.6 \times 10^{6} M^{-2}s^{-1}$	Schmidt et al., 1997
6	$NO_2^{\cdot}$ + urate $\rightarrow$ NO_2^{-} + $urate^{\bullet}$ + H^+	$k_6 = 2 \times 10^{7} M^{-1}s^{-1}$	Augusto et al., 2002; Ford et al., 2002

$$\frac{d[SPER/NO]}{dt} = -k_1[SPER/NO]$$

$$\frac{d[NO]}{dt} = k_1[SPER/NO]x - k_2[CPTIO][NO] - k_3[NO_2][NO] - k_5[O_2][NO]^2$$

$$\frac{d[NO_2]}{dt} = k_2[CPTIO][NO] - k_3[NO_2][NO] - 2k_4[NO_2]^2 - k_6[urate][NO_2] + k_5[O_2][NO]^2$$

$$\frac{d[CPTIO]}{dt} = -k_2[CPTIO][NO]$$

$$\frac{d[urate]}{dt} = -k_6[urate][NO_2]$$

The equations were solved numerically using the adaptive Runge-Kutta algorithm in Mathcad (version 2001i; Adept Scientific, Letchworth, Herts, UK). Calculation of the NO concentrations registered by the electrode was carried out by multiplying the derived NO concentration by the factor $[1 - exp(-k_e t)]$, where k_e is the rate constant of the electrode (0.116 s^{-1}) (Griffiths and Garthwaite, 2001).

Results

Theoretical Considerations. To obtain clamped NO concentrations after release from a donor, the following simple consecutive reaction scheme can be considered:

$$Donor \xrightarrow{k_a} NO \xrightarrow{k_b} products$$

The equation describing the change in NO concentration over time is

$$[NO] = x[Donor]\frac{k_a}{k_b - k_a}(e^{-k_a t} - e^{-k_b t}) \quad (1)$$

where x represents the number of moles of NO released per mole of donor. For an effectively constant rate of NO release (required to achieve steady NO concentrations), the donor must decompose slowly relative to the duration of the experiment. For the present purposes, NO concentrations that were steady over the time scale of a few minutes were desired, so SPER/NO, which has a half-life of 39 min at 37°C (Keefer et al., 1996), was selected. The corresponding rate constant for NO release from SPER/NO (k_a) is $2.96 \times 10^{-4} s^{-1}$.

To achieve rapid steady-state, the k_b value needs to be much greater than k_a, but not too large or the resulting NO concentrations would be too low to exert biological effects. When $k_b \gg k_a$, the steady-state NO concentration is given by

$$[NO] = \frac{k_a}{k_b} \times [SPER/NO] \quad (2)$$

A k_b value on the order of 1 s^{-1} (imposing on NO a half-life of 0.7 s) would give rapid attainment of steady state (roughly within the mixing time of an experiment conducted manually) and would provide NO concentrations in the nanomolar range when micromolar concentrations of SPER/NO are added. Such a value of k_b renders insignificant any loss of NO through autoxidation (e.g., at 100 nM NO, autoxidation would consume NO at a rate of only 25 pM/s, giving an NO half-life of 66 min). The sink needs to have the capacity to consume NO over the requisite time scale without significant exhaustion. With 100 μM SPER/NO and assuming $x = 1$ (see below), the initial rate of NO release (and consumption) would be approximately 1.8 μM/min, so sink concentrations in the 100 μM range are required. These considerations indicate that the sink needs to consume NO with a bimolecular rate constant of around $10^{-4} M^{-1} s^{-1}$.

The nitronyl nitroxides 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide and its carboxylated derivative (CPTIO) are stable radicals that scavenge NO at approximately the required rate (Akaike et al., 1993). We chose to use CPTIO, the less cell-permeant of the two, to reduce possible unwanted intracellular effects when used with intact cells (although, to our knowledge, none has yet been described). Despite the NO being largely consumed extracellularly, the resulting extracellular and intracellular NO concentrations would be in dynamic equilibrium because of the fast rate of NO diffusion in lipid and aqueous environments. The reaction between NO and CPTIO forms the NO_2 radical, which is undesirable because it is a reactive oxidizing species that undergoes various reactions, including rapid combination with other radicals such as NO (Augusto et al., 2002). Therefore, NO_2 needs to be scavenged. Urate, an endogenous antioxidant (Becker, 1993) that converts NO_2 into NO_2^- ($k = 2 \times 10^7 M^{-1} s^{-1}$) (Ford et al., 2002), was used for the purpose.

To analyze the reactions quantitatively, a more complex mathematical model was constructed using the rate constants given in Table 1. According to the model, with an initial CPTIO concentration of 200 μM, the addition of 300 μM SPER/NO results in a rapid increase of the NO concentration to 15 nM and the NO_2 concentration to 3 nM, both being stable over several minutes (Fig. 1). The inclusion of urate at the concentration found in plasma (300 μM) (Becker, 1993) leads to a doubling of the NO concentration (eliminat-

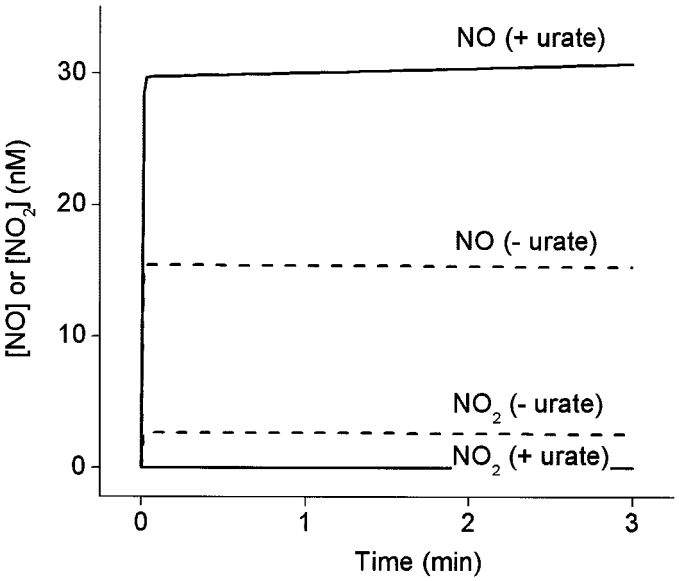

Fig. 1. Predicted profiles of the NO and NO_2 concentrations obtained by mixing 300 μM SPER/NO with 200 μM CPTIO in the absence (broken lines) and presence (solid lines) of 300 μM urate using the kinetic model described under *Materials and Methods*.

ing loss caused by a reaction with NO_2) and a reduction in the NO_2 concentration to 16 pM. Although included in the model for the sake of completion, the reaction of NO with O_2 is negligible compared with the reaction with CPTIO. In the presence of urate, therefore, the complex model reduces to the simple scheme outlined above (eqs. 1 and 2), providing that there is no significant depletion of NO donor or CPTIO. The mixture of urate, CPTIO, and SPER/NO (with the addition of 1000 U/ml of superoxide dismutase to scavenge any superoxide ions that would otherwise react with NO) was used for the experimental tests.

Evaluation of the Method for Delivering Clamped NO Concentrations. By measuring the initial rate of increase of the NO concentration after the addition of SPER/NO, the NO release rate was found to be 19.1 ± 0.8 nM NO/min/μM SPER/NO (n = 3). Assuming a SPER/NO half-life of 39 min, this value signifies 1.08 mol of NO released per mole of SPER/NO (x in eqs. 1 and 2 and in the more complex model). It is important to note that the rate of NO release from NONOates (including SPER/NO) can vary between different batches and between different suppliers (results not shown). Equation 2 predicts a linear relationship between the steady-state NO concentration and the donor concentration. To test this, SPER/NO was added in a range of concentrations, and the resulting profile of the NO concentration was measured using an electrochemical probe. The probe responds too slowly to register the rising phase accurately (see below), but increasing the SPER/NO concentration between 100 and 1000 μM produced graded increases in steady-state NO concentration from 10 to 100 nM (Fig. 2a). The amplitude of the plateau NO concentration (measured as the average recorded between 45 and 75 s after addition of SPER/NO) was directly proportional to the SPER/NO concentration (Fig. 2b). From the gradient of the line (10^{-4} M NO/M SPER/NO) and an x value of 1.08, the value of k_b comes to 3.2 s^{-1}. Dividing this pseudo–first-order rate constant by the CPTIO concentration (200 μM) gives a rate constant for the reaction of CPTIO with NO of 1.6×10^4 $M^{-1}s^{-1}$ at 37°C, a value compatible with the published value of 1.01×10^4 $M^{-1}s^{-1}$ at 25°C (Akaike et al., 1993).

A k_b value of 3.2 s^{-1} implies that 95% of the plateau NO concentration would be attained 1 s after the addition of SPER/NO (Fig. 2c), which is much faster than indicated by the electrode (Fig. 2a). If the time constant of the electrode (8.6 s) (Griffiths and Garthwaite, 2001) was incorporated into the model (see *Materials and Methods*), the shape of the recorded response was well approximated (Fig. 2c), suggesting that the sluggishness of the electrode accounts for the slow recorded rise time.

To explore the limitation of the method with respect to the time over which clamped NO concentrations can be maintained, recordings were made for more prolonged periods. With 100 μM SPER/NO, the NO concentration (initially approximately 10 nM) remained low for at least 50 min (Fig. 2d). With 300 μM SPER/NO, NO remained fairly steady for 10 to 15 min (at 30–40 nM) but then rose at a progressively increasing rate. With 1000 μM SPER/NO, the secondary increase in NO concentration was accelerated to the extent that there was initially more of a shoulder than a plateau (Fig. 2d). The recorded profiles of the NO concentration resemble those predicted by the more complex model (Fig. 3a), which suggests that the time over which NO can be maintained is a function both of the CPTIO concentration, which declines as it is used up (Fig. 3b), and the NO release rate, which reduces as the donor decomposes (Fig. 3c). At high SPER/NO concentrations, the former predominates, and as the CPTIO becomes depleted, the NO concentration eventually increases steeply (until curtailed by autoxidation to the low micromolar range) (data not shown). At low SPER/NO concentrations, on the other hand, the decreasing rate of NO release results in a gradually diminishing NO concentration. For example, at 100 μM SPER/NO, NO is predicted to decrease from 10 to 5.5 nM over 1 h. The measurement of such a change, however, is beyond the capability of the recording apparatus (Fig. 2d) whose quantifiable limit of detection is approximately 10 nM, depending on the particular electrode being used.

Application of the Method to Activation of GC-Coupled NO Receptors. To explore the usefulness of the method for biological purposes, we first investigated the kinetics of activation by NO of its GC-coupled receptor purified

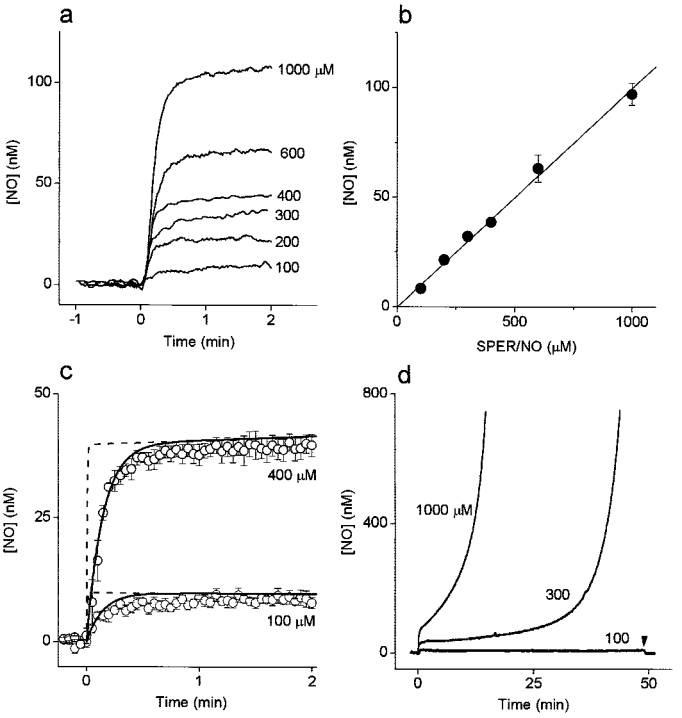

Fig. 2. Experimentally determined profiles of the NO concentrations resulting from the addition of SPER/NO in different concentrations to a mixture of 200 μM CPTIO and 300 μM urate. a, SPER/NO (100–1000 μM) was added at time = 0. The traces are representative of at least three individual experiments and were smoothed by adjacent averaging (5-s bins). b, amplitude of the steady-state NO concentration (measured as the average concentration between 45 and 75 s after the addition of SPER/NO) at different SPER/NO concentrations. Data are means ± S.E.M. (n = 3–6) and are fitted by a linear function, which gives a slope of 10^{-4} M NO/M SPER/NO. c, comparison of the measured NO concentration profile obtained by adding 100 or 400 μM SPER/NO (data are means ± S.E.M., n = 3–4) with the profile predicted by the theoretical model with (solid line) or without (broken line) correction for the electrode response time. d, NO profiles over prolonged time intervals after the addition of 100, 300, and 1000 μM SPER/NO. In the case of 100 μM SPER/NO, hemoglobin (10 μM) was added at the arrowhead to remove NO. Records are single traces from one experiment. With 300 μM SPER/NO, the precise time at which NO started its secondary (almost vertical) ascent varied from test to test, even in the same experiment; this presumably reflects the cumulative effect of small variations in the initial CPTIO concentration and NO release rate on the time at which the CPTIO becomes exhausted (see Fig. 3).

from bovine lung. Despite the more complex reaction mixture used for measuring GC activity (see *Materials and Methods*), the SPER/NO-CPTIO couple generated stable NO concentrations over a 2-min period that were linearly related to the SPER/NO concentrations (Fig. 4a). The slight difference in the slope compared with the simple buffer used previously (Fig. 2b) is probably attributable to the use of different batches of both SPER/NO and CPTIO. NO concentrations in the GC reaction mixture were unaffected by the addition of receptor protein (data not shown).

Possible untoward effects of the new approach were investigated by comparing the time course of GC activity over 2 min at a maximally effective NO concentration produced by the SPER/NO-CPTIO couple (50 nM; see below) with that occurring on the addition of a supramaximal concentration of a donor used frequently in the past, DEA/NO (1 μM). The GC activity in each case was linear with time, and the slopes were not significantly different (approximately 10 μmol of cGMP/mg of protein/min; $P > 0.05$) (Fig. 4b). With DEA/NO, receptor activity cannot be monitored usefully at submaximal concentrations because the NO concentration changes rapidly and continuously (Bellamy et al., 2002). In contrast, using the SPER/NO-CPTIO couple, GC activity remained linear with time at the low NO concentration of 2 nM (Fig. 4b). The addition of a further 2 nM NO after 1 min increased the rate from 4.4 to 6.4 μmol/mg of protein/min, whereas addition of hemoglobin to scavenge NO led to an immediate cessation of GC activity (Fig. 4c), indicating that if any biologically significant variation in the NO concentration had occurred over time, it would have been detected.

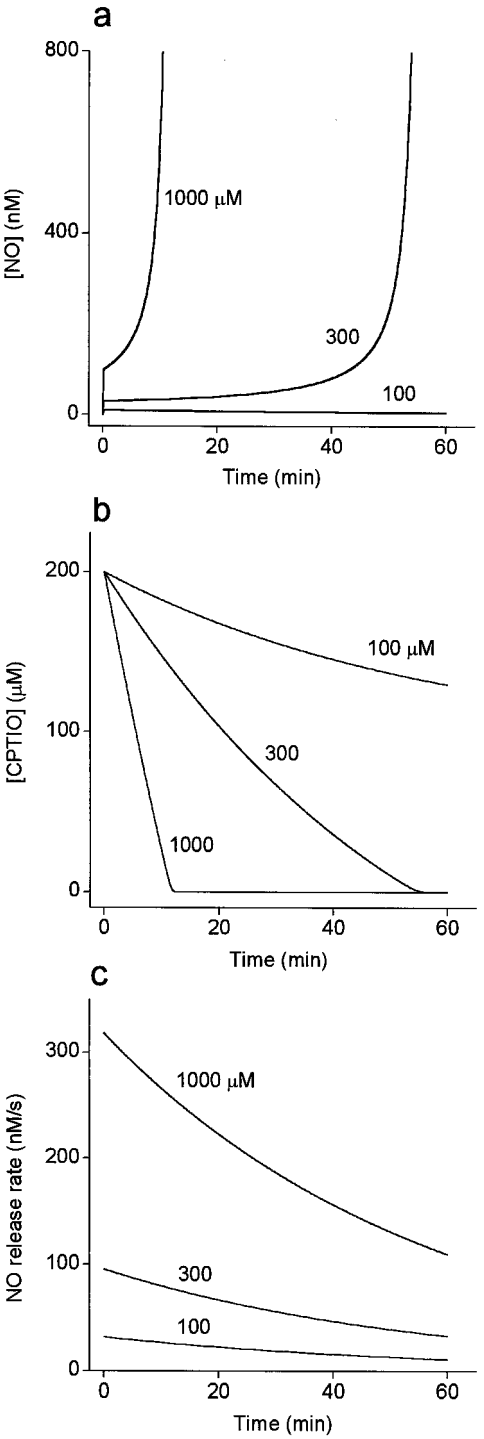

Fig. 3. Predicted profiles of the NO concentration (a), the CPTIO concentration (b), and the NO release rate (c) after the addition of 100, 300, and 1000 μM SPER/NO for comparison with the experimental data shown in Fig. 2d. The curves were derived from the model described under *Materials and Methods*.

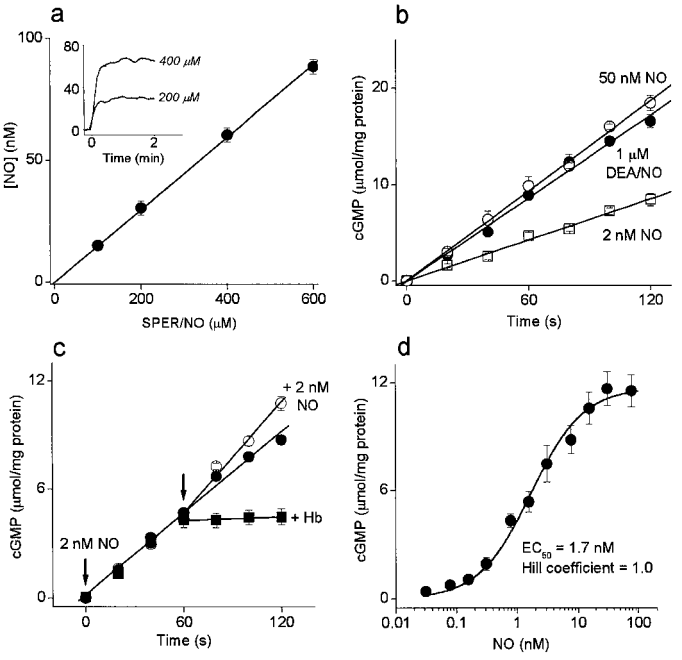

Fig. 4. Activation of the GC-coupled NO receptor under steady-state conditions. a, the mean NO concentrations present 45 to 75 s after the addition of SPER/NO in a range of concentrations to GC assay buffer containing CPTIO (200 μM) are plotted against the SPER/NO concentration and fit with a linear function. Data are means ± S.E.M. ($n = 4$). Inset, representative traces of the NO concentration profile with 200 and 400 μM SPER/NO, smoothed by adjacent averaging (5-s bins). b, time course of cGMP production by the receptor protein after exposure either to a SPER/NO-CPTIO mixture giving steady-state NO concentrations of 2 nM (□) and 50 nM (○) or to 1 μM DEA/NO (●). Data are fit with a linear function and are the means ± S.E.M. of three independent runs. c, cGMP accumulation after exposure of the receptor protein to 2 nM NO without further addition (●) or with addition after 60 s (arrow) of either 25 μM hemoglobin (Hb) (■) or a further 2 nM NO (○). Data are means ± S.E.M. ($n = 11$–12). d, equilibrium concentration-response curve for NO on the GC activity of the purified receptor protein. Data (means ± S.E.M., $n = 4$) were obtained after a 2-min exposure to a range of NO concentrations generated by adding various SPER/NO concentrations to a reaction mixture containing 200 μM CPTIO. Data are fit to the Hill equation, giving an EC_{50} of 1.7 nM and a Hill coefficient of 1.0.

The concentration-response relationship was studied using 2-min exposures. The curve had a threshold of approximately 0.1 nM NO and displayed maximal activity at approximately 20 nM (Fig. 4d). It was well fitted by the Hill equation, with an EC_{50} of 1.7 nM and a Hill coefficient of 1.0.

With such low NO concentrations being effective, it is necessary to question whether the depletion of ligand through receptor binding could have distorted the results. Assuming a molecular mass of 150 kDa for GC and a single heme binding site on each protein (Denninger and Marletta, 1999; Koesling and Friebe, 2000), the total concentration of available binding sites at the protein concentration used (50 ng/ml) amounts to 0.33 nM, which is approximately 3-fold higher than the lowest effective NO concentration for GC activation (Fig. 4d). With normal methods of ligand application, therefore, ligand depletion would be significant and would have to be accounted for. To examine this issue using the present method of NO delivery, we incorporated reversible receptor binding into the model and assumed that the resultant GC activity was dependent on the concentration of the NO-bound species (see Fig. 5 legend for parameters). At 0.33 nM receptor, there would be negligible ligand depletion because the amount bound to the receptor is rapidly restored by NO release from the donor (Fig. 5a). Significant slowing of the attainment of the steady-state NO concentration and a resulting underestimate of GC activity is predicted to occur only with the receptor at concentrations 100-fold higher or more (Fig. 5, a and b).

To check that the concentration-response curve obtained for the purified receptor (Fig. 4b) was not peculiar to the use of SPER/NO as the donor, we evaluated the combination of diethylenetriamine/NO (DETA/NO; half-life = 20 h) and CPTIO for the same purpose. To avoid the use of excessive DETA/NO concentrations, the concentration of CPTIO was decreased to 50 μM, allowing steady-state NO concentrations to be achieved in 4 s. As with SPER/NO, there was a linear relationship between the concentrations of DETA/NO and NO, the gradient being 4.6×10^{-5} M NO/M DETA/NO (data not illustrated). When the mixture was used to investigate the concentration-response curve for NO on purified lung GC (2-min exposure), the results (EC_{50} = 1.4 nM, Hill coefficient = 1.0; data not illustrated) were indistinguishable from those obtained using SPER/NO.

The established GC-coupled NO receptors are $\alpha\beta$ heterodimers, and the lung may contain both known isoforms, $\alpha1\beta1$ and $\alpha2\beta1$, with the former predominating (Mergia et al., 2003). Accordingly, the response of the purified lung protein may be a composite one. To examine the sensitivity of the separate isoforms to NO, they were expressed in COS-7 cells, and the NO-evoked GC activity was followed in cell lysates. The resultant maximal activity of the two isoforms was similar (Fig. 6, a and b). Moreover, the EC_{50} values for NO were also comparable (0.9 nM for $\alpha1\beta1$ and 0.5 nM for $\alpha2\beta1$). The slopes of both curves were described by a Hill coefficient of 1.1.

Discussion

Methodological Considerations. The method described here for delivering clamped NO concentrations is simple to use and requires chemicals that are available commercially, so it should find wide applications in NO research in which, until now, it has been very difficult to administer this key signaling molecule in a manner that would be taken for granted in corresponding studies of other chemical messengers. There are, nevertheless, a number of real and potential limitations.

First, it is necessary to consider the biological reactivity of the ingredients and products other than NO. CPTIO has frequently been used as an NO scavenger to test for its participation in various biological phenomena, and we are unaware of any unrelated side effects. SPER/NO belongs to the much-used class of NONOate donor, but the carrier molecule, spermine, is an endogenous polyamine with biological activity (Bachrach et al., 2001). The rate of NO release from SPER/NO may also depend on constituents of the medium and on donor concentration (Davies et al., 2001), although we have not yet observed such inconsistencies (Figs. 2 and 4) and have obtained identical results with another donor (DETA/NO) not reported to exhibit this anomalous behavior (Davies et al., 2001). Other NONOates such as NOC-5 or NOC-12

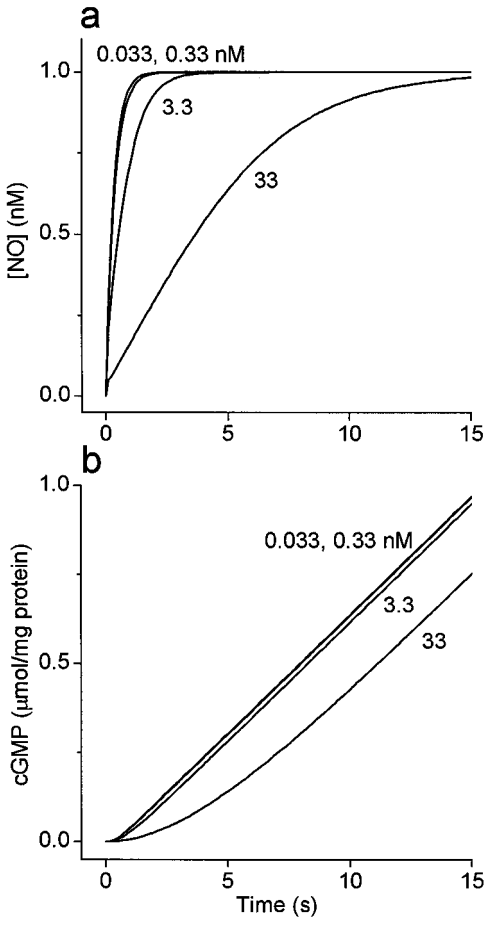

Fig. 5. Evaluation of the effect of ligand depletion on the measured activity of the GC-coupled NO receptor. NO delivery was modeled using 10 μM SPER/NO and 200 μM CPTIO, giving a steady-state NO concentration of 1 nM (see *Materials and Methods*). To investigate the depletion of NO through receptor binding, we assumed that NO binds with a bimolecular rate constant of 2.5×10^9 $M^{-1}s^{-1}$ (Zhao et al., 1999) and dissociates from the NO receptor complex with a rate constant of 3.7 s^{-1} (Bellamy and Garthwaite, 2001), giving an apparent K_d of 1.5 nM. The profiles of the NO concentration at receptor concentrations of 0.033 to 33 nM are shown in a. In each case, the corresponding profile of GC activity (b) was calculated by assuming that cGMP is formed from the NO receptor complex at a maximal rate (when all the receptors are occupied) equivalent to 10 μmol/mg of protein/min (Fig. 4b).

with half-lives of 25 and 100 min, respectively, could be used instead (http://www.dojindo.com/newprod/1/no/nodonors/nocsb.html). To deplete NO_2, urate was used at the concentration found in plasma (Becker, 1993) and so it can be regarded as a physiological ingredient. The fate and reactivity of the resulting urate radicals, however, are unclear, and it seems wise to limit their production. Finally NO_2^-, produced by the reaction of NO_2 with urate, will be formed at the same rate at which CPTIO is consumed. The NO_2^- concentration range found in human bodily fluids is 0.5 to 210 μM (Augusto et al., 2002), and it is relatively unreactive at neutral pH and therefore unlikely to create problems. Because of an overall lack of anticipated side effects, we recently used the method to analyze the kinetics of the NO-cGMP-phosphorylation pathway in suspensions of intact platelets (E. Mo, H. Amin, and J. Garthwaite., unpublished results) without encountering any problems.

Second, the method is limited in the range and duration of the NO concentrations obtainable. These two parameters are linked to the concentration and half-life of the donor and to the capacity of the sink. The immediate aim was to have a method that delivers fixed NO concentrations rapidly and maintains them over a time scale of minutes. With the combination of the donor and sink concentrations chosen, this objective was met for NO concentrations up to 100 nM. At this upper limit, the NO concentration was not constant but increased at a sufficiently slow rate to remain usable. For other types of experiments, exposures to NO of longer than a few minutes may be desirable. In this case, a donor with a longer half-life, such as DETA/NO (half-life 20 h), would be preferred. In this scenario, it is unlikely that the final NO concentration needs to be attained as rapidly as described here, so both donor and sink can be diluted to reduce the chemical flux. To illustrate the scope of such an application, Fig. 7 displays the predicted profile of the NO concentration obtained with a combination of 2 μM CPTIO and 3 to 10 μM DETA/NO. The time required for the initial equilibration of the NO concentration is approximately 2 min. With 3 μM DETA/NO, NO can be maintained at nearly 1 nM for at least 10 h, whereas at lower DETA/NO concentrations, the NO concentration decreases slowly as the donor decays. With 6 μM DETA/NO, the NO concentration is reasonably stable (2–3 nM) for approximately 5 h, but with 10 μM, the usable duration falls to approximately 1 h (3–4 nM NO). The duration can be extended quite easily by timely supplementation with fresh CPTIO, as illustrated for 6 μM DETA/NO in Fig. 7. Using essentially the same method, therefore, long exposures to physiological NO concentrations could be achieved.

The third methodological issue is variability. The precise value of the NO concentration obtained, and its duration, relies critically on the purity of the CPTIO and the release rate of NO from the donor. As mentioned earlier (see *Results*), we have noticed significant variation in the rate of NO released from NONOates depending on the particular batch and supplier used. In addition, the rate of decomposition of the NONOates depends on both temperature and pH (Davies et al., 2001). For this reason, it is essential that the pH of the buffers is adjusted at the temperature used for the experiment and that the buffers have a sufficient capacity to tolerate the addition of alkaline solutions (used to dissolve uric acid and the NONOates) or the production of protons (by the reaction of NO_2 with urate) without a change in pH. Finally, it should be noted that some cells can avidly consume NO (Griffiths and Garthwaite, 2001), which may necessitate the calibration of the NO delivery system in the presence of the cells under study.

Activity of GC-coupled NO Receptors Under Steady-State Conditions. In the past, a lack of control over NO concentrations has led to widely differing estimates of the potency of NO on its GC-coupled receptors. An initial estimate of the EC_{50} value, derived from the addition of NO from concentrated solutions, was ≤250 nM (Stone and Marletta, 1996), and the similar potency found for the NONOate DEA/NO in standard GC assays (approximately 300 nM)

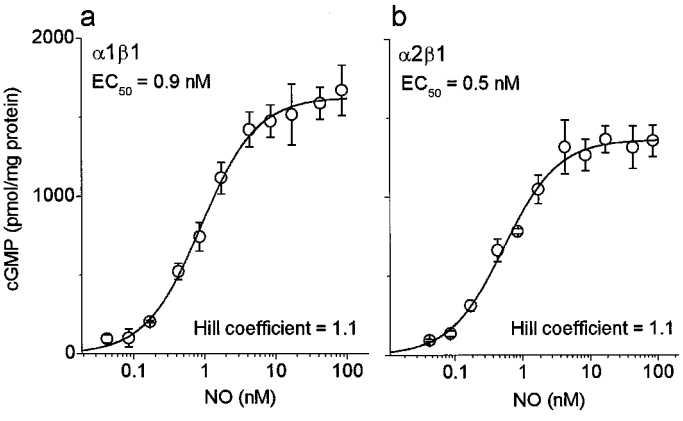

Fig. 6. Equilibrium concentration-response curves for NO on the GC activity of the $\alpha1\beta1$ (a) and $\alpha2\beta1$ (b) receptor isoforms in lysates of transfected COS-7 cells. Data (means ± S.E.M., n = 3) were obtained after a 2-min exposure to a range of NO concentrations generated by adding various SPER/NO concentrations to a reaction mixture containing 200 μM CPTIO. Data are fit to the Hill equation, giving EC_{50} values of 0.9 nM for $\alpha1\beta1$ and 0.5 nM for $\alpha2\beta1$ and a Hill coefficient of 1.1 for both.

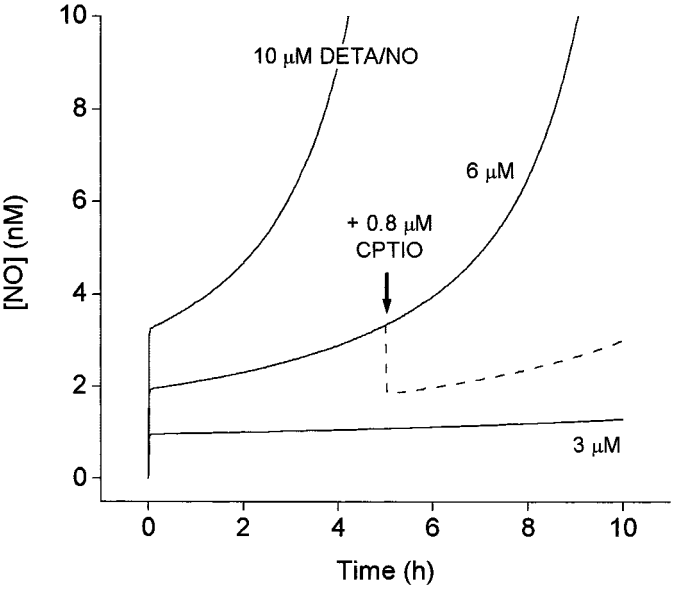

Fig. 7. Theoretical NO concentration profiles over periods of hours produced by a combination of 2 μM CPTIO (instead of the usual 200 μM) and 3, 6, and 10 μM of the NONOate DETA/NO, which has a half-life of 20 h. In the case of 6 μM DETA/NO, the effect of adding a CPTIO supplement (0.8 μM) after 5 h (arrow) is illustrated by the broken line. Although at this time CPTIO is predicted to be 50% depleted, less than 50% of the starting concentration is needed to restore the NO concentration because the donor is also partially degraded. Curves were generated using the model described under *Materials and Methods*.

(Russwurm et al., 1998) sustained the concept that physiological NO signaling involved NO concentrations in the 100 nM range. By monitoring the profile of NO release during the course of such assays, however, we found that a measured EC_{50} value of 300 nM for DEA/NO is compatible with the true potency of NO being in the low nanomolar range that had been suggested by a series of studies on intact cells from the brain (Bellamy et al., 2002). Furthermore, by using red blood cells to maintain constant NO concentrations, we obtained an EC_{50} of 4 nM for the purified lung receptor (Bellamy et al., 2002), suggesting that the high potency of NO in cells did not reflect some peculiarity of the protein in an intracellular environment. In addition, the slope of the concentration-response curve was unexpectedly steep (Hill coefficient of 2) which, if correct, would have important mechanistic implications for receptor activation in that it implies cooperative binding of two or more molecules of NO to each receptor.

Re-examination of this issue in the present study using the new NO delivery system supports the potency of NO for its receptor in lung being in the low nM range, although the actual EC_{50} value (~1.5 nM) was approximately 2-fold lower than that obtained using the red blood cell method (Bellamy et al., 2002). More importantly, the Hill coefficient was of the value (1) predicted for a single NO binding site. The discrepancy is best attributed to differences in the methodology and, in particular, to the former use of red blood cells. Any lysis of cells in the suspension would result in the release of free hemoglobin, which binds and inactivates NO far more avidly than when encapsulated in red blood cells (Liu et al., 1998). Although there was no evidence for free hemoglobin at the NO concentrations that were measurable (≥5–10 nM) significant cell lysis (calculated to be 0.1% or greater) would preferentially impact the lower NO concentrations that could not be measured. Should this occur, the lower half of the concentration-response curve, which relied on predicted NO concentrations, would be artificially steepened, giving rise to an overestimate of the Hill coefficient. Such an effect would also explain the higher EC_{50} value obtained beforehand.

There had been no previous examination of the relative or absolute NO sensitivity of the individual $\alpha1\beta1$ and $\alpha2\beta1$ receptor isoforms in cell-free preparations. Concentration-response curves to DEA/NO were reported to be similar (Russwurm et al., 1998), but this result is equivocal (Bellamy et al., 2002). Nevertheless, a direct comparison using the new method indicated that the EC_{50} values for NO are closely comparable with each other (approximately 1 nM) and with the value obtained for the purified receptor protein from lung. We had previously found similar absolute potencies of NO toward the two isoforms when expressed in COS-7 cells (Gibb et al., 2003), but these estimates were complicated by receptor desensitization and bell-shaped concentration-response curves observed with the receptors in intact cells.

In conclusion, the kinetic parameters for activation of the GC-coupled NO receptor derived in this study are likely to be more reliable than those determined previously using the red blood cell method. The modified parameters obtained will simplify the development of models of receptor activation because they eliminate the need for incorporating cooperative binding of NO to its receptor. More generally, the results support the usefulness of the new method for delivering physiological concentrations of NO to biological preparations in a reliable and reproducible manner, which should assist the analysis of NO signal transduction.

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