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NO Release from NO Donors and Nitrovasodilators: Comparisons between Oxyhemoglobin and Potentiometric Assays

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Unraveling the biology, pharmacology, and toxicology of NO depends on accurate NO assays, two of the more common being the oxyHb (oxyhemoglobin) assay and potentiometric detection using a Clark-type NO-selective electrode. Comparison of the specificity and sensitivity of the oxyHb and potentiometric methods was carried out using a broad series of nitrovasodilators, including organic nitrates, nitrites, thionitrates, nitrosothiols, and diazenium diolates. Only with the more labile diazenium diolates was a linear relationship observed between the rates of NO release measured potentiometrically and the rate of oxyHb oxidation from the oxyHb assay. The nonlinear plots indicate that N,O-species other than NO itself are capable of oxidizing oxyHb.

Introduction

The intensity of research on the pharmacology, biology, and toxicology of nitric oxide (NO) has continued unabated since the discovery of the significant biological roles of NO (*1*−*3*). NO is involved in the neurotoxic response to cerebral ischemia, and the potential toxicity of NO and its byproducts, in particular peroxynitrite, is associated with various disease states (*3*−*7*).

In addition to endogenous sources of NO, exogenous NO donors, in particular the nitrovasodilators, are important cardiovascular therapeutics with potential as cerebrovascular drugs (*8*). The organic nitrate, nitroglycerin (GTN[1]), has been employed for more than a century in treatment of angina (*9*, *10*), with earlier studies on the organic nitrite, amyl nitrite, inspiring the therapeutic use of GTN. Nitrosothiols such as *S*-nitrosoglutathione (SNOG) and protein nitrosothiols have been proposed as biological pools of NO (*11*, *12*), whereas diazenium diolate salts (popularly termed NONOates) have been examined as potential vasodilators that circumvent nitrate tolerance (*13*). Organic nitrates and nitrites have also been proposed as intermediates in toxicological mechanisms

[1] Abbreviations: GTN, glyceryl trinitrate; SNOG, *S*-nitrosoglutathione; GCase, guanylyl cyclase; oxyHb, oxyhemoglobin; TSA, thiosalicyclic acid; DTT, dithiothreitol; DEA/NO, $(CH_3CH_2)_2N(NONO)Na$; SNAP, *S*-nitroso-*N*-acetylpenicillamine; FeTPP, Fe−tetraphenylporphyrin-bis(*N*-methylimidazole); NAC, *N*-acetylcysteine; ISDN, isosorbide dinitrate; NOC9/NO, $CH_3NH_2(CH_2)_6N(NONO)CH_3$; SPER/NO, $H_2N(CH_2)_3NH(CH_2)_4N(NONO)(CH_2)_3NH_3$; DETA/NO, $H_2N(CH_2)_2N(NONO)(CH_2)_2NH_3$; GDN, glycerol 1,2-dinitrate; GMN, glycerol 1-mononitrate; GT009, 2,3-dinitratopropyl thiosulfonate, sodium salt; GT016, 2-nitrato-1,3-propyl sultone; GT017, 2-nitrato-1,3-propyl sultine; ClDN, 3-chloropropyl 1,2-dinitrate; GT002, 3-fluoropropyl 1,2-dinitrate; GT027, 3-(1,1,2,2-tetrafluoroethoxy)propyl 1,2-dinitrate.

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of action of peroxynitrite, itself, the product of reaction between NO and superoxide (*14*).

It is widely held that all four nitrovasodilator families, organic nitrates ($RONO_2$), nitrites (RONO), nitrosothiols (RSNO), and diazenium diolates [$R_2N(NO)_2^-$], exert their biological activity through release of NO (*15*) which activates the enzyme guanylyl cyclase (GCase) (*10*). Diazenium diolates release NO spontaneously at neutral pH, whereas simple nitrosothiols require trace amounts of Cu^I and/or free thiol (*16*). Both organic nitrates and nitrites are proposed to require biotransformation to liberate NO (*15*). Nitrosothiols and organic thionitrates ($RSNO_2$) have been proposed as intermediates in these biotransformations (*8*).

Detection and quantification of nitric oxide (NO) remain a challenge (*17*), despite the large number of methods available (*18*−*22*). In the oxyhemoglobin (oxyHb) assay, oxyHb is oxidized to Fe^{III}−methemoglobin, which can be simply monitored spectrophotometrically (*21*, *22*). The oxyHb assay has been widely used to quantify NO release in studies on NO donors and nitrovasodilators and for the enzyme assay of NO synthase (*15*, *23*−*25*). Schmidt et al. (*18*) first reported that the oxyHb assay could not distinguish between peroxynitrite and NO, using an NO-selective electrode for comparison. Feelisch and co-workers (*20*), who were largely responsible for the initial acceptance of the oxyHb assay, have recently expanded the assay to propose simultaneous detection of superoxide and NO (*22*). Herein, we report comparisons between rates of NO release using the oxyHb assay and an NO-selective electrode, from a wide range of putative NO donors, including organic nitrates, nitrites, thionitrates, nitrosothiols, and diazenium diolates.

Experimental Section

Organic nitrites (*26*), organic nitrates (*27*), nitrosothiols (*28*, *29*), FeTPP (*30*), tBu-thionitrate (*31*), and GTN were synthesized as described in the literature. Diazenium diolate salts were obtained from RBI (Natick, MA) or Calbiochem (La Jolla, CA). Bovine hemoglobin and bovine plasma were obtained from Sigma (St. Louis, MO), and human plasma was obtained from Kingston Blood Bank (Kingston, ON). All other chemicals were obtained from Aldrich Chemicals (Milwaukee, WI) or BDH (Toronto, ON). Spectrophotometric kinetics were measured on a Beckman DU 7400 or Hewlett-Packard 8452A instrument. NO was detected using a Clark-type NO-sensitive electrode (ISO-NO, World Precision Instruments Inc., Sarasota, FL) attached to a chart recorder. The reaction volume for potentiometric analysis was 1−2 mL, in a 7 mL unsealed reaction vessel. Reaction vessels for all measurements were water-jacketed or pelletier-controlled to maintain temperature. *tert*-Butyl thionitrate (CH_3CN), organic nitrites (dioxane), SNAP and SNOG (CH_3CN), and GTN (CH_3CN or EtOH) were added in organic solvent to phosphate-buffered solutions (concentration and pH described in Table 1 and Figure 1), to give final concentrations of <5% organic component. The diazenium diolates were added as a freshly prepared stock solution in 10 mM NaOH to phosphate-buffered solutions (*32*). Various reaction temperatures and pHs were used to improve detection (i.e. slow NO releasers studied at 37 °C) and for comparison with literature data.

Rate constants determined spectrophotometrically for the hydrolysis or aqueous degradation of the NO donors were measured at 21 or 37 °C. Rate constants for the breakdown of diazenium diolate salts and nitrosothiols, and hence generation of NO, were determined by monitoring at 250 (DEA/NO and NOC9/NO), 252 (sper/NO and DETA/NO), or 339 nm (SNAP and SNOG), under pseudo-first-order conditions, except for DETA/NO for which the method of initial rates was used (*32*−*34*). In all cases, results were the average of triplicate experiments.

The maximum voltage detected potentiometrically was linearly related to the NO donor concentration (Figure 1A); thus, the electrode was calibrated by calculating the rate of NO release from DEA/NO using the spectrophotometrically determined rate constant for breakdown of DEA/NO and the reported molar yield of 1.5 equiv of NO (literature value for the rate constant of release of NO from DEA/NO is 5.5×10^{-3} s^{-1} at pH 7.4 and 37 °C) (*32*). Spectrophotometric reaction conditions mimicked potentiometric conditions as much as possible. This calibration must be performed for each set of assay conditions (i.e., reaction medium, reaction volume, pH, temperature, mixing conditions, reaction headspace, dissolved oxygen, degassing, etc.), since the reaction vessel is unsealed and the rate of NO loss is consistent only under constant conditions. All potentiometric measurements were performed in triplicate.

The reaction of GTN with Fe^{II}TPP (insoluble in water) was monitored in a biphasic 1:1 system of dichloromethane and phosphate buffer. Fe^{II} oxidation, detected spectrophotometrically, occurred over the space of minutes. Reaction of GTN with Fe^{II}TPP is sufficiently rapid that despite the nonanaerobic conditions, generation of NO is easily and reproducibly observed potentiometrically. Conversely, no response is observed in control experiments involving combinations of the individual components of this system, or with Fe^{III}TPP. The reaction of GTN with Fe^{II}TPP in CH_2Cl_2 in a sealed system under O_2-free N_2 is stoichiometric not catalytic, and complete within 2.5 min, yielding GDN isomers and GMN at higher concentrations of Fe^{II}-TPP.

The oxyHb assay was performed using conditions similar to those described by Feelish and Noack (*21*), monitoring oxidation of oxyHb to metHb at 405 nm. Bovine Hb was used exclusively since other Hb's give very different reactions with GTN. In assays with added thiol, equilibration of thiol with oxyHb for 15 min was initiated before the reaction.

Results and Discussion

Benchmarking of NO release assays can be achieved by using gaseous NO, but use of diazenium diolate salts, which spontaneously release up to 2 equiv of NO in aqueous solution at physiological pH, has significant advantages, including stability and ease of handling, and is especially appropriate when NO donors are being assayed (*32*, *34*). Diazenium diolates possess varying rates of NO release, which can be measured, under the assay conditions of interest, by spectrophotometrically monitoring diazenium diolate degradation (*32*, *34*).

Detection of NO release from organic nitrates has not been reported using Clark-type, NO-selective electrodes, even in the presence of thiol (*8*, *27*). However, both NO and GTN activate GCase, the latter only in the presence of specific thiol additives [e.g., Cys and thiosalicylic acid (TSA), but not dithiothreitol (DTT)]. Fung and co-workers (*35*) have detected low levels of NO release from GTN and thiol in phosphate buffer (5% of that detected in plasma), using chemiluminescence detection under anaerobic conditions with the addition of superoxide dismutase. In this work, nitrosothiols and organic nitrates were examined with and without thiol additives.

Potentiometric Assay. The potentiometric response of the ISO/NO NO-specific electrode to solutions of NO donors (pH 7.6 or 7.4, phosphate buffer, 21 or 37 °C) was measured by the maximal voltage response, which was both linear with respect to the concentration of NO donor and highly reproducible (Figure 1A). DEA/NO was used

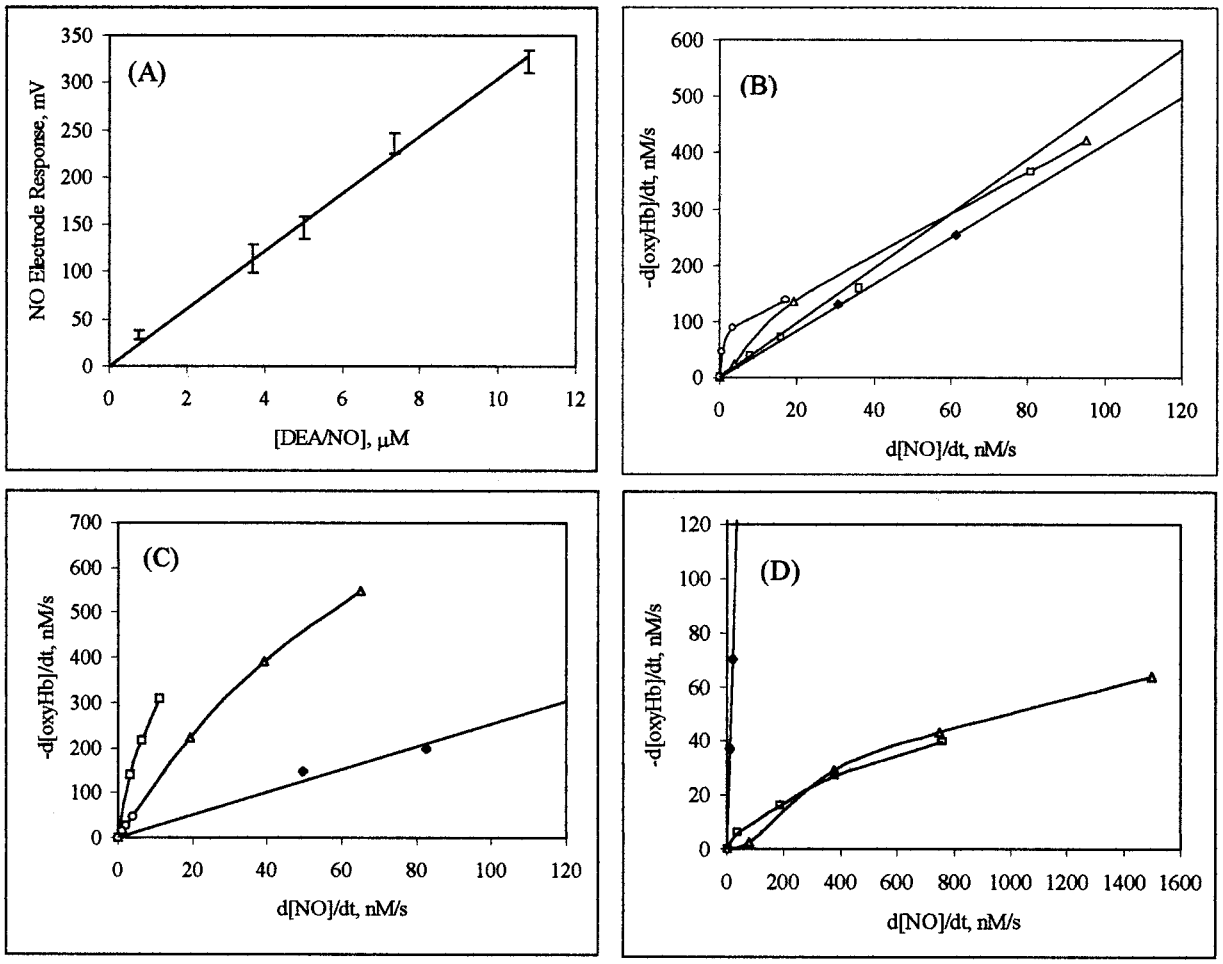

Figure 1. Comparison of potentiometric and colorimetric responses to nitrovasodilators. (A) Calibration of the NO electrode by measurement of NO production from DEA/NO in 100 mM phosphate buffer at pH 7.4 and 37 °C. (B) Rates of NO production vs initial rates of oxyHb oxidation for NOC9/NO (□), DEA/NO (◆), Sper/NO (△), and DETA/NO (○) in 100 mM phosphate buffer at pH 7.4 and 21 °C (rates of DETA/NO and Sper/NO NO production determined spectrophotometrically). (C) Rates of NO production vs initial rates of oxyHb oxidation for DEA/NO (◆), $ClCH_2CH_2ONO$ (□), $CH_3CH_2CH_2ONO$ (△), and *t*-Bu-ONO (○) in 100 mM phosphate buffer at pH 7.4 and 37 °C. (D) Rates of NO production vs initial rates of oxyHb oxidation for DEA/NO (◆), SNOG (□), and SNAP (△) in 100 mM phosphate buffer at pH 7.4 and 21 °C (rates of SNOG NO production determined spectrophotometrically). OxyHb assay monitored δA(404nm−408nm), corrected using $10^4 \times \delta\epsilon = 4.11$ (21 °C), 5.20 (25 °C), and 8.48 (37 °C). The concentration of the oxyHb assay solutions was 3.8 mM diluted in oxygenated buffer. All rates are the result of triplicate experiments.

for calibration of the electrode using the rate constant for NO release derived spectrophotometrically (*32*). The potentiometric method is rapid and reproducible, but requires recalibration with DEA/NO if changes to the reaction conditions are made. Rate constants for NO release were measured for a range of nitrovasodilators (Table 1).

Spontaneous release of NO from organic thionitrate and nitrites can be observed in aqueous solution, at slow rates and uninfluenced by the presence of EDTA (Table 1A). NO release is not observed from GTN (≤2 mM) and thiol (Cys, TSA ≤50 mM), or any other nitrate studied, in aqueous buffer, above the electrode detection threshold (Table 1B). NO release from both DEA/NO and SNAP was observed, even in the presence of large concentrations of Cys (≤50 mM), negating the unlikely possibility that excess Cys somehow scavenges NO produced from reaction with GTN. NO release was also observed from solutions of GTN and TSA where plasma replaced phosphate buffer as the reaction medium, in accordance with chemiluminescence measurements (Table 1B) (*35*). Importantly, NO release was observed from the rapid reaction of GTN with $Fe^{II}TPP$ (Table 1B).

OxyHb Assay. An important early use of the oxyHb assay by Feelisch and Noack reported NO release rates for solutions of organic nitrates with added thiols in phosphate buffer (*21*). We find the reported data for oxyHb oxidation entirely reproducible, but under no conditions (in the absence of oxyHb) is NO detected potentiometrically (Table S1 in the Supporting Information). OxyHb oxidation rates are linearly dependent on NO donor concentration for the two most reactive diazenium diolates, DEA/NO and NOC9/NO, with the stoichiometry of 4 equiv of NO per mole of metHb produced (Figure 1B). If the oxyHb assay represents a simple direct measure of NO production, all data points for NO donors should lie on this line (Figure 1). The less reactive polyamine diazenium diolates, SPER/NO and DETA/NO, oxidize oxyHb at a rate slightly higher than that expected from rates of NO release in the absence of oxyHb (Figure 1B), although the deviation is much more dramatic for the organic nitrites (Figure 1C). Conversely, both nitrosothiols release sufficient NO to oxidize oxyHb at a much greater rate than observed (Figure 1D). Furthermore, nitrite ion, which is a primary hydrolysis product of organic nitrites and thiolysis product of nitrates, oxidizes

Table 1. Potentiometrically Determined Rate Constants (A) and Rates (B) for NO Release from Nitrovasodilators, Calibrated against Spectrophotometric Determinations for DEA/NO

A	k_{NO} (s^{-1})[a]	T[b] (°C)	B[g]	adjuvant	d[NO]/dt (nM/s)[h]	medium[i]
DEA/NO	8.3×10^{-3}	37	1 mM GTN	1 mM Fe^{II}TPP[j]	9.2	21 °C[j]
DEA/NO	1.2×10^{-3}	21	1 mM GTN	50 mM TSA	$\gg 0$[k,l]	37 °C
NOC9/NO	4.0×10^{-3}	21	500 mM GTN	1 mM TSA	37	BP, 37 °C
*n*PrONO[c]	1.3×10^{-4}	37	500 mM GTN	−	$\gg 0$[l]	BP, 37 °C
*n*PrONO	1.2×10^{-4}	37	250 mM GTN	500 mM TSA	61	HP, 37 °C
*n*PrONO[d]	1.4×10^{-4}	37	250 mM GTN	−	9.1	HP, 37 °C
$ClCH_2CH_2ONO$[c]	2.2×10^{-5}	37	1 mM GTN	1 mM Cys	$\gg 0$[l]	HP, 37 °C
*t*BuONO[c]	8.0×10^{-6}	37	250 mM GT027	500 mM TSA	51	HP, 37 °C
*t*BuSNO$_2$	8.0×10^{-6}	21	250 mM ClDN	500 mM TSA	15	HP, 37 °C
SNAP[e]	1.5×10^{-3} [e]	21	500 mM GT002	1 mM TSA	3.1	HP, 37 °C
*t*BuSNO[f]	6.2×10^{-5}	21				

[a] Determined from plots of observed rate vs concentration for at least three nitrovasodilator concentrations in triplicate. [b] Phosphate buffer at 37 °C, 100 mM, and pH 7.4 or at 21 °C, 50 mM, and pH 7.6, except DEA/NO, NOC9/NO (100 mM and pH 7.4). [c] [EDTA] = 10 mM. [d] [Cys] = 5 mM. [e] [Cys] = 0.4 mM; spectrophotometric k(degradation) = 2.0×10^{-3} s^{-1}, and k_{NO}(SNOG) is below detection limits. [f] Contains *t*-BuSH, and *t*-Bu-SS-*t*-Bu. [g] No NO was observed in HP from GT016 and TSA, GT017 and TSA, GT009 and TSA, GTN and DTT, and GTN and GSH. [h] Calibration by DEA/NO ($k_{NO} = 2.2 \times 10^{-3}$ in 100 mM phosphate buffer at pH 7.86 and 37 °C). [i] Phosphate buffer (100 mM) at pH 7.4, HP (human plasma), or BP (bovine plasma). [j] In 1:1 CH_2Cl_2/aqueous phosphate (pH 7.6 and 50 mM). [k] With or without SOD. [l] Below detection limits.

oxyHb (Table S2 in the Supporting Information).

Rates of oxyHb oxidation by the organic nitrates in the presence of thiols are low compared to those of other NO donors, but for the more reactive nitrates at 37 °C, NO should be potentiometrically detectable if oxyHb oxidation is a true assay of NO release. For example, oxyHb rates for organic nitrates in the presence of thiol correspond to NO release rates of up to 30 nM s^{-1}, well above threshold of detection [e.g., −d[oxyHb]/dt(pH 7.4, 37 °C) = 32, 65, and 58 nM/s for GTN (1 mM) and TSA (2 mM), GT009 (1 mM) and Cys (2 mM), and GT016 (1 mM) and TSA (2 mM), respectively]. In addition, rates of oxyHb oxidation by GTN and ISDN (isosorbide dinitrate), in the presence of DTT, are relatively high, although neither ISDN nor GTN will activate GCase, and therefore presumably release NO, in the presence of DTT [−d[oxyHb]/dt(pH 7.9, 25 °C) = 1.1 nM/s when [nitrate] = 1 mM and [thiol] = 5 mM; cf. 0.49 and 1.5 nM/s for ISDN and Cys, and GTN and Cys, respectively].

There is a rich chemistry of reactions between NO-containing molecules and heme units. First, Doyle (*36*) has shown that organic nitrites directly oxidize deoxyHb and oxyHb and that reactions with the cysteinyl thiols of hemoglobin (Hb-SH) are possible. Second, it has been proposed that nitrosothiols may activate GCase by directly binding to the heme NO-binding site, and transnitrosation with Hb-SH is possible (*37*). Finally, GTN has been shown to react readily with deoxyHb to yield inorganic nitrite (but not NO) (*38*), and heme proteins, especially cytochrome P450, have been proposed as catalysts of GTN biotransformation to NO (*10*). Of course, GTN with added Cys activates GCase, which is itself an Fe^{II}−heme protein (*10*). Further, we have seen herein that GTN reacts rapidly with the heme model, Fe^{II}TPP, to yield NO. Given this rich chemistry, it is perhaps not surprising that the interactions of NO donors with oxyHb are not limited to discrete release and subsequent trapping of NO.

Conclusions. The oxyHb assay continues to be used as a direct measure of NO release. Recent studies have conflictingly described both the applicability and unsuitability of the oxyHb assay for in situ measurements of cellular NO release, the unsuitability arising partly from interference from other chemicals in the medium (*39*, *40*). Potentiometric detection unambiguously and reproducibly measures NO release, albeit with a relatively high detection threshold, whereas the oxyHb assay measures oxidation of Fe^{II}−oxyHb by NO and other N- and O-containing species. Nevertheless, the detailed mechanisms of interaction of nitrosothiols, thionitrate, and nitrate esters with oxyHb require further study (i) because of the biological significance of hemoglobin itself and (ii) because oxyHb provides a model for interactions of these biologically and therapeutically important molecules with other ferrous−heme proteins, such as GCase.

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Supporting Information Available: Tables of rates of oxyHb oxidation by nitrates and added thiols (1 page). Ordering information is given on any current masthead page.

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