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Nitric Oxide ($NO^{\bullet}$), the Only Nitrogen Monoxide Redox Form Capable of Activating Soluble Guanylyl Cyclase

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ABSTRACT. In the present study, we determined that of the redox forms of nitrogen monoxide, NO^-, $NO^{\bullet}$ and NO^+, only NO· significantly activates soluble guanylyl cyclase (GTP pyrophosphate-lyase cyclizing, EC 4.6.1.2). Neither of the NO^- donors tested, Angeli's salt ($Na_2N_2O_3$) or Piloty's acid ($C_6H_5SO_2NHOH$), caused a change in the guanylyl cyclase activity relative to the basal activity level. Interference by other reaction products was eliminated as a possible explanation for the lack of activation. To the extent that NO^+ could be stabilized in aqueous solution, by dissolution of the nitrosonium salt $NOPF_6$ in dry organic solvent prior to addition to the enzyme in buffer, NO^+ had no effect on the activity of soluble guanylyl cyclase. The counter-ion, PF_6^-, had a minimal effect on the enzyme activity and, therefore was, not responsible for the lack of activation by NO^+. These observations suggest that NO· is the natural activator of soluble guanylyl cyclase and is reasonably identical with endothelium-derived relaxing factor, the physiological regulator of soluble guanylyl cyclase activity. BIOCHEM PHARMACOL 51;12:1593–1600, 1996.

KEY WORDS. soluble guanylyl cyclase; nitric oxide; nitroxyl; nitrosonium; endothelium-derived relaxing factor (EDRF)

sGC‡ (GTP pyrophosphate-lyase cyclizing, EC 4.6.1.2) is the cytosolic enzyme that controls vascular smooth muscle relaxation and platelet aggregation/deaggregation through interaction with biologically produced nitrogen monoxide [1, 2]. This enzyme catalyzes the conversion of GTP to cGMP and pyrophosphate in the presence of excess Mg^{2+} (the physiological cofactor) or Mn^{2+} [2, 3]. sGC is a 150-kDa heterodimer that is composed of subunits of similar but not identical size [4, 5]. It contains one mole of heme (Fe(II)PPIX) per mole of heterodimer [6–8]; the heme moiety is not required for sGC function and is quite labile [3, 6, 9]. The heme-containing sGC is activated by NO· and NO·-donors in a manner dependent on both heme and NO· or NO·-donor concentration [9]. Spectroscopic studies have shown that a five-coordinate nitrosyl-heme is formed in NO·-activated sGC [6, 8, 10, 11]. As there is still some skepticism as to whether the free radical NO· is the biologically relevant signaling species, we thought it valuable to explore whether other nitrogen monoxide species were capable of activating sGC.

The biological production of NO· is not well understood and the mechanism of the NO-synthase reaction and identity of the product are currently the subjects of intensive investigation. Because of the technical difficulties involved in conclusive identification of the radical, NO·, and the mechanistic uncertainties in the odd electron chemistry required for formation of a radical product, there is still skepticism as to whether NO· is the exclusive product of the enzyme NO-synthase. Spin-trapping experiments have demonstrated a 1:1 stoichiometry of NO· and citrulline formation from NO-synthase, suggesting that NO· is in fact the oxygenated nitrogen product released [12]. Recently, however, it was discovered that NO-synthase produces *S*-nitrosylated proteins. Typically, formation of *S*-nitrosothiols results from the reaction of thiols with NO^+; however, it does appear that in certain cases NO· can react directly with thiols [13–15]. The aforementioned spin-trapping experiments cannot distinguish between free NO· and *S*-nitrosothiols; therefore, the possibility remains that NO^+ (or its equivalent) is produced by NO-synthase and subsequently reacts to form *S*-nitrosothiols. It is known that *S*-nitrosothiols, either on proteins or other biologically relevant thiols, activate sGC, and such *S*-nitroso species have been proposed as biological carriers of NO· [13]. As the vast majority of nitrosation reactions proceed via the interme-

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‡ *Abbreviations:* sGC, soluble guanylyl cyclase; NO·, nitric oxide; NO^-, nitroxyl anion; NO^+, nitrosonium cation; TEA, triethanolamine hydrochloride; DTT, D,L-dithiothreitol; SNP, sodium nitroprusside; SNAP, *S*-nitrosyl-*N*-acetylpenicillamine; EDRF, endothelium-derived relaxing factor; PEG, polyethylene glycol; cGMP, guanosine 3′,5′-monophosphate; PEI-F, polyethyleneimine cellulose with fluorescent indicator; HNO, nitrosyl hydride; BZSA, benzenesulfinic acid; Fe(II)PPIX, Fe(II)protoporphyrin IX; and HRsGC, heme-reconstituted sGC.

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diacy of NO^+, this species must be considered as a possible product of the NO-synthase reaction.

Recent studies suggest that HNO or its conjugate base, NO^-, may be a viable product of the oxygenation of arginine. A recent chemical study demonstrated that either NO· or HNO could be released from *N*-hydroxyguanidines, including *N*-hydroxy-L-arginine, depending on the oxidative conditions of the reaction; oxidants that proceed via radical mechanisms form NO·, while those oxidants that proceed via other mechanisms form HNO [16]. Importantly, NO^- has been identified as a product in the hydrogen peroxide-mediated oxidation of *N*-hydroxy-L-arginine by NO-synthase [17]. As *N*-hydroxy-L-arginine is an obligate intermediate in the oxidative conversion of arginine to NO· by NO-synthase [18–20], these observations raise the possibility that HNO or NO^- could be the primary product of this enzyme.

These considerations raise the question of what particular nitrogen monoxide species is actually responsible for the physiological effects medicated via activation of sGC. There are some physical characteristics of the physiological activator of sGC, i.e. EDRF, that do not match those of NO·. The fact that EDRF does not pass through an anion exchange column as does NO· would suggest that EDRF is an anion [21]. Furthermore, when EDRF was reacted with deoxyhemoglobin, the expected ESR spectrum of NO-heme indicative of NO· reaction with ferrous heme was not observed [22]. These facts, together with other recent studies that show that NO^--donors are vasodilators [23, 24], raise the possibility that NO^- is the natural activator of sGC. Stamler and coworkers have also raised questions about the role of NO^+ in vasodilatation and sGC activation [25], although Feelisch and coworkers have reported that NO^+ is not a vasodilator [26]. In the present study, we have addressed this question by studying the activation of sGC by the nitrogen monoxide species, NO^-, NO· and NO^+ (Scheme 1), to determine which of these species is competent to activate the enzyme. We report herein data revealing that only the radical NO· is an activator of sGC.

MATERIALS AND METHODS

Materials

TEA, DTT, SNP ($Na_2Fe(CN)_5NO$), EDTA, Q Sepharose Fast Flow, PEG, benzamidine, and hemin chloride were all purchased from the Sigma Chemical Co., St. Louis, MO. NO· (99+%) was obtained from Liquid Carbonic, Milwaukee, WI, and purified by passage through a solid NaOH column and a 6 M NaOH solution prior to use. The sodium dithionite was from Fluka Chemical, Ronkonkoma, NY, and was of the highest purity available. NaCl and NaOH were purchased from Fisher Scientific, Itasca, IL. $MgCl_2$ and $NaNO_2$ were obtained from the Mallinckrodt Chemical Co., St. Louis, MO. [α-^{32}P]GTP was obtained from NEN-Dupont, Boston, MA. EM Science PEI-F cellulose TLC plates were from VWR Scientific, Chicago, IL, and prepacked Sephadex G25 columns (PD-10) were from Pharmacia, Uppsala, Sweden. BZSA ($C_6H_5SO_3H$) and DMSO were obtained from the Aldrich Chemical Co., Milwaukee, WI. $NaPF_6$ and $NOPF_6$ were from Alfa Aesar, Ward Hill, MA. Piloty's acid ($C_6H_5SO_2NHOH$), Angeli's salt ($Na_2N_2O_3$), and SNAP were gifts from Dr. Jon Fukuto, UCLA School of Medicine.

$$NO^+ \xrightarrow{e^-} NO^\cdot \xrightarrow{e^-} NO^-$$

Scheme 1

Isolation of Partially Purified sGC

sGC was partially purified by modification of the method reported previously [27]. Bovine lung (1 kg) was processed as described through the low speed centrifugation step [27]. Following centrifugation at 13,000 *g*, the supernatant was stirred with 1% PEG for 1 hr, and the 1% PEG-protein solution was centrifuged for 1 hr at 17,000 *g*. The supernatant was brought to 15% PEG and stirred for 1 hr before centrifugation for 2 hr at 17,000 *g*. The pellet was redissolved at room temperature in 1 L of 25 mM TEA buffer (pH 7.8) containing 5 mM DTT and 0.2 mM benzamidine. The redissolved pellet was batch-adsorbed to the Q Sepharose Fast Flow resin. sGC was eluted from the column with a 1.5 L linear salt gradient (0 to 0.7 M NaCl) in 25 mM TEA buffer containing 5 mM DTT. The partially purified sGC was stored at −80°. The biuret method was used for determining protein concentration [28]. The partially purified sGC contained no detectable BSA upon western blotting.

sGC Assay

sGC activity was determined as reported previously [9, 27] by measurement of the formation of [^{32}P]cGMP from [α-^{32}P]GTP separated by anion exchange TLC. Assays were performed in 40 mM TEA, pH 7.4, 10 mM DTT, 1 mM GTP, 3 mM Mg^{2+}, 0.3 mM 1-methyl-3-isobutylxanthine [α-^{32}P]GTP (2.5 pmol, 800 Ci/mol) in a total reaction volume of 0.2 mL and incubated for 10 min at 37°. Enzyme reactions were initiated by the addition of substrate and terminated by the addition of 10 μL of 0.5 M EDTA at 0°. [^{32}P]cGMP was isolated on PEI-cellulose and quantitated by scintillation counting on a Beckman LS-6000 in 10 mL of Biosafe II fluid. Specific activities are reported as nanomoles cGMP formed per minute per milligram protein and are the means of triplicate determinations.

Heme Reconstitution of sGC

Partially purified sGC was reconstituted anaerobically with Fe(II)PPIX, and the unbound heme was removed by gel filtration chromatography as reported previously [9]. Hemin chloride was reduced under an N_2 atmosphere with sodium dithionite, and a molar excess of reduced heme was added

TABLE 1. Effect of NO•-donors on sGC activity

Protein sample	Specific activity (nmol cGMP formed/min/mg protein)
sGC	0.551
sGC + NO•	15.6
sGC + SNP	12.0
sGC + SNAP	18.7
HRsGC	0.277
HRsGC + NO•	35.7
HRsGC + SNP	36.5
HRsGC + SNAP	39.4

Partially purified sGC and HRsGC were assayed after a 5-min preincubation (25°) with 100 μM NO• (concentration determined by dilution of an anaerobically saturated NO• solution, 1.8 mM at 25°, 1 atm NO•, pH 2–13), 100 μM SNP, or 100 μM SNAP in 25 mM TEA buffer (pH 7.8). The reported data are averages of triplicate determinations.

to the protein. The solution was stirred under N_2 for 15 min at room temperature, and then unbound heme was removed by desalting over a PD-10 column.

Effect of NO·-Donors (NO·, SNAP, SNP) and NO⁻-Donors (Angeli's Salt, Piloty's Acid) on sGC Function

Aliquots of partially purified sGC and HRsGC were mixed with 100 μM NO·-donor in 25 mM TEA buffer (pH 7.8) and incubated at room temperature in air or under N_2. All of the samples were then assayed for enzyme activity. Those samples incubated with NO^--donors were also assayed in the presence of 100 μM SNAP in 25 mM TEA buffer, pH 7.8. The concentration dependence of the response to NO^--donors was examined at 20, 50, 100 and 500 μM NO^--donor. The concentration of 100 μM NO·- or NO^--donor was chosen for comparison based on previous studies that have shown maximal activation of sGC at 100 μM NO· or NO·-donor [9].

Effect of NO^+ on sGC Function

Aliquots of sGC were chromatographed on a PD-10 column pre-equilibrated with 25 mM TEA buffer (pH 7.8) to remove DTT, as DTT could potentially reduce NO^+ to NO·. The desalted sGC was stirred gently under N_2 for 15 min at 25° with either 100 μM $NOPF_6$ or $NaPF_6$ in 25 mM TEA buffer, pH 7.8. Buffer was added to pre-weighed $NOPF_6$, and the solution was added to the protein sample within seconds of mixing. Following incubation, the sGC was desalted again on a PD-10 column pre-equilibrated with 25 mM TEA buffer (pH 7.8), to remove PF_6^-, and assayed for enzyme activity in the absence and presence of 100 μM SNAP in 25 mM TEA buffer, pH 7.8.

To maximize the likelihood that NO^+ reached the enzyme, the experiment was repeated using a solution of $NOPF_6$ prepared in dry organic solvent. Under N_2, $NaPF_6$ and $NOPF_6$ were dissolved in either 25 mM TEA buffer (pH 7.8) or dry CH_2Cl_2; when buffer was used, the solution was added to the protein sample as rapidly as possible, as described above. Desalted sGC (*vide supra*) was stirred gently under N_2 for 15 min with either $NaPF_6$ or $NOPF_6$ in either aqueous or organic solution. The sGC was then loaded onto a PD-10 column pre-equilibrated with 25 mM TEA buffer (pH 7.8) to remove PF_6^- and CH_2Cl_2. All samples were then assayed for enzyme activity.

RESULTS

NO· and NO·-donors are both potent activators of sGC and vasodilators [1]. Partially purified sGC and HRsGC were substantially activated by aqueous NO·, SNP, and SNAP, as shown in Table 1. Both SNAP and SNP are vasodilators and are NO·-donors, although the mechanism of NO· release by SNP is not fully understood [29–33]. As expected, the HRsGC was activated to a much greater extent than the unreconstituted enzyme, indicating that the enzyme is isolated in a partially heme-deficient form. SNAP consis-

TABLE 2. Effect of Angeli's salt on sGC activity in the presence and absence of O_2

Protein sample + activator	Specific activity (nmol cGMP formed/min/mg protein)	Specific activity in the presence of SNAP (nmol cGMP formed/min/mg protein)
sGC	0.756	14.2
Anaerobic sGC + Angeli's salt	0.801	16.1
Aerobic sGC + Angeli's salt	0.764	17.7
HRsGC	0.620	24.3
Anaerobic HRsGC + Angeli's salt	0.711	24.2
Aerobic HRsGC + Angeli's salt	0.656	21.8

Partially purified sGC and HRsGC were preincubated for 15 min at 25° with 100 μM Angeli's salt in 25 mM TEA buffer (pH 7.8). One set of samples was prepared in degassed buffer under an atmosphere of nitrogen, while another was prepared aerobically. Following preincubation, all samples were assayed as described in the text. The data reported are averages of triplicate determinations.

TABLE 3. Effect of Angeli's salt (NO^- + NO_2^-) and NO_2^- alone on sGC activity

Protein sample + activator	Specific activity (nmol cGMP formed/min/ mg protein)	Specific activity in the presence of SNAP (nmol cGMP formed/min/ mg protein)
sGC	0.715	14.6
sGC + Angeli's salt	0.752	14.3
sGC + NO_2^-	0.565	15.1
HRsGC	0.570	20.1
HRsGC + Angeli's salt	0.587	19.1
HRsGC + NO_2^-	1.342	18.9

Aliquots of partially purified sGC and HRsGC were stirred gently under N_2 for 25 min at 25° with either 100 μM Angeli's salt or 100 μM $NaNO_2$ in 25 mM TEA buffer (pH 7.8). All samples were assayed in the absence and presence of 100 μM SNAP, and the data reported are the averages of triplicate determinations.

tently activated both the unreconstituted and the reconstituted sGC to a greater extent than either the SNP or the NO·-saturated solution. NO· release from SNP is likely substoichiometric, accounting for the weaker activation versus other NO·-donors [33]. The difference in the extent of activation by equimolar NO·-saturated buffer and SNAP is possibly a consequence of more effective delivery of NO· to the enzyme during the decomposition of SNAP.

Tissue studies with NO^- sources have indicated that NO^- is a vasodilator and, therefore, might activate sGC [23, 24]. To explore this hypothesis, reactive salts known to release NO^- were tested for their ability to activate sGC. Both Angeli's salt and Piloty's acid release NO^- under physiological conditions [34–37]. Angeli's salt decomposes in aqueous solution to release NO^- and NO_2^- [34–36, 38]. Solutions of Angeli's salt did not activate either unreconstituted sGC or HRsGC (Table 2), although the same NO^--treated samples were readily activated by equimolar SNAP, verifying that Angeli's salt had not damaged the enzyme. Variation of the concentration of Angeli's salt from 20 to 500 μM had no effect; the enzyme was not activated under any of the conditions studied. The presence or absence of oxygen in the solution had no effect on the level of activity in response to NO^- (Table 2). Since Angeli's salt releases NO_2^- as well as NO^-, the effect of NO_2^- on unreconstituted sGC and HRsGC was compared with that of Angeli's salt (Table 3). NO_2^- has been reported to be a vasodilator as well as a mild activator of sGC, although only at concentrations of 10 mM or greater [39]. Assays of both the unreconstituted and reconstituted sGC showed little or no activation by either NO_2^- or Angeli's salt, demonstrating that neither NO_2^- nor NO^- is an activator. Again, the samples were responsive to activation by the NO·-donor SNAP, demonstrating that the presence of NO_2^- was not deleterious to the enzyme.

To confirm the failure of NO^- to activate sGC without the possible interference of NO_2^-, Piloty's acid was tested for activation of sGC. Piloty's acid decomposes to give NO^- and $C_6H_5SO_2^-$ (BZSA) [37]. Comparison of the effects of Piloty's acid on unreconstituted sGC and HRsGC in either aerobic or anaerobic solutions shows that Piloty's acid had little or no effect on sGC activity (Table 4) [40]. As in the case of Angeli's salt, no activation was observed over a range (20–500 μM) of concentrations of Piloty's acid. The Piloty's acid-treated sGC was activated by SNAP, indicating that the sGC was still functional. Since Piloty's acid also releases BZSA, the effect of BZSA on sGC function was also explored. BZSA weakly inhibited the basal activity of both the unreconstituted and reconstituted sGC but did not affect the activation by SNAP (Table 5). Piloty's acid is reported to release NO^- slowly, with a half-life of approximately 47 min [37]; therefore, unreconstituted sGC and HRsGC were incubated with Piloty's acid for 90 min, and the enzyme activity was measured at intervals to de-

TABLE 4. Effect of Piloty's acid on sGC activity in the presence and absence of O_2

Protein sample + activator	Specific activity (nmol cGMP formed/min/ mg protein)	Specific activity in the presence of SNAP (nmol cGMP formed/min/ mg protein)
sGC	1.35	18.2
Anaerobic sGC + Piloty's acid	1.51	18.6
Aerobic sGC + Piloty's acid	1.38	17.0
HRsGC	0.986	45.8
Anaerobic HRsGC + Piloty's acid	1.66	37.8
Aerobic HRsGC + Piloty's acid	0.957	33.4

Solutions of Piloty's acid were prepared in 25 mM TEA buffer (pH 9.0) containing 1% DMSO under aerobic and anaerobic conditions. Aliquots of partially purified sGC and HRsGC were stirred gently for 25 min at 25° with 100 μM Piloty's acid either in the air or under N_2. All samples were assayed in the absence and presence of 100 μM SNAP; the data reported are averages of triplicate determinations. Small amounts of DMSO (<2%) have been shown previously to have no effect on sGC function [40].

TABLE 5. Effect of Piloty's acid and BZSA on sGC activity

Protein sample + activator	Specific activity (nmol cGMP formed/min/mg protein)	Specific activity in the presence of SNAP (nmol cGMP formed/min/mg protein)
sGC	0.776	18.9
sGC + Piloty's acid	0.495	18.6
sGC + BZSA	0.414	18.9
HRsGC	0.534	41.2
HRsGC + Piloty's acid	0.299	40.9
HRsGC + BZSA	0.288	40.7

Aliquots of partially purified sGC and HRsGC were stirred gently under N_2 with either 100 μM Piloty's acid or 100 μM BZSA in 25 mM TEA buffer (pH 9.0) containing 1% DMSO. All samples were assayed for activity in the absence and presence of 100 μM SNAP; the data reported are averages of triplicate determinations.

termine whether the effect of Piloty's acid would change over time. Over the course of 90 min (almost two half-lives), little or no effect on sGC activity was observed; the only observable change was a slight decrease in sGC activity (<10%). In all cases, the enzyme could be fully activated by SNAP, indicating that it was still functional.

Stamler and coworkers have raised the possibility that NO^+ may activate sGC [25], although evidence from Feelisch and coworkers suggests that NO^+ is not a vasodilator [26]. The question of whether or not NO^+ is a potential physiological regulator of sGC is controversial because NO^+ reacts very rapidly with H_2O to give HNO_2 [41]. Although a precise half-life has not been reported, NO^+ is not expected to have a significant lifetime in aqueous environments. When sGC was treated with nitrosonium salts in aqueous solution, slight and inconsistent activation of the enzyme was observed. Aqueous $NOPF_6$ activated sGC 2.5-fold or less over the basal activity; neither $NOPF_6$ nor $NaPF_6$ interfered with activation of sGC by SNAP (Tables 6 and 7). The PF_6-counter-ion had little effect on the basal activity of sGC; slight activation (Table 6) or inhibition (Table 7) was observed. To extend the lifetime of NO^+, $NOPF_6$ was dissolved in dry CH_2Cl_2. The $NOPF_6/CH_2Cl_2$-treated sGC was activated slightly (1.4-fold) over the starting basal activity; however, CH_2Cl_2 itself inhibited the basal activity. In comparison with the CH_2Cl_2-inhibited basal activity, the $NOPF_6/CH_2Cl_2$-treated sGC was activated 2.8-fold. The slight activation of sGC by $NOPF_6$ was much less than that observed with SNAP (20 to 60-fold over the basal activity). Although the likelihood that NO^+ is reaching sGC is not known, these experiments seem to indicate that NO^+ is not a significant activator of sGC.

Together these data suggest that only nitric oxide is capable of activating sGC; the other redox forms of nitrogen monoxide are not effective modulators of enzyme activity. A graphical summary of the activation of sGC by $NO^\cdot$, NO^- and NO^+ is given in Fig. 1.

TABLE 6. Effect of aqueous $NOPF_6$ and $NaPF_6$ on sGC activity

Protein sample + activator	Specific activity (nmol cGMP formed/min/mg protein)	Specific activity in the presence of SNAP (nmol cGMP formed/min/mg protein)
sGC	0.971	55.0
sGC + $NOPF_5$	2.51	51.7
sGC + $NaPF_6$	1.31	55.5

Aliquots of partially purified sGC were preincubated under N_2 for 15 min at 25° with either 100 μM $NOPF_6$, prepared immediately before use, or 100 μM $NaPF_6$ in 25 mM TEA buffer, pH 7.8. Following incubation, the sGC was desalted on a PD-10 column and assayed for enzyme activity in the absence and presence of 100 μM SNAP in 25 mM TEA buffer, pH 7.8. The reported data are averages of triplicate determinations.

DISCUSSION

Although for years $NO^\cdot$ has been known to relax vascular smooth muscle and to activate sGC [1], the redox state of the true biological activator of sGC has not been established conclusively. Since the late 1980s, $NO^\cdot$ has been thought to be EDRF, the factor released from endothelial cells upon stimulation by acetylcholine or bradykinin that diffuses into vascular smooth muscle causing it to relax [42, 43]. $NO^\cdot$ is uncharged and would easily diffuse through the phospholipid membrane; this property of $NO^\cdot$ is consistent with the behavior of EDRF. $NO^\cdot$ is also known to bind directly to the heme group in sGC to form a five-coordinate NO-heme and activate the enzyme [6, 8, 10, 11]. Since the physiological response to EDRF is mediated via an increase in cGMP levels [44], sGC activation by $NO^\cdot$ is a likely step in this process. As a radical, $NO^\cdot$ is fairly unreactive [25, 45]; it does not dimerize readily nor does it react directly with most organic molecules. $NO^\cdot$ has a surprisingly long lifetime in fully oxygenated solutions because its reaction with dioxygen is termolecular (bimolecular in NO) and,

TABLE 7. Comparison of the effect of organic and aqueous nitrosonium cation on sGC activity

Protein sample + activator	Specific activity (nmol cGMP formed/min/mg protein)
sGC	3.32
sGC + SNAP	76.7
sGC + aqueous $NOPF_6$	1.99
sGC + aqueous $NaPF_6$	2.06
sGC + CH_2Cl_2	1.65
sGC + organic $NOPF_6$	4.68
sGC + organic $NaPF_6$	1.75

Under N_2, $NaPF_6$ and $NOPF_6$ were dissolved in either 25 mM TEA buffer (pH 7.8) or dry CH_2Cl_2; when buffer was used the solution was prepared and used immediately. Aliquots of sGC were stirred gently under N_2 for 15 min at 25° with either $NaPF_6$ or $NOPF_6$ in either aqueous or organic solution. Following desalting, samples were assayed for activity in 25 mM TEA buffer (pH 7.8). The reported data are averages of triplicate determinations.

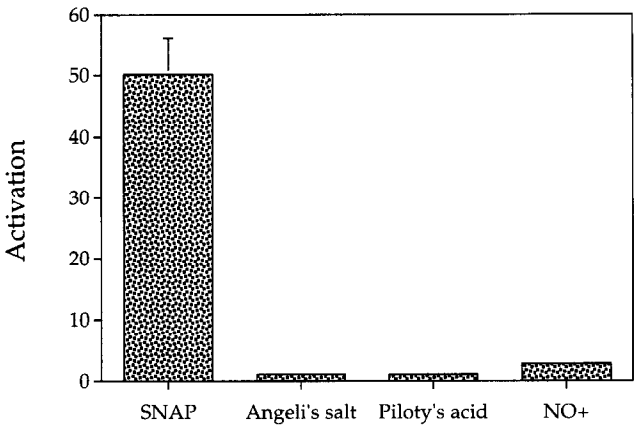

FIG. 1. Comparison of the activation of sGC by NO·, NO^- and NO^+. The activation of sGC by the NO· donor SNAP is compared with that of the NO^- donors Angeli's salt and Piloty's acid, and the NO^+ source $NOPF_6$. Activation is the specific activity of the enzyme in the presence of activator divided by the specific activity in the absence of activator. The plotted data are a summary of the aerobic experiments reported in the tables. Values are means ± SD of triplicate measurements; for the activation by NO^+ and NO^-, the error bars were smaller than the scale of the plot.

therefore, slow at physiological concentrations of NO and O_2 [46]. The lifetime, diffusibility, and ability to activate sGC are all characteristics that support the identification of NO· as EDRF.

Despite the functional similarity between NO· and EDRF, there are a number of observations that do not support the identity of these two species. Chemical evidence suggests that EDRF behaves as an anion [21] and does not react directly with heme groups as would be expected for NO· [22]. These observations, coupled with the formation of NO^- in model oxidations of *N*-hydroxyguanidines [16], have raised the possibility that NO^- is EDRF. Further support for this hypothesis has been obtained in physiologically relevant systems where NO^--donors have been shown to cause vasodilatation [23, 47]. Additionally, *S*-nitrosylated proteins, most likely formed by reaction of either NO^+ or NO^+-donors with proteins, have been detected after the NO-synthase reaction, raising the possibility that NO^+ is the natural activator of sGC [13].

This study confirms that NO· is the only redox form of nitrogen monoxide (Scheme 1) that activates sGC, and therefore supports the identification of EDRF as NO·. Although NO^- is a vasodilator [23, 47], it did not appear to activate sGC significantly or consistently. Furthermore, reaction of sGC with NO^- did not interfere with subsequent NO· activation of the enzyme. These observations support the conclusion that only NO· is capable of activating sGC and suggest that NO^- does not become oxidized to NO· upon interaction with sGC or under the reaction conditions employed for the sGC assay. In previous studies, the reaction of NO^- with O_2^- has been shown to produce NO· [48]; however, activation of sGC by NO^- was never observed, regardless of the availability of dioxygen as a potential source of superoxide anion. Direct oxidation of NO^- to NO· by O_2 is not expected; singlet NO^- produced in the decomposition of Angeli's salt does not react directly with dioxygen [49], and triplet NO^-, formed upon relaxation of singlet NO^-, reacts with dioxygen to give peroxynitrite [49]. These observations do not necessarily rule out the identification of NO^- as EDRF, but they do suggest that oxidation of EDRF, if it were NO^-, would be necessary for sGC activation. There are mechanisms by which NO^- could react directly with ferrous or ferric heme proteins to form NO-heme, which in turn would activate sGC [36, 50, 51]. Such mechanisms are clearly not operative in sGC itself as evidenced by the failure of NO^- to activate the enzyme. Reaction of NO^- with other heme proteins may be responsible for vasodilatation in *in vitro* tissue studies and *in vivo*, since many heme proteins are present in tissues.

It is considerably more difficult to assess the possibility that EDRF is NO^+. NO^+ is hydrolyzed rapidly in water to nitrite and, although the rate of this reaction has never been directly measured, it is assumed to be extremely rapid, presumably at or near the diffusion controlled bimolecular limit [41]. Feelisch and coworkers did not observe vasodilatation with NO^+ in tissue preparations [26], but whether the NO^+ cation actually reached the critical cellular receptor in these experiments in unclear. *In vitro* monitoring of sGC activity in response to NO^+ provides a somewhat better method for assessing the potential function of NO^+ as EDRF. The experiments reported herein were carried out in a manner designed to maximize the stability of NO^+; the nitrosonium salt was dissolved in dry organic solvent, and thiol reducing agents were removed from the protein solution. $NOPF_6$ was observed to activate sGC slightly in organic solution and inconsistently in aqueous solution. Both the PF_6^- counter-ion and CH_2Cl_2 inhibited the basal activity of sGC, but neither interfered with subsequent NO· activation of sGC. It is difficult to determine whether the slight activation is actually due to NO^+ as it is not certain that NO^+ exists long enough to interact with sGC; however, the results suggest that NO^+ has little effect on the activity of the enzyme. The small amount of activation could be due either to direct activation by NO^+, reduction of NO^+ to NO· during incubation with sGC, or by activation by NO_2^-, the hydrolysis product of NO^+. NO^+ could be reduced by a protein thiol or by the Fe(II)PPIX present in the enzyme, resulting in activation of the enzyme in proportion to the amount of NO· produced. Were NO^+ produced by NO-synthase *in vivo*, it would most likely react with protein thiols to form *S*-nitrosothiol adducts or else be hydrolyzed rapidly to NO_2^-. Both *S*-nitrosothiols and NO_2^- can activate sGC; the former, of which SNAP is an example, are potent activators and act via release of NO· [45]. NO_2^- only activates sGC at concentrations of 10 mM or greater [39], conditions that are not physiologically relevant. While these reactions provide potential mechanisms by which the labile nitrosonium ion could ultimately activate sGC, the more reasonable conclusion remains that EDRF is NO·.

In summary, it seems clear that the oxidation state of nitrogen monoxide is important in sGC regulation. Only $NO^{\cdot}$ consistently and substantially activates sGC; NO^{+} and NO^{-} are weak activators of sGC at best. Should EDRF or the product of NO-synthase prove to be something other than $NO^{\cdot}$, there will likely be a physiological pathway for converting it to $NO^{\cdot}$ or NO-heme in order to activate sGC.

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