Communications

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, nitrates, 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 (GTN1), 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, thiosalicylic acid; DTT, dithiothreitol; DEA/NO, (CH3CH2)2N(NONO)Na; SNAP, S-nitroso-N-acetylpenicillamine; FeTPP, Fe–tetraphenylporphyrin-bis(N-methylimidazole); NAC, N-acetylcysteine; ISDN, isosorbide dinitrate; GT009, 2,3-dinitratopropyl thiosulfonate, sodium salt; GT016, 2-nitroso-1,3-propyl sultone; GT017, 2-nitroso-1,3-propyl sulfone; CIDN, 3-chloropropanol, 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 (RONO₂), nitrates (RONO₂), nitrosothiols (RSNO₂), and diazenium diolates [R₂N.NO(−)], 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 Cu²⁺ and/or free thiol (16). Both organic nitrates and nitrates are proposed to require biotransformation to liberate NO (15). Nitrosothiols and organic thionitrates (RSNO₂) 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 (oxy-Hb) assay, oxy-Hb is oxidized to Fe³⁺–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, nitrates, thionitrites, nitrosothiols, and diazenium diolates.

**Experimental Section**

Organic nitrates (26), organic nitrates (27), nitrosothiols (28, 29), FeTPP (30), tBu-thionitrite (31), and GTN were synthesized as described in the literature. Diazonium 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 measured using a Clark-type NO-sensitive electrode (ISO/NO NO-specific electrode to solutions of NO (21, 22). The oxyHb assay was performed using conditions similar to those described by Feelisch 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). Diazonium 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 dithiobitol (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
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 Fe²⁺TPP (Table 1B).

OxyHb Assay. An important early use of the oxyHb assay by FeeDish 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 nitrates (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...
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 \(\sim\) 2.2 \times 10\(^{-3}\) 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 \(-\sim\) (10) Bennett, B. M., McDonald, B. J., Nigam, R., and Simon, W. C. (1994) Role of NO in vascular smooth muscle and cardiac muscle function. Trends Pharmacol. Sci. 15, 255–259.


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

<table>
<thead>
<tr>
<th>A</th>
<th>(k_{NO} (s^{-1})) (^{a})</th>
<th>T (°C)</th>
<th>B</th>
<th>(d[NO]/dt (nM/s)^{b})</th>
<th>medium (^{c})</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEA/NO</td>
<td>(8.3 \times 10^{-4})</td>
<td>37</td>
<td>1 mM GTN</td>
<td>1 mM Fe(^{II})TPP</td>
<td>9.2</td>
</tr>
<tr>
<td>DEA/NO</td>
<td>(1.2 \times 10^{-3})</td>
<td>37</td>
<td>1 mM GTN</td>
<td>50 mM TSA</td>
<td>(&gt;0)</td>
</tr>
<tr>
<td>NOCs9/NO</td>
<td>(4.0 \times 10^{-3})</td>
<td>37</td>
<td>500 mM GTN</td>
<td>1 mM TSA</td>
<td>37</td>
</tr>
<tr>
<td>nPrONO (^{c})</td>
<td>(1.3 \times 10^{-3})</td>
<td>37</td>
<td>500 mM GTN</td>
<td>–</td>
<td>BP, 37 °C</td>
</tr>
<tr>
<td>nPrONO (^{d})</td>
<td>(1.2 \times 10^{-4})</td>
<td>37</td>
<td>250 mM GTN</td>
<td>500 mM TSA</td>
<td>61</td>
</tr>
<tr>
<td>nPrONO (^{d})</td>
<td>(1.4 \times 10^{-4})</td>
<td>37</td>
<td>250 mM GTN</td>
<td>–</td>
<td>BP, 37 °C</td>
</tr>
<tr>
<td>Cl(CH(_2)CH(_2)ONO) (^{c})</td>
<td>(2.2 \times 10^{-3})</td>
<td>37</td>
<td>1 mM GTN</td>
<td>1 mM Cys</td>
<td>(&gt;0)</td>
</tr>
<tr>
<td>tBuONO (^{c})</td>
<td>(8.0 \times 10^{-5})</td>
<td>37</td>
<td>250 mM GT027</td>
<td>500 mM TSA</td>
<td>51</td>
</tr>
<tr>
<td>tBuSNONO (^{c})</td>
<td>(8.0 \times 10^{-5})</td>
<td>37</td>
<td>250 mM C1DN</td>
<td>500 mM TSA</td>
<td>61</td>
</tr>
<tr>
<td>SNAP (^{e})</td>
<td>(4.0 \times 10^{-5})</td>
<td>37</td>
<td>250 mM GT002</td>
<td>1 mM TSA</td>
<td>3.1</td>
</tr>
<tr>
<td>tBuSNONO (^{b})</td>
<td>(6.2 \times 10^{-5})</td>
<td>21</td>
<td>250 mM GT002</td>
<td>1 mM TSA</td>
<td>3.1</td>
</tr>
</tbody>
</table>

\(^{a}\) Determined from plots of observed rate vs concentration for at least three nitrovasodilator concentrations in triplicate. \(^{b}\) Phosphate buffer at pH 7.4, 37 °C. \(^{c}\) Contains t-BuSH, and t-Bu-SS-t-Bu. \(^{d}\) No NO was observed in HP from GT016 and TSA, GT017 and TSA, GTN and DTT, and GTN and GSH. \(^{e}\) Calibration by DEA/NO (\(k_{NO} = 2.2 \times 10^{-3}\)) in 100 mM phosphate buffer at pH 7.86 and 37 °C. \(^{f}\) Phosphate buffer (100 mM) at pH 7.4, HP (human plasma), or BP (bovine plasma). \(^{g}\) In 1:1 CH\(_2\)Cl\(_2\)/aqueous phosphate (pH 7.6 and 50 mM). \(^{h}\) With or without SOD. \(^{i}\) Below detection limits.

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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.


(23) Kelm, M., Feilisch, M., Krebber, T., Deussen, A., Motz, W., and Strauer, B. E. (1995) Role of nitric oxide in the regulation of coronary vascular tone in hearts from hypertensive rats. Main-This study was supported by Grant NM-1603-2000 from the National Science Foundation. The authors thank Dr. E. J. Feilisch for his assistance in the preparation of this manuscript.


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