In Vitro Activation of Soluble Guanylyl Cyclase and Nitric Oxide Release: A Comparison of NO Donors and NO Mimetics†

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ABSTRACT: Nitric oxide (NO) performs a central role in biological systems, binding to the heme site of soluble guanylyl cyclase (sGC), leading to enzyme activation and elevation of intracellular levels of cGMP. Organic nitrates, in particular, nitroglycerin (GTN), are clinically important nitrovasodilators that function as NO-mimetics in biological systems. Comparison of sGC activation data with electrochemically measured rates of NO release for genuine NO donors, NONOates and nitrosothiols, yields an excellent correlation between the EC_{50} for sGC activation and the rate constant for NO release, k_{NO}. However, activation of sGC by GTN and the nitrates has very different characteristics, including the requirement for specific added thiols, for example, cysteine. The reaction of GTN with cysteine in anaerobic solution yields NO slowly, and NO release, measured by chemiluminescence detection, is quenched by added metal ion chelator. The generation of NO under aerobic conditions is 100-fold slower than the anaerobic reaction. Furthermore, NO release from the reaction of GTN with cysteine in phosphate buffer is too slow to account for sGC activation by GTN/cysteine. The slow rate of the chemical reaction to release NO suggests that nitrates can activate sGC by an NO-independent mechanism. In contrast to the genuine NO donors, GTN behaves as a partial agonist with respect to sGC activation, but in the presence of the allosteric sGC activator, YC-1, GTN exhibits full agonist activity.

The intensity of research into the pharmacology, biology, and toxicology of nitric oxide (NO)[1] has continued unabated since the discovery of the significant biological roles of endogenous NO (*1*−*5*). A key to the understanding of NO biology is the enzyme soluble guanylyl cyclase (sGC), which converts GTP to the secondary messenger molecule, cGMP (*6*, *7*). The Fe^{II}-heme site of sGC is a high affinity NO receptor, and formation of sGC-NO by binding of one or two molecules of NO to this site induces conformational changes that result in a several 100-fold increase in enzyme activity (*8*, *9*). Although carbon monoxide also activates sGC, the different structure at the heme of sGC-CO results in a much smaller increase in enzyme activity. The NO-independent sGC activator, 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1), is thought to bind to an allosteric site on sGC, possibly in the region of Cys238 and Cys243 on the α_1 subunit (*10*). The combination of YC-1 allosteric binding and CO binding to the heme site conformationally triggers an increase in enzyme activity approaching that for sGC-NO, although the potency of CO remains orders of magnitude below that of NO.

Physiologically, NO is produced from L-arginine by the various isoforms of nitric oxide synthase (*11*). In addition to endogenous sources of NO, various exogenous NO donors have been discovered, including several classes of nitrovasodilators. Of these, the organic nitrates hold special significance, as important therapeutic agents in the treatment of angina pectoris and congestive heart failure and as potential drugs in other disease states including pain, inflammation and neurodegeneration (*12*−*14*). The prototypical nitrate, nitroglycerin (GTN), has been employed for over a century in treatment of angina pectoris, its utility resulting, in large part, from significant differences in hemodynamic properties from other nitrovasodilators (*15*). Organic nitrates ($RONO_2$), organic nitrites (RONO), nitrosothiols (RSNO), diazeniumdiolates [NONOates; $RR'N(NO)_2-$], sodium nitroprusside (SNP), and molsidomine (SIN-1) represent distinct chemical families of nitrovasodilators. NONOates, nitrosothiols, SNP, and SIN-1 are genuine NO donors, that is, they undergo spontaneous (or light induced) reaction at physiological pH in simple aqueous buffered media to release NO. In contrast, organic nitrates have been proposed to require enzymic biotransformation, in vivo, to liberate NO (*5*, *16*), with nitrites, nitrosothiols and organic thionitrates ($RSNO_2$) variously proposed as intermediates in these biotransformation reactions (*17*−*19*). It is important

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[1] Abbreviations: GTN, glyceryl trinitrate; SNOG, *S*-nitrosoglutathione; sGC, soluble guanylyl cyclase; DTT, dithiothreitol; DEA/NO, $(CH_3CH_2)_2N(NONO)Na$; SNAP, *S*-nitroso-*N*-acetylpenicillamine; FeTPP, Fe-tetraphenylporphyrin-bis(*N*-methylimidazole); ISDN, isosorbide dinitrate; NOC9/NO, $CH_3NH_2(CH_2)_6$-N(NONO)-CH_3; Sper/NO, $H_2N(CH_2)_3NH(CH_2)_4$-N(NONO)-$(CH_2)_3NH_3$; DETA/NO, $H_2N(CH_2)_2$-N(NONO)-$(CH_2)_2NH_3$; GDN, glyceryl dinitrate; GMN, glyceryl mononitrate; DFP, 1,2-dinitrooxy-3-fluoropropane; YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole, SNP, sodium nitroprusside; DTPA, diethylenetriaminepentaacetic acid; ODQ, 1H-(1,2,4)oxadiazole(4,3-a)quinoxaline-1-one).

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to note that GTN and the nitrates differ from all other nitrovasodilators in that (a) in vitro, they will not activate sGC without thiol adjuvants such as cysteine (*20*, *21*), and (b) evidence for simple chemical reaction to give NO (that is, a reaction in a simple medium, not containing proteins or other complex biomolecules) is either lacking or problematic (*12*, *22*).

In the 25 years of research on sGC, encompassing Murad's early work to his group's recent report on recombinant human sGC, a large number of papers have appeared on in vitro activation of crude preparations of sGC by nitrates, in particular GTN (*16*, *20*, *21*, *23*−*31*). Although, when purified, activity per milligram of protein is obviously considerably greater, there is good compatibility between activation and inhibition data from work on crude preparations and on pure, recombinant enzyme, demonstrating the general reliability of data obtained with these preparations of sGC (*9*, *30*, *32*, *33*). However, none of these studies asked or have provided an unequivocal answer to the question of whether the in vitro activation of sGC by GTN and cysteine is mediated via a chemical reaction releasing NO. The early work of Feelisch and Noack is still cited as evidence that the chemical reaction of GTN with cysteine in phosphate buffer generates NO (*31*). This study had used oxyHb oxidation as an assay for NO release. We have subsequently confirmed that the interaction of GTN with thiols, including cysteine, does lead to oxidation of Fe^{II}-oxyHb to Fe^{III}-methemoglobin (metHb), but the rate of oxidation is too high to be accounted for by NO release from the reaction of GTN with cysteine (*34*). Clearly, a definitive answer is needed to the question of whether reaction mixtures of GTN and cysteine in simple aqueous media release NO.

In this paper, we set out to determine the mechanism of activation of sGC by GTN, by measuring rates of NO release and sGC activity, in comparison with genuine NO donors and the NO-independent activator YC-1. We correlate rate constants for NO release, with concentration-activity curves for aortic sGC activation, for six genuine NO donors (NONOates and nitrosothiols). An excellent correlation exists between k_{NO} and EC_{50} for these NO donors. In contrast, although GTN does react with cysteine in simple aqueous buffer to release NO, this slow rate of NO release cannot account for the potency of GTN as an activator of sGC. Maximal enzyme activity for nitrates is 4−5-fold lower than for NO donors (i.e., they behave as partial agonists), and in further contrast to NO donors, the addition of YC-1 increases maximal activation 5-fold, to that seen with genuine NO donors. These data suggest two alternative mechanisms of interaction of nitrates with sGC: (1) nitrates react with an sGC cysteine residue to yield NO, leading to activated sGC-NO, or (2) nitrates activate sGC by an NO-independent mechanism, that triggers conformational change to an activated sGC*. However, in both cases, a corollary invoking sGC inhibition or inactivation by nitrates must be added in order to account for the observed partial agonist activity of nitrates.

MATERIALS AND METHODS

Materials. Nitrosothiols, GTN and YC-1 were synthesized as described in the literature (*35*−*37*). NONOate salts were obtained from RBI (Natick, MA) or Calbiochem (La Jolla, CA). All other chemicals were obtained from Sigma-Aldrich Chemicals (Milwaukee, WI) or BDH (Toronto, ON). 2,3-Dinitrooxy-1-fluoropropane (DFP) was synthesized by standard synthetic procedures from epifluorohydrin, via acid-catalyzed hydrolysis, and nitration of the resulting diol in HNO_3/H_2SO_4(aq)/CH_2Cl_2, yielding the product as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 5.34−5.57 (1H, dm, J_{HF} = 20.6 Hz), 4.53−4.87 (4H, superposition of multiplets, $O_2NO-CH_2 + CH_2F$, J_{HF} = 46.7 Hz; J_{HF} = 0.66 Hz); ^{13}C NMR ($CDCl_3$) δ 79.47 (d, J_{CF} = 177 Hz), 76.73 (d, J_{CF} = 20.6 Hz), 67.84 (d, J_{CF} = 6.87 Hz).

Enzyme Activation. Activation of soluble sGC by nitrates, nitrosothiols and NONOates was assayed employing enzyme freshly prepared from the 105000*g* supernatant fraction of rat aorta homogenates, as described. (*23*, *24*). Aortic homogenate supernatant fractions contained Tris (50 mM, pH 7.6), DTT (0.5 mM), EDTA (1 mM), and sucrose (0.25 M). Enzyme assay solutions contained Tris (50 mM, pH 7.6), *i*-butylmethylxanthine (0.5 mM), creatine phosphate (3.5 mM), creatine phosphokinase (1 units), GTP (1 mM), $MgCl_2$ (4 mM), aortic supernatant (5−8 μg of protein) and other reagents as indicated and were incubated at 37 °C for 10 min. Aortae from four to eight rats were used as the source of sGC: each activator was assayed in duplicate, at each concentration, in each aortic preparation. To assess the inhibitory effect of GTN on sGC activation by DEA/NO, enzyme assay solutions were incubated with GTN (0.1−1 mM) for 10 min at 37 °C prior to the addition of DEA/NO (10 nM to 1 mM) for a further 10 min. All reactions were terminated by dilution and heating at 90 °C for 3 min. Aliquots were assayed for cGMP by radioimmunoassay and aortic supernatant protein was determined by the Bradford protein assay using bovine serum albumin as standard. Data are presented as means ± sem or sd, as indicated. EC_{50} values were determined from nonlinear fitting of activation data by a sigmoidal dose−response curve with variable slope using GraphPad Prism 2.01/3.0.

Rate Measurements. Spectrophotometric kinetic analysis was performed on a Beckman DU 7400 or Hewlett-Packard 8452A. HPLC analysis of GTN and reaction products was performed on a Shimadzu LC-10A, using detection at 215 nm (correcting for relative extinction coefficients of GTN and GDN), with separation achieved on an analytical RP C-18 column with MeOH/water as eluant. For the NONOates and nitrosothiols, NO was detected using a Clark-type NO selective electrode (ISO-NO, World Precision Instruments Inc., Sarasota, FL), using the methodology previously reported (*34*). For GTN, NO was detected using a chemiluminescence detector (Sievers Research Inc., Boulder, CO, model 207B), using the general method of Brien et al. (*38*). Much greater sensitivity is achieved through headspace gas sampling. It is important with this method that separate reactions be sampled at each time point, because sampling of the same reaction at multiple time points perturbs the system by removal of NO from the system. To reliably measure the concentration of NO generated in solution by this method, known quantities of purified NO gas (Scott Air Products, Plumsteadville, PA) were injected directly into the reaction solution, with stirring of the solution, and the headspace was sampled. For rigor, a calibration curve must be obtained for each set of reaction conditions, i.e., varied temperature, solvent, and vessel volume, since partitioning

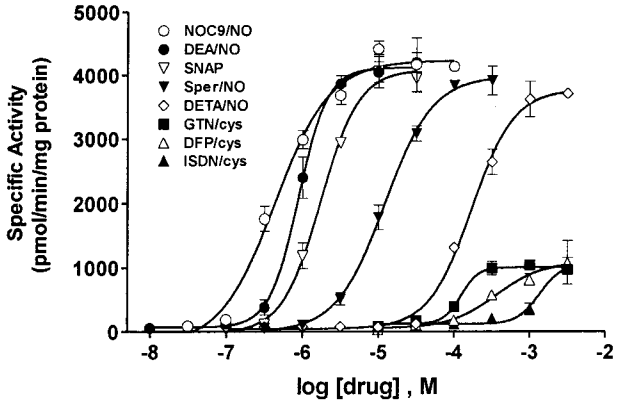


FIGURE 1: Activation of sGC as a function of concentration of added NO donor: GTN + Cys (2 mM) (■), NOC9/NO (○), DEA/NO (●), DETA/NO (◇), Sper/NO (▼), DFP + Cys (2 mM) (△), SNAP (▽), ISDN + Cys (2 mM) (▲) (data for SNOG are left out for clarity), after 10 min of incubation at 37 °C. Data points shown are mean ± sem.

Table 1: Values for the Rate Constant of NO Release (37 °C, pH 7.4, 100 mM Phosphate Buffer) versus EC_{50} for Guanylyl Cyclase Activation Plot for NONOates and Nitrosothiols[a]

	k_{NO}, 37 °C (s^{-1})	EC_{50} (M)
NOC9/NO	1.37×10^{-2}	4.71×10^{-7}
DEA/NO	8.10×10^{-3}	8.81×10^{-7}
Sper/NO	5.62×10^{-4}	1.21×10^{-5}
DETA/NO	1.92×10^{-5}	1.59×10^{-4}
SNAP	3.01×10^{-3} [a]	1.73×10^{-6}
SNOG	3.85×10^{-3} [a]	1.50×10^{-6}

[a] Contains 1.0 mM CuCl.

between gas and solution phases is determined by these factors. Reaction vessels for all measurements, spectroscopic, HPLC, and NO-detection, were water-jacketed or pelletier-controlled to maintain temperature. SNAP and SNOG (in DMSO or CH_3CN) and nitrates (in DMSO, CH_3CN, or EtOH) were added in organic solvent to aqueous phosphate buffer (100 mM) at pH 7.4, to give final concentrations of <5% organic component. The NONOates were added as freshly prepared stock solutions in 10 mM NaOH to phosphate buffered solutions (*39*).

RESULTS

Activation of sGC was assayed in the presence of NONOates (DEA/NO, Sper/NO, DETA/NO, NOC9/NO) and nitrosothiols (SNAP, and SNOG), employing a crude enzyme preparation from rat aorta, under aerobic conditions. Specific activity was measured as picomoles of cGMP produced per minute per milligram of total protein for a 10 min incubation, and curves were fitted to a binding hyperbola to obtain EC_{50} values (Figure 1, Table 1). Activity was measured, in a similar fashion, for nitrates in the presence of cysteine (2 mM). An increase in activity of up to approximately 20% only is observed at higher cysteine concentrations (data not shown). Basal activity in all assays ranged from 50 to 80 pmol/min/mg of protein. No activity above basal levels was seen for nitrates in the absence of cysteine nor in the presence of DTT (2 mM), in the concentration range studied (data not shown). Activation of sGC over basal levels was 50−100-fold for the NO donors: NONOates and nitrosothiols. Activation by the nitrates in the presence of cysteine was only 10−20-fold over basal. Thus, the nitrates behave as partial agonists with respect to sGC activation, whereas the NONOates and nitrosothiols act as full agonists.

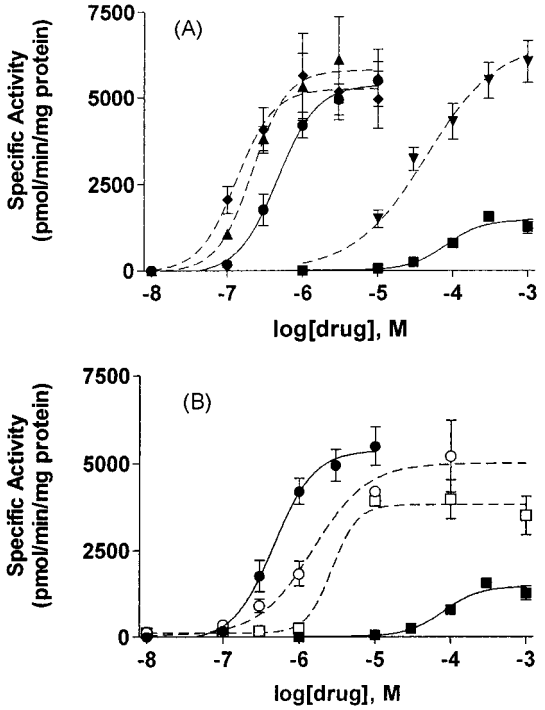


FIGURE 2: (A) Activation of sGC as a function of concentration of (1) DEA/NO and (2) GTN (+Cys), showing potentiation by YC-1: solid lines without adjuvants; dashed lines with added YC-1. (1) DEA/NO [without (●) and with YC-1 (10 μM (▲), 100 μM (♦))]. (2) GTN + Cys (2 mM) [without (■) and with YC-1 (100 μM (▼)). Values for enzyme activity in the presence of YC-1 alone have been subtracted in order to show only the potentiation by YC-1 of NO-dependent and GTN-dependent activation of sGC. (B) Activation of sGC as a function of concentration of (1) DEA/NO and (2) GTN (+Cys), showing inhibition by GTN: solid lines without adjuvants; dashed lines with added GTN. (1) DEA/NO (without (●) and with GTN [100 μM (○) and 1 mM (□)]. (2) GTN + Cys (2 mM) (■). Data points shown are mean ± sem.

In further experiments, using separate preparations of sGC from rat aorta, activation of sGC by GTN (in the presence of cysteine) was compared to activation by DEA/NO, in the absence and presence of YC-1. YC-1 is independently a modest activator of sGC; therefore, sGC activity in the presence of YC-1 (10, 100 μM) was subtracted from the activity data obtained in the presence of YC-1 (10, 100 μM) and either GTN (+cysteine) or DEA/NO, to represent solely the combined or cooperative actions of YC-1:GTN/Cys or YC-1:DEA/NO. For both DEA/NO and GTN, YC-1 caused an approximate 3-fold leftward shift in the concentration-activity curve for sGC (Figure 2A). YC-1 had no effect on the maximal activity of sGC-NO. In marked contrast however, YC-1 caused an approximate 4−5-fold increase in the maximal activation of sGC by GTN, such that it conferred full agonist activity to this compound.

A third series of experiments examined the influence of GTN on the activation of sGC by DEA/NO, under conditions in which GTN itself had no effect on enzyme activity (i.e., in the absence of cysteine). Enzyme activation by DEA/NO was inhibited in a concentration dependent manner, with GTN (1 mM) causing a 10-fold increase in the EC_{50} for sGC activation (Figure 2B). The inhibitory effect of GTN was

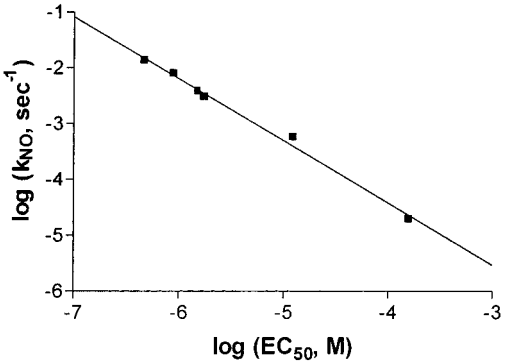


FIGURE 3: Correlation of rate constants for NO release determined electrochemically or spectrophotometrically for NO donors (4 NONOates, 2 nitrosothiols) (37 °C, pH 7.4, 100 mM phosphate buffer) versus EC_{50} values for sGC activation.

most dramatic at 1 μM DEA/NO, which in the absence of GTN resulted in near maximal activation of sGC, but in its presence was almost completely inhibited.

Rate constants for NO release from NONOates and nitrosothiols were determined both by spectrophotometric monitoring of aqueous degradation and by measurement of NO release rates in aqueous phosphate buffer (100 mM) at pH 7.4, using the electrochemical method described previously (*34*). Nitrosothiols will activate sGC without adjuvants. However, since it is known that the primary mechanism of NO release from nitrosothiols is a reaction initiated and catalyzed by Cu^{I} (provided by either added Cu^{I}/Cu^{II} or trace Cu^{II} reduced by added thiol), cysteine or a Cu^{I} salt should be added to ensure consistency between preparations (*40*). Reactions were performed under aerobic conditions in unsealed reaction vessels, to closely mimic the conditions of the sGC incubations. No release of NO from GTN was observed over a wide range of GTN and thiol concentrations using the electrochemical method (*34*). However, the detection threshold of this method would not preclude that GTN reacted to release NO at a subnanomolar per second rate. Rate constants obtained for NO release from the NONOates and nitrosothiols were plotted against EC_{50} values for sGC activation by these NO donors, yielding an excellent linear correlation for the log−log plot (Figure 3). Given this excellent relationship between rate and activation, the data was further transposed, calculating the total concentration of NO generated in the 10 min. incubation period ($[NO]_{tot}$), for each NO donor at each concentration, thus yielding an apparent EC_{50} of 1.6 μM for NO itself (Figure 4). However, since these are unsealed, aerobic systems, actual NO concentration will be lower than $[NO]_{tot}$, because of NO effusion.

Chemiluminescence detection was used to measure NO in the headspace gas from reaction solutions of GTN and cysteine in phosphate buffer. The detection threshold for this method is reported as 8 pmol of NO, which, using a 2 mL reaction volume, corresponds to a NO concentration of 4 nM (*38*). Using an anaerobic sealed system, deoxygenated by bubbling a stream of N_2 gas through an O_2 scavenger cartridge and through the reaction solutions, NO loss from the headspace via reaction or leakage at low concentrations of NO is minimal, thus rates of NO release of subpicomolar per second (1×10^{-9} mM s^{-1}) are reliably measured. The method can be used to measure solution NO concentrations

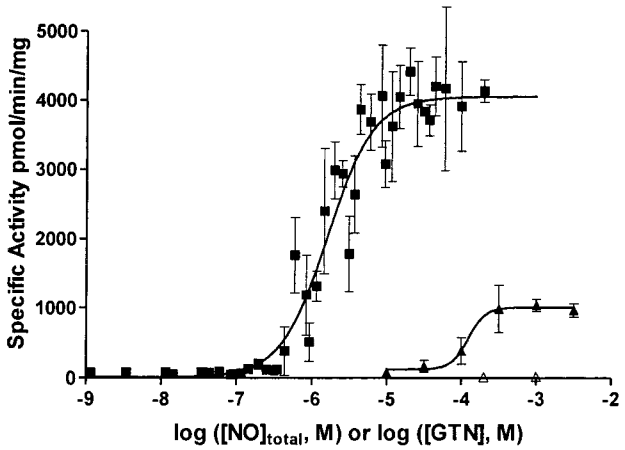


FIGURE 4: Plot of (1) specific activity of sGC after 10 min incubation with NO donors versus total theoretical NO concentration generated by NO donors after 10 min incubation [(■) using data shown in Figure 1 and rate constants described in Table 1]; (2) specific activity of sGC: (▲) after incubation with GTN + cysteine (2 mM); (△) calculated concentration-activity data for GTN acting as an NO donor, based upon [NO] measured by chemiluminescence under aerobic conditions from GTN + cysteine (2 mM). Data points shown are mean ± sd.

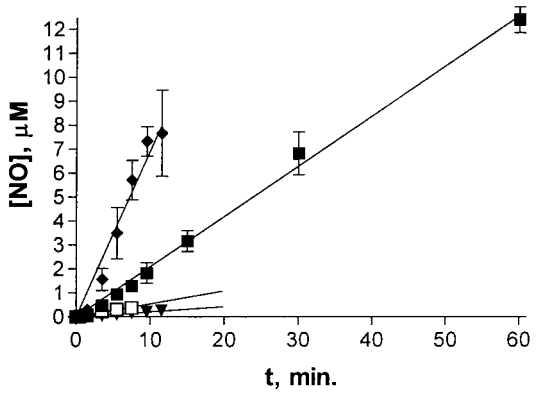


FIGURE 5: Plot of time-course for NO release measured by chemiluminescence detection: GTN [2 mM (♦)], GTN [1 mM (■)], GTN [0.2 mM (▼)], GTN (1 mM) + DTPA (1 mM) (□), all in the presence of cysteine (2 mM), at 37 °C, anaerobically in N_2-degassed phosphate buffer (100 mM, pH 7.4).

by direct injection of known quantities of gaseous NO into the injector and application of gas/liquid partition coefficients. However, we chose to rigorously calibrate by direct injection of known quantities of NO gas into the phosphate buffer reaction medium and sampling of the headspace. NO release was indeed detected from GTN in phosphate buffer, after addition of cysteine, and in reasonable agreement with one published observation using the same chemiluminescence method (*38*). The rate of release of NO in solutions of GTN and cysteine was independent of the source of GTN, which included a commercial, pharmaceutical preparation, Tridil, synthesized GTN purified by flash silica gel chromatography, and GTN further purified by reversed-phase HPLC. Reaction rates were dependent upon substrate concentration, and rate was approximately linear with time over an extended period (Figure 5).

The rate of NO release from 1 mM GTN in the presence of 2 mM cysteine, under anaerobic conditions, at 37 °C in 100 mM phosphate buffer at pH 7.4, was approximately 3 nM s^{-1}. The yield of NO (2 μM; 0.2% yield) in the initial

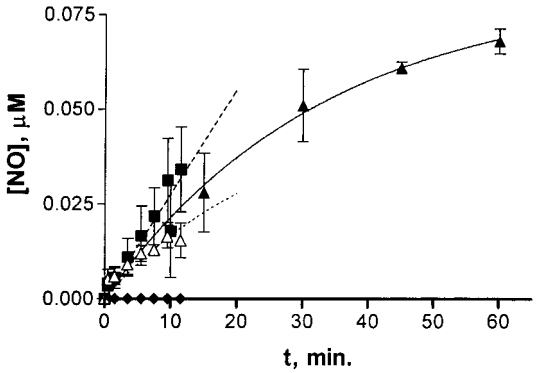


FIGURE 6: Plot of time-course for NO release measured by chemiluminescence detection: GTN (0.2 mM) + DTPA (1 mM) (♦), GTN (1 mM) in aerated solution [(■) dashed line], KNO_2 [100 μM (▲), solid line], KNO_2 (100 μM) + DTPA (1 mM) [(△) dotted line), all in the presence of cysteine (2 mM), at 37 °C, in phosphate buffer (100 mM, pH 7.4), under anaerobic conditions, except where otherwise stated.

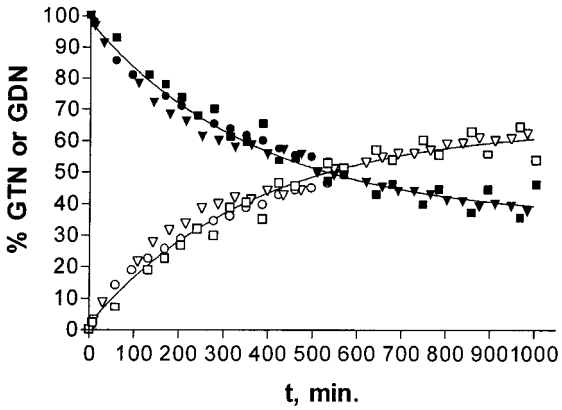


FIGURE 7: Time course for reaction of GTN (1 mM) with cysteine (2 mM) (37 °C, pH 7.4, 100 mM phosphate buffer) determined by UV−HPLC quantitation of relative concentrations of GTN reactant and GDN products: (▼) GTN and (▽) GDN in air-equilibrated solutions; (●) GTN and (○) GDN with DTPA (1 mM) added; and (■) GTN and (□) GDN in Ar-flushed anaerobic solutions. Each data point shown is the average of triplicate or duplicate experiments.

10 min reaction period compares with 2−3% overall reaction observed by UV−HPLC analysis. Thus, comparison with the time course for the overall reaction of GTN with cysteine under these conditions reveals that approximately 10% of the reaction flux proceeds to give NO as product (Figure 7). In the presence of the metal ion chelator, DTPA (1 mM), the reaction evolving NO was quenched (Figure 6), although the Cu^I-specific chelator neocuproine (1 mM) had no significant effect on NO release (data not shown). Since inorganic nitrite is a primary reaction product of nitrate ester hydrolysis, KNO_2 was also studied with cysteine under anaerobic conditions. NO release from NO_2^- required added cysteine, was only slightly retarded by added DTPA, and was at a rate substantially below that from GTN+Cys (Figures 5 and 6). The sGC assays reported herein are performed under aerobic conditions; therefore, NO release was measured in air equilibrated solutions. The rate of NO release from the reaction of GTN (1 mM) with cysteine (2 mM) was reduced 100-fold under aerobic conditions (Figure 6). The yield of NO from 0.2 and 1 mM GTN, in aerobic reaction with cysteine (2 mM) after 10 min was very low, approximately 10 and 25 nM, respectively.

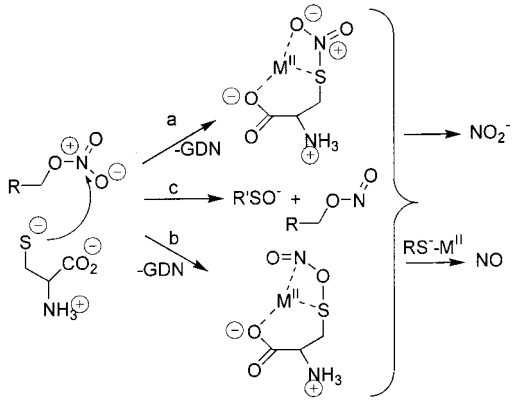
DISCUSSION

Nitrates, NO Donors, and NO Detection. Quantitative correlation of the effects, resulting from NO generated by genuine NO donors, to NO concentration is complicated because reaction rates and reaction products are strongly influenced by the reaction medium. For example, the mechanism of nitrosothiol degradation is complex, dependent on Cu^I, fortuitous metal chelators, varies with thiol concentration, and can lead to N-containing products other than NO (*40*−*42*) Furthermore, many NONOates undergo hydrolytic degradation to release far less than the theoretical yield of 2 M equiv of NO. Indeed, DEA/NO, the most widely used NONOate, is reported to yield only 1.5 equiv of NO (*39*). Since it is unreasonable to assume that reaction rate and product distribution will be independent of the reaction medium and environment, measurement of NO release becomes crucial. We have shown that electrochemical detection of NO, using a Clark-type electrode, is ideally suited to measurements of NO release rates in aerobic solution, although the method is limited by its detection threshold (*34*). In addition, we have demonstrated that the oxyhemoglobin (oxyHb) assay is not reliable and specific for NO detection (*34*). The advantage of the chemiluminescence detection method lies in its high sensitivity, especially when the reaction headspace gas is assayed.

GTN and other nitrates are routinely classified as NO donors, and while the evidence is unambiguous that GTN degradation leads to NO release in biological media, the support for a simple chemical reaction of GTN, releasing NO, is uncertain (*12*). NO release from the reaction of GTN with cysteine is undetectable, electrochemically, even at high substrate concentration (*34*). Reports of NO release from the chemical reaction of GTN with cysteine using chemiluminescence detection have appeared. In a rigorous analysis by Fung and co-workers of NO release from GTN and thiols in plasma, it was reported that NO was not detectable from the reaction of GTN with cysteine in phosphate buffer, but that low concentrations of NO were detectable after addition of superoxide dismutase (SOD) (*22*). Recently, SOD has been shown to participate in reactions other than removal of superoxide, for example, reacting with nitrosothiols to generate NO (*43*). In another study, Brien et al. measured NO concentration at 5 min from the anaerobic reaction of GTN (0.2 mM) with cysteine (5 mM) in phosphate buffer and did observe NO (*38*).

In contrast to many NO donors, nitrates are relatively stable toward reaction with nucleophiles in aqueous solution. Intermolecular reactions with thiols are slow, often with half-lives of several hours: the reaction between GTN (1 mM) and cysteine (2 mM), for example, having progressed only 2−3% after 10 min (Figure 7). However, we observed that a small portion of the reaction flux (10%) does lead to NO as product (Figure 5), although, under aerobic conditions, the detected yield of NO is considerably reduced (Figure 6). It is now accepted that trace metal catalysis by Cu^I ions is responsible for the "spontaneous" liberation of NO from nitrosothiols, since the Cu^I chelator, neocuproine, quenches this reaction (*40, 44*). Neocuproine (up to 1 mM) had no inhibitory effect on liberation of NO from the reaction between cysteine and GTN (which also strongly argues against *S*-nitrosocysteine as a reaction intermediate). How-

Scheme 1: Reaction of Thiolate with Nitrate May Yield a Number of Possible Intermediates, (a) Thionitrate, (b) Sulfenyl Nitrite, (c) Nitrite, That May Partition toward NO or Nitrite Ion as Products



ever, DTPA, which has selectivity for iron, effectively quenches NO release from this reaction (Figures 5 and 6), suggesting a role for trace metal ion catalysis in cysteine-mediated NO formation from GTN. It is noteworthy that we have previously reported reduction of GTN to NO by an Fe^{II}-porphyrin complex (Fe-TPP) (*34*).

Reaction of a nitrosothiol to generate thiol and NO is a $1e^-/1H^+$ reduction, which with metal-ion catalysis yields disulfide and 2 equiv of NO, whereas reduction of a nitrate to NO is formally a $3e^-/3H^+$ reduction. A balanced equation may be drawn to include metal ion catalysis:

$$6R'SH + 6M^{n+} \rightleftharpoons 3R'SSR' + 6M^{(n-1)+} + 6H^+$$

$$6RONO_2 + 6M^{(n-1)+} + 12H^+ \rightleftharpoons 6ROH + 6NO + 6H_2O + 6M^{n+}$$

$$6RONO_2\ (GTN) + 6R'SH + 6H^+ \rightleftharpoons 6ROH\ (GDN) + 3R'SSR' + 6NO + 6H_2O$$

Since the rate of NO release from GTN is significantly affected by addition of DTPA, but the overall rate of degradation of GTN to GDN is unaffected (Figure 7), the simplest explanation of the data would have partitioning of a common reaction intermediate, yielding, on one hand, NO via a metal ion catalyzed reduction and, on the other, NO_2^- via hydrolysis (Scheme 1). The exact chemical nature of the reaction intermediate is a focus of our continuing research. We have previously reported experimental and computational studies identifying as possible intermediates, sulfenyl nitrite, thionitrate, and nitrite esters (Scheme 1) (*12*, *17*, *18*, *45*). Known products from the reaction of GTN with thiol are the glyceryl dinitrates (GDN), disulfide, and NO_2^- (*12*). The NO reaction product, observed in this study, does not result indirectly from the NO_2^- primary reaction product, since we observe that cysteine does react with NO_2^- to yield NO, but the rate is too low for NO_2^- to be a kinetically competent reaction intermediate (Figures 5 and 6).

sGC Activation by NO Donors. Genuine NO donors, including the nitrosothiols, SNAP and SNOG, and four NONOates, can be seen to activate sGC with varying potency (Figure 1), but with the same maximal enzyme activation of up to 100-fold above basal levels, which is of similar magnitude to values observed for purified enzyme (*9*). That these compounds are activating sGC via release of NO can be seen by the excellent linear correlation between the measured rate constants for NO release and the EC_{50} values for sGC activation (Figure 3). The sGC activation data for the NO donors can be transformed to provide a concentration response curve for activation of sGC by NO itself, yielding an EC_{50} for NO of 1.6 μM. The effect of YC-1 on sGC activation by NO donors, including DEA/NO, has been reported previously (*33*, *46*−*48*). YC-1 is an NO-independent activator of sGC, with modest potency and efficacy in vitro (*47*, *49*), and exerts a small effect on NO-dependent activation of sGC; the concentration−response curves, in Figure 2, show the typical, small increase in potency from the combined effects of YC-1 and an NO donor (*46*)

sGC Activation by Nitrates. Classical nitrates, that is, those not possessing other reactive functional groups, activate sGC above basal levels only in the presence of "active thiols" (*20*, *21*). Active thiols, including cysteine, *N*-acetyl cysteine, and thiosalicylic acid, all contain a β-mercaptocarboxylic acid group, thus sGC is not activated in the presence of GTN + DTT (an important side isssue, since DTT is present in most sGC preparations). From a variety of possible classical nitrates, three were chosen as representative, GTN, 1,2-dinitrooxy-3-fluoropropane (DFP), and isosorbide dinitrate (ISDN). GTN and ISDN are clinically relevant, whereas GTN and DFP are similar, structurally and electronically, both possessing vicinal nitrates and an electron-withdrawing group at the 3-position and which might therefore be expected to interact with sGC by similar mechanisms.

Classical nitrates, in the presence of cysteine (1−10 mM; 2 mM in this study), activate sGC with low potency, but more remarkably, maximal enzyme activation is 4−5-fold less with nitrates than with NO (Figures 1, 2, and 4). EC_{50} values for the NO donor, DETA/NO, and for GTN/Cys are the same, 100−200 μM, yet GTN is unable to fully activate sGC (Figure 1). The effects of YC-1 are also more profound on nitrate activation of sGC than on NO activation (Figure 2A). YC-1 potentiates activation of sGC by GTN/Cys such that activity is observed at the maximal levels seen for NO and NO donors, although this potentiation is achieved with little or no increase in the potency of GTN. Thus, YC-1 potentiates the activation of sGC by GTN/Cys 5-fold. This large value is similar to that reported for potentiation of the activity of sGC-CO (3−5-fold), but not that for potentiation of sGC-NO (1.0−1.2-fold) (*30*, *46*, *48*). It is interesting to note that the high activity of sGC-CO, in the presence of YC-1, has been argued to support a physiological role for CO in cGMP signaling, presumably for sGC-CO in the presence of an endogenous YC-1-like potentiator (*49*). Since the activity of sGC-CO is entirely matched by that of sGC/GTN/Cys, in the presence of YC-1, an endogenous sGC potentiator, should it exist, may have a role in sGC activation by GTN in vivo.

sGC Inhibition by Nitrates. The potential inhibitory properties of GTN toward sGC-NO were studied by incubation of sGC with the NO donor, DEA/NO, after preincubation with GTN alone (Figure 2B). At 100 μM GTN, a rightward shift in the concentration response curve for sGC activation by DEA/NO was observed. Furthermore, at the higher concentration of 1 mM GTN, inhibition of the maximal

activity of sGC-NO was also observed. The rightward shift in the concentration−activity curve for DEA/NO in the presence of GTN, with modest decrease in maximal activation (Figure 2B), does suggest a reversible, competitive antagonist action for GTN, but these data cannot rule between (i) nitrate binding to and deactivation of sGC-NO or (ii) inhibition of NO binding to sGC.

There is substantial support for an oxidative mechanism for nitrate inhibition of sGC activation. Oxidative inhibition of sGC by 1H-(1,2,4)oxadiazole(4,3-a)quinoxaline-1-one (ODQ), the best studied inhibitor of sGC, results from binding of ODQ at the ferrous-heme and oxidation of the Fe^{II}-center (*50*, *51*). Reactions of N,O compounds, nitrites and nitrosothiols, with the reactive Fe^{II}/Fe^{III} sites in hemoglobin are documented (*52*, *53*). GTN itself reacts very rapidly with deoxyHb leading to oxidized metHb, GDN, and NO_2^- (*54*). In addition, nitrates, including GTN will oxidize Fe^{II}-oxyHb to Fe^{III}-metHb in the presence of cysteine, at a rate much greater than can be accounted for by any chemical reaction between the nitrate and cysteine yielding NO (*34*). Oxidation of a cysteine residue of sGC by GTN can be proposed since modification of cysteine residues of the β_1 subunit has been shown to inhibit NO-dependent activation of sGC (*55*). However, we disfavor this mechanism, since nitrosothiols, which would be expected to show even greater oxidative reactivity toward cysteine, act as full agonists toward sGC (Figure 1).

NO-Dependent Activation of sGC by Nitrates. Interpolation of the plot obtained for NO concentration versus sGC activity for genuine NO donors (Figure 4), requires micromolar NO concentrations to be generated within the 10 min incubation, to attain the maximal activity seen for GTN/Cys. This concentration is far greater than that observed from the reaction of GTN with cysteine under aerobic conditions (Figures 5 and 6), and to emphasize this point, the calculated enzyme specific activity resulting from the measured NO release from GTN, acting as an NO donor in reaction with cysteine, is shown in Figure 4 (open triangles). Under aerobic conditions, NO release from GTN was in the low nanomolar range, concentrations that are insufficient to activate sGC above basal levels.

These data show that NO derived from reaction of GTN with cysteine cannot account for the observed GTN-induced increase in sGC activity. A remaining potential NO-dependent mechanism for activation via GTN-derived NO, is an alternative reaction of GTN in the assay medium producing NO. Purified, recombinant sGC and crude enzyme preparations are similar in their responses to GTN, that is, GTN alone does not stimulate sGC above basal levels, but in the presence of cysteine, GTN acts as a partial agonist.[2] This fact negates the possibility that components of the enzyme preparation, other than sGC, accelerate the degradation of GTN to NO, leaving the sole possible source of GTN-derived NO as a reaction between GTN and sGC itself.

There is ample evidence of the reactivity of hemoproteins toward N,O compounds, with reactions occurring at both Fe-heme and cysteine sites (see above), and we have previously postulated similar reactivity toward nitrates (*12*, *52*−*54*). There are several conserved cysteine residues in the α_1-heme domain and in the β_1-subunit of sGC (*55*). The reaction of nitrates with protein-thiol to yield NO is chemically reasonable, since we have recently shown that nonclassical nitrates containing a thiol group generate significant amounts of NO (*56*). If GTN does react with sGC to liberate NO, the question of the absolute requirement for cysteine must be answered. The lack of activation of sGC by GTN alone may be readily explained by a requirement for cysteine to reduce either oxidized protein-thiol or oxidized ferric-heme. Interestingly, nonclassical nitrates containing a disulfide linkage release significant NO only on addition of thiols, including cysteine (*56*). As an alternative, initial reaction of sGC with nitrate may yield an intermediate (i.e., a sulfenyl nitrite, thionitrate, or nitrite ester) which requires subsequent chemical reaction with cysteine to yield NO.

Mechanisms for in Vitro sGC Activation by Nitrates. The foregoing enzyme activity data demonstrate two distinct properties of classical nitrates: (1) in the presence of cysteine, they are partial agonists of sGC, and (2) they inhibit sGC activation by NO. Kinetic data for NO release demonstrate that classical nitrates, in the presence of cysteine, will undergo reaction to NO, but not at a rate sufficient to explain the observed concentration-activity curves for sGC activation. Two discrete mechanisms can be postulated to account for in vitro nitrate activation of sGC: (1) nitrates react with a sGC cysteine residue to yield NO, NO binds to sGC giving activated sGC-NO, or (2) nitrates activate sGC by an NO-independent mechanism, that triggers conformational change to an activated sGC*. Both these mechanisms must carry a corollary to explain the observed partial agonist behavior, which may derive from one of a number of causes: nitrates are antagonists of NO binding; sGC* is inherently 4−5-fold less active than sGC-NO; or, nitrates inhibit sGC-NO or sGC*.

Any mechanism must be compatible with the potentiation of nitrate activity observed for YC-1. *The observation that YC-1 potentiation of sGC/GTN/Cys activity closely resembles that seen for potentiation of sGC-CO activity, but not that seen for sGC-NO, leans toward an NO-independent mechanism for nitrate activation of sGC in vitro.* An NO-independent mechanism may involve interaction of GTN with the heme site or a protein-thiol, for which there is some support (see above). But, binding of GTN at a hydrophobic, allosteric site on sGC, akin to that proposed for binding of YC-1, cannot be discounted, because there is no pertinent published data on protein binding of nitrates. One reasonable NO-independent mechanism that includes oxidative inhibition, chosen because there is some precedent in the literature, would have the nitrate binding at the heme-site of sGC forming a complex which would partition between oxidative reaction, leading to inhibition of NO binding and activation, or in the presence of cysteine, diversion down a path to an activated sGC*. In simile with sGC-CO, sGC* is partially activated, but binding of YC-1 results in full conformational activation. Clearly, further research on both the chemical reactions and the binding interactions of nitrates is required to unequivocally define the most fundamental biochemical mechanism of nitrates, viz., activation of sGC.

Mechanisms of in Vivo sGC Activation by Nitrates. GTN is a clinically important, highly potent vasodilator, effecting tissue relaxation with nanomolar potency. The low potency and partial agonist activity observed for nitrate activation of

[2] Marletta M. A., and J.D.A. (2000) Private communication.

sGC in broken cell preparations contrasts with its potent vasodilator activity in intact tissues. For example, in the isolated rat aorta GTN is 2.5- and 7.5-fold more potent than DEA/NO and SNOG, respectively (*57*). The function of nitrates as NO donors through biotransformation to NO, is likely to be important both in vivo and in intact cellular systems. Cytochromes P-450 and glutathione-S-transferases, have been most often cited as enzymes responsible for biotransformation (*5*), although no evidence exists for reaction of GTN with a purified enzyme system leading to a significant yield of NO. Clearly, the chemical conversion of GTN to NO must be greatly accelerated, or NO-independent activation must be strongly potentiated, to explain the potency of GTN observed in vivo. The observations of metal ion catalysis and the greatly increased rate of liberation of NO under anaerobic conditions may be relevant to biotransformation of GTN in vivo since GTN is well-known to show selectivity for relaxation of the more hypoxic venous over arterial tissue.

SUMMARY

The research presented herein demonstrates unequivocally that the archetypal nitrate, GTN, reacts with cysteine, even in simple aqueous buffer, in a metal ion catalyzed reaction to release NO at a slow rate. The slow rate of NO release is not compatible with the potency of GTN as an activator of sGC, in vitro, by comparison with genuine NO donors, which show an excellent correlation between EC_{50} for sGC activation and rate constants for NO release. Furthermore, the activity of sGC in the presence of GTN/Cys/YC-1, is equivalent to that of sGC-CO in the presence of YC-1 and to that of sGC-NO itself. These and other data, including the antagonist behavior of GTN toward sGC activation by NO, suggest that nitrates can act as both NO-independent activators of sGC and inhibitors of sGC activation. Thus nitrates are NO mimetics that may also function as NO donors.

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