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Inactivation of Soluble Guanylate Cyclase by Stoichiometric *S*-Nitrosation

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ABSTRACT

Dysfunction of vascular nitric oxide (NO)/cGMP signaling is believed to contribute essentially to various cardiovascular disorders. Besides synthesis and/or bioavailability of endothelial NO, impaired function of soluble guanylate cyclase (sGC) may play a key role in vascular dysfunction. Based on the proposal that desensitization of sGC through *S*-nitrosation contributes to vascular NO resistance (*Proc Natl Acad Sci U S A* **104:**12312–12317, 2007), we exposed purified sGC to dinitrosyl iron complexes (DNICs), known as potent nitrosating agents. In the presence of 2 mM GSH, DNICs stimulated cGMP formation with EC_{50} values of 0.1 to 0.5 μM and with an efficacy of 70 to 80% of maximal activity measured with 10 μM 2,2-diethyl-1-nitroso-oxyhydrazine (DEA/NO). In the absence of GSH, the efficacy of DNICs was markedly reduced, and sGC stimulation was counteracted by the inhibition of both basal and DEA/NO-stimulated cGMP formation at higher DNIC concentrations. Inactivation of sGC was slowly reversed in the presence of 2 mM GSH and associated with stoichiometric *S*-nitrosation of the protein (2.05 $\pm$ 0.18 mol S-nitrosothiol per mol of 143-kDa heterodimer). *S*-Nitrosoglutathione and sodium nitroprusside caused partial inhibition of DEA/NO-stimulated sGC that was prevented by GSH, whereas nitroglycerin (0.3 mM) had no effect. Our findings indicate that nitrosation of two cysteine residues in sGC heterodimers results in enzyme inactivation. Protection by physiologically relevant concentrations of GSH (10 μM to 3 mM) suggests that *S*-nitrosation of sGC may contribute to vascular dysfunction in inflammatory disorders associated with nitrosative and oxidative stress and GSH depletion.

Soluble guanylyl cyclase (sGC; EC E.C.4.6.1.2), which catalyzes the conversion of GTP into the cyclic nucleotide cGMP, is the major effector enzyme of NO in mammalian cells (Friebe and Koesling, 2003). Binding of NO to a regulatory heme group at the β-subunit of the protein results in ~100-fold stimulation of cGMP formation. The NO/cGMP signaling pathway is essentially involved in a number of biological processes, including vasodilation, penile erection, platelet function, and cell-to-cell communication in the brain (Moncada et al., 1991).

Dysfunction of vascular NO/cGMP signaling, which is believed to contribute to a wide variety of cardiovascular disorders, may be caused by several not mutually exclusive mechanisms, including impaired NO synthesis as a result of reduced NO synthase substrate or cofactor availability, decreased NO bioavailability as a result of oxidative inactivation of endothelium-derived NO, down-regulation of sGC protein expression, dysfunction of vascular sGC, up-regulation of phosphodiesterase activity, and impaired signaling downstream of cGMP (cGMP-dependent protein kinases, Ca^{2+} homeostasis, etc.). Although impaired NO release and/or bioavailability ("endothelial dysfunction") has been studied extensively in the past decade, little is known about the pathways leading to reduced vascular sensitivity to NO, also termed as NO resistance (Chirkov and Horowitz, 2007). Impaired sGC function has been suggested to contribute to vascular dysfunction in several experimental models of cardiovascular disorders, including atherosclerosis (Schmidt et al., 1991; Laber et al., 2002), hypertension (Jacke et al., 2001), diabetes (Witte et al., 2002), and chronic lung disease (Bland et al., 2003). A better knowledge of the mechanisms

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ABBREVIATIONS: sGC, soluble guanylate cyclase; BSA, bovine serum albumin; DEA/NO, 2,2-diethyl-1-nitroso-oxyhydrazine; DNIC, dinitrosyl iron complex; GSNO, *S*-nitrosoglutathione; GTN, glyceroltrinitrate (nitroglycerin); PBS, phosphate-buffered saline; SNP, sodium nitroprusside; TEA, triethanolamine; YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole; RSNO, *S*-nitrosothiol.

underlying sGC inactivation would aid in the interpretation of data obtained in these disease models and may pave the way for the design of studies exploring the in vivo relevance of sGC dysfunction.

It has been reported that *S*-nitrosation of sGC causes desensitization of the enzyme to NO (Sayed et al., 2007). Based on these and more recent observations (Sayed et al., 2008), the authors suggested that *S*-nitrosation of sGC could constitute a crucial element in the development of cardiovascular disease and nitrate tolerance. *S*-Nitrosothiols may represent a relatively stable transport and storage form of NO in tissues and plasma. Through the release of NO, these compounds may be vasoprotective, but they also contribute to tissue injury involving oxidative and nitrosative stress (Ridnour et al., 2004). In cells and tissues, low and high molecular weight *S*-nitrosothiols are formed by transnitrosation, that is, transfer of NO^+ equivalents from another nitrosating species to low molecular weight thiols, in particular GSH, or exposed cysteine residues of proteins (Scharfstein et al., 1994). Because free NO radical does not nitrosate thiols, nitrosation requires oxidation of NO by either a trace metal ion such as Cu^{2+} in ceruloplasmin (Inoue et al., 1999) or molecular oxygen, resulting in the formation of the autoxidation products NO_2 radical and N_2O_3 as reactive intermediates (Schrammel et al., 2003). Alternatively, the nitrite reductase activity of deoxyhemoglobin has been proposed to yield an *S*-nitrosothiol precursor that produces SNO-hemoglobin as nitrosating agent upon exposure to O_2 (Angelo et al., 2006).

Similarly to *S*-nitrosothiols, DNICs were identified as biologically relevant storage forms of NO (Mülsch et al., 1991). These compounds are formed in cytokine-activated macrophages expressing high levels of inducible NO synthase (Lancaster and Hibbs, 1990) but also in agonist-stimulated vascular endothelial cells (Mülsch et al., 1991, 1993) and lipopolysaccharide-exposed blood vessels (Muller et al., 1996). DNICs, which are stored in a protein-bound form in the vasculature and released by thiols (Mülsch et al., 1991; Muller et al., 1996), were shown to trigger vasodilation through NO-mediated stimulation of sGC (Mülsch et al., 1991; Severina et al., 2003). As NO^+ carriers, these compounds are potent nitrosating agents (Boese et al., 1995; Hughes, 1999) that trigger caspase- and Bcl-2-independent apoptosis (Kleschyov et al., 2006). In the present study, we exposed purified sGC to mono- and binuclear DNICs with thiosulfate as ligands (Fig. 1). Solutions of the mononuclear compound DNIC-G contain 18-fold molar excess of free thiosulfate, whereas no free thiosulfate is present in solutions of the binuclear complex DNIC-Y. In the presence of high concentrations of GSH, these compounds acted as NO donors, resulting in the expected stimulation of cGMP formation. However, in the absence or at low concentrations of GSH, NO-mediated sGC activation was counteracted by the inhibition of basal and NO-stimulated cGMP formation. Inactivation of sGC was accompanied by *S*-nitrosation of two cysteine residues of the heterodimeric protein, suggesting that sGC becomes inactivated by selective *S*-nitrosation.

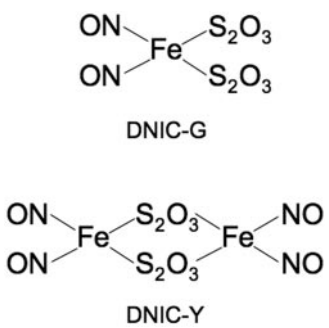

Fig. 1. Structures of the mono- and binuclear DNICs used in this study. The letters G and Y refer to the colors of the complexes, green and yellow, respectively.

Materials and Methods

Materials. Bovine lung sGC was purified as described previously (Russwurm and Koesling, 2005). [α-^{32}P]GTP (400 Ci/mmol) was from PerkinElmer Life and Analytical Sciences (Vienna, Austria). DEA/NO (Alexis Corporation, Lausen, Switzerland) was purchased via Eubio (Vienna, Austria) and dissolved and diluted in 10 mM NaOH. Nitro Pohl ampules (G. Pohl-Boskamp GmbH. and Co, Hohenlockstedt, Germany), containing 4.4 mM GTN in 250 mM glucose, were obtained from a local pharmacy; dilutions were made in 50 mM TEA/HCl buffer. Mononuclear DNIC-G (green) and the binuclear dimer DNIC-Y (yellow) were prepared with thiosulfate as anionic ligand as described previously (Giannone et al., 2000). All other chemicals were from Sigma (Vienna, Austria).

Determination of sGC Activity. Purified bovine lung sGC (50 ng) was incubated at 37°C for 10 min in a final volume of 0.1 ml in the presence of 50 mM TEA/HCl, pH 7.4, 0.5 mM [α-^{32}P]GTP (~250,000 cpm), 3 mM $MgCl_2$, 1 mM cGMP, and 0.1 mM diethylene triamine pentaacetic acid. Drugs were present as indicated in the text and figures. Reactions were terminated by addition of 450 μl of zinc acetate (120 mM) and 450 μl of sodium bicarbonate (120 mM), followed by isolation of [^{32}P]cGMP as described previously (Schultz and Böhme, 1984). Blank values were determined in the absence of sGC. The enzyme preparations used for this study exhibited maximal specific activities of 15 to 20 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ (determined in the presence of 10 μM DEA/NO). DEA/NO controls were included in each assay to determine the effects of DNICs in relation to maximal sGC activity. Because sGC was purified with buffer containing 2 mM dithiothreitol (Russwurm and Koesling, 2005), trace amounts of the thiol (~7 μM) were endogenously present in all experiments described in this article.

To test for reversibility of sGC inactivation, purified sGC (150 ng) was preincubated for 10 min at 37°C in 150 μl of 50 mM TEA/HCl, pH 7.4, in the presence of 0.5 mg/ml bovine serum albumin (necessary to avoid the loss of sGC activity during the preincubation period) and 10 μM DNIC-G, followed by the addition of 40 μl of a reaction mixture containing [α-^{32}P]GTP (2.5 mM, ~150,000 cpm), $MgCl_2$ (15 mM), and cGMP (5 mM) with or without GSH (10 mM; 2 mM final) and 10 μl of a solution of DEA/NO (200 μM). Samples were incubated for 5 to 20 min at 37°C and processed as described above.

Determination of NO Release. NO release from DNICs was measured with a Clark-type electrode (World Precision Instruments, Berlin, Germany) calibrated daily with acidified nitrite as described previously (Mayer et al., 1995). After equilibration at 37°C with PBS, pH 7.4, DEA/NO, DNIC-G, or DNIC-Y were added to give the final concentrations indicated in Fig. 2 (1–10 μM).

Determination of DNIC-Triggered *S*-Nitrosation of sGC. Purified sGC (3 μl of stock solution containing 156 pmol of protein) was incubated in PBS (137 mM NaCl, 2,7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4) with 20 μM DNIC-G for 3 min at 37°C in the dark in a final volume of 200 μl. Samples were transferred on ice, diluted with 2.8 ml of chilled PBS, and desalted by chromatography over Econo-Pac 10 DG columns (Bio-Rad Laboratories, Hercules, CA). Protein recovery, determined with bovine serum albumin, was 57%. *S*-Nitrosothiol content was determined as $HgCl_2$-releasable nitrite by the Saville method (Saville, 1958) with modifications pub-

lished previously (Ng et al., 2004). Samples were incubated in the absence or presence of $HgCl_2$ (200 μM final) at room temperature in the dark for 30 min, followed by the addition of 50 μl of a 2,3-diaminonaphtalene solution (50 μg/ml in 0.62 M HCl) and incubation at 37°C for 15 min in the dark. To maximize fluorescent signals, 100 μl of 2.8 M NaOH was added, and samples were kept at room temperature for a further 10 min. Fluorescence was determined with a Luminescence Spectrometer (LS50B; PerkinElmer) at excitation and emission wavelengths of 363 and 407 nm, respectively. RSNO content was calculated as $HgCl_2$ releasable nitrite corrected for column recovery (57%) after subtraction of blank levels (31 ± 7 nM; n = 4). The detection limit of the method was 10 nM nitrite.

The *S*-nitrosylated protein detection kit from Cayman (purchased through Biozol, München, Germany) was used to demonstrate *S*-nitrosation of sGC by the biotin switch assay (Jaffrey et al., 2001). Fatty acid-free BSA (0.1 mg, corresponding to 1.5 nmol) or purified sGC (7.4 μg corresponding to 0.106 nmol) were incubated in a final volume of 0.1 ml of 50 mM Tris buffer, pH 7.4, at 37°C for 3 min in the dark with and without 20 μM DNIC-G in the absence and presence of 2 mM GSH. To facilitate protein precipitation, 94 μg of myosin was added, and samples were processed according to the instructions of the manufacturer. Biotinylated protein pellets were resuspended in 50 μl of buffer, diluted 1:2 with Laemmli buffer, and separated by SDS electrophoresis on 8% polyacrylamide gels followed by Western blot analysis.

Results

DNIC-G and DNIC-Y rapidly released NO when dissolved in PBS at pH 7.4. As shown in Fig. 2, the initial rates of NO release obtained with 2 μM DNIC-G were identical with the rates obtained with the same concentration of the established NO donor DEA/NO, which exhibits a half-life of ~3 min at 37°C (Maragos et al., 1991; Schmidt et al., 1997). The NO signals derived from both DNICs decayed more rapidly than DEA/NO-derived NO, suggesting that autoxidation does not fully account for the decay of DNIC-derived NO.

As shown in Fig. 3, in the absence of GSH, both DNICs caused biphasic effects on the rates of cGMP formation. Maximal enzyme activity determined in the presence of 10 μM DEA/NO under identical conditions was 14.1 ± 0.63 μmol · min^{-1} · mg^{-1}. At maximally effective concentrations of 1 and 3 μM, DNIC-G (Fig. 3A) and DNIC-Y (Fig. 3B) stimulated the enzyme to 46.5 and 32.3% of the DEA/NO maximum, respectively, whereas higher concentrations led to the inhibition of cGMP formation. In the presence of 0.1 mM DNIC-Y, basal sGC activity was reduced from 0.11 ± 0.013 μmol · min^{-1} · mg^{-1} to a value that was hardly detectable under the assay conditions (0.012 ± 0.018 μmol · min^{-1} · mg^{-1}). In the presence of 2 mM GSH, DNIC-G and DNIC-Y acted as potent sGC activators with EC_{50} values of approximately 0.1 and 0.5 μM and maximal effects amounting to approximately 83 and 72% of the DEA/NO maximum, respectively. The effect of GSH was concentration-dependent with maximal effects occurring at 0.3 to 1 mM. Figure 4 shows the effects of 0.03 to 3 mM GSH on sGC activity measured in the presence of 3 and 30 μM DNIC-G and DNIC-Y, respectively, which inhibit DEA/NO-stimulated sGC by 70 to 80% (see Fig. 6 below).

Figure 5 shows that YC-1 (0.2 mM), a drug that activates sGC in the absence of added NO and sensitizes the enzyme for NO stimulation (Friebe et al., 1996), potentiated sGC activation by DNICs, apparent as pronounced leftward shifts of the concentration-response curves (EC_{50} values of ~5 nM and ~0.5 μM for DNIC-G and DNIC-Y, respectively) and an increase in maximal activity. However, YC-1 did not protect the enzyme from inactivation, which occurred at similar DNIC concentrations in the absence and presence of the drug (Figs. 3 and 5). Note that basal sGC activity, which was stimulated approximately 15-fold in the presence of YC-1 (Figs. 2 and 5), was completely inhibited by 10 and 100 μM DNIC-G and DNIC-Y, respectively.

The inactivation of sGC was more clearly apparent when DNIC concentration-response curves were recorded in the presence of DEA/NO (10 μM). As shown in Fig. 6A, DNIC-G and DNIC-Y inhibited the NO-stimulated enzyme with IC_{50} values of approximately 1 and 10 μM, respectively. Inhibition was completely prevented when GSH (2 mM) was present

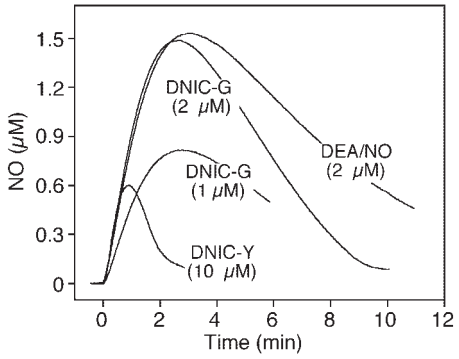

Fig. 2. Release of NO from DNICs and DEA/NO. At time point 0, release of NO from the indicated final concentrations of DNIC-G, DNIC-Y, and DEA/NO was measured with a Clark-type electrode as described under *Materials and Methods*. The traces shown are representative of four similar experiments.

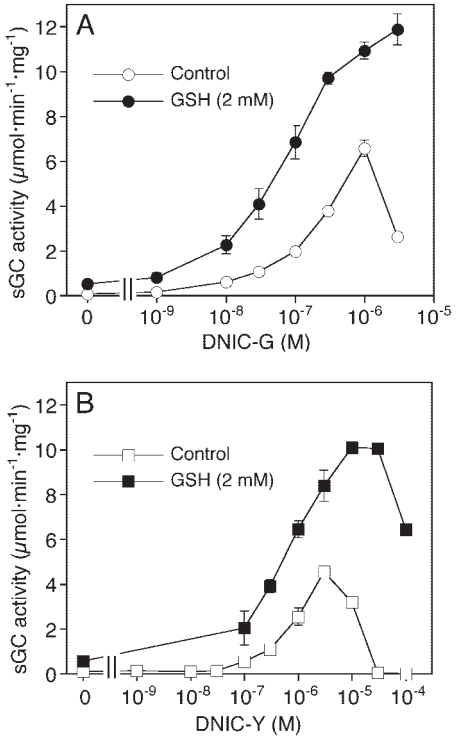

Fig. 3. Effects of DNICs on the activity of purified sGC in the absence and presence of GSH. Purified sGC (50 ng) was incubated at 37°C for 10 min in the presence of the indicated concentrations of DNIC-G (A) or DNIC-Y (B) with and without 2 mM GSH, followed by isolation and quantification of cGMP as described under *Materials and Methods*. Shown are mean values ± S.E of three independent triplicate determinations.

(Fig. 6B). The effects of DNICs were compared with those of GSNO, SNP, and GTN. At relatively high concentrations (1 mM), GSNO and SNP inhibited NO-stimulated sGC to approximately 45 and 75% of control, respectively, whereas GTN (0.3 mM) caused no significant inhibition. The inhibitory effect of GSNO was attenuated in the presence of 2 mM GSH (Fig. 6B).

To test for reversibility of sGC inactivation, the enzyme was preincubated with 10 μM DNIC-G for 10 min, followed by the determination of the NO-stimulated enzyme activity in the absence and presence of 2 mM GSH. As shown in Fig. 7, DNIC-treated sGC was virtually inactive over the incubation period of 20 min, whereas GSH (2 mM) caused a significant reactivation of the enzyme. After 20 min of incubation, DNIC-treated sGC exhibited a specific activity of 8.2 $\pm$ 2.86 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, corresponding to approximately 50% of controls that had been preincubated in the absence of DNIC-G under otherwise identical conditions.

We considered that DNICs may inactivate sGC by *S*-nitrosation of critical cysteine residues essential for catalytic activity and, therefore, quantified the RSNO content of the DNIC-treated enzyme. Based on a molecular mass of 143 kDa (Friebe and Koesling, 2003), we found that heterodimeric sGC treated for 3 min with 20 μM DNIC-G contained 2.05 $\pm$ 0.18 (n = 4) mol of RSNO per mol of protein, suggesting nitrosation of 1 cysteine residue per subunit. *S*-Nitrosation was not detectable when sGC was treated either in the absence of DNICs or with DNIC-G in the presence of 2 mM GSH under otherwise identical conditions. Figure 8 illustrates *S*-nitrosation of BSA (as positive control) and sGC by the biotin switch assay. Again, *S*-nitrosation of the pro-

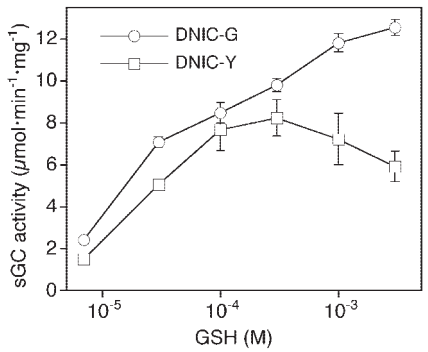

Fig. 4. Effect of GSH concentration on sGC activity in the presence of DNICs. Purified sGC (50 ng) was incubated at 37°C for 10 min with DNIC-G (3 μM) or DNIC-Y (30 μM) in the presence of increasing concentrations of GSH. Shown are mean values $\pm$ S.E. of three independent triplicate determinations.

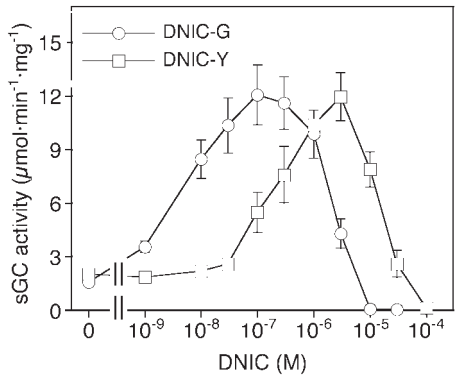

Fig. 5. Effects of DNICs on sGC activity in the presence of the NO-sensitizing drug YC-1. Purified sGC (50 ng) was incubated at 37°C for 10 min with the indicated concentrations of DNIC-G and DNIC-Y in the presence of 0.2 mM YC-1. Shown are mean values $\pm$ S.E of three independent triplicate determinations.

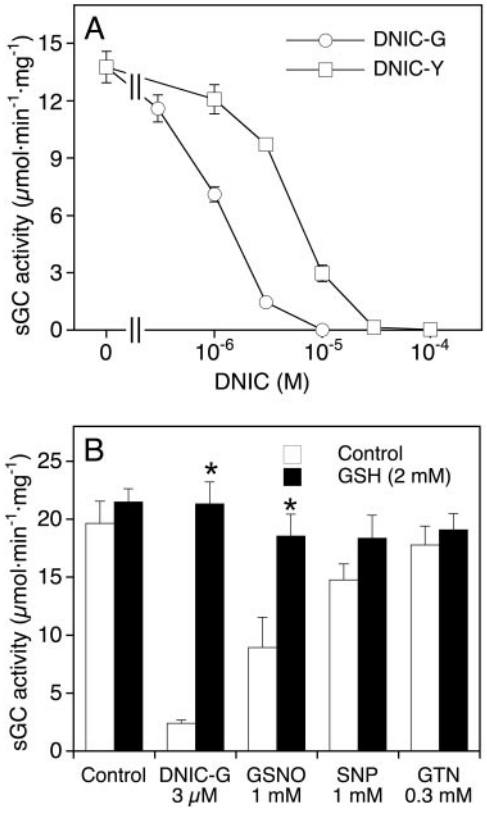

Fig. 6. Effects of DNICs, GSNO, SNP, and GTN on the activity of NO-stimulated sGC. Purified sGC (50 ng) was incubated at 37°C for 10 min in the presence of 10 μM DEA/NO and DNICs, GSNO, SNP, or GTN as indicated. A, concentration-dependent inhibition of NO-stimulated cGMP formation by DNIC-G and DNIC-Y in the absence of GSH. B, effects of DNIC-G (3 μM), GSNO and SNP (1 mM each), and GTN (0.3 mM) in the absence and presence of 2 mM GSH. Shown are mean values $\pm$ S.E of three independent triplicate determinations. Significant ($p < 0.05$) protection by GSH is indicated by an asterisk (analysis of variance with post hoc Bonferroni-Dunn test).

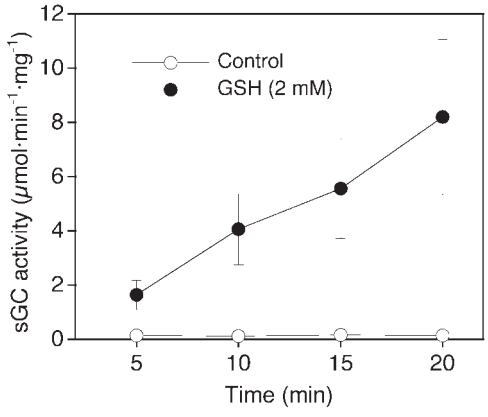

Fig. 7. Reversibility of DNIC-induced sGC inactivation in the presence of GSH. Purified sGC (150 ng) was preincubated for 10 min at 37°C with 10 μM DNIC-G followed by the addition of 40 μl of a reaction mixture containing [α-^{32}P]GTP (2.5 mM, ~150,000 cpm), $MgCl_2$ (15 mM), and cGMP (5 mM) with or without GSH (10 mM, 2 mM final) and 10 μl of a solution of DEA/NO (200 μM, 10 μM final). At the indicated time points, [^{32}P]cGMP was isolated and quantified as described under *Materials and Methods*. Shown are mean values $\pm$ S.E. of three independent triplicate determinations.

teins was completely prevented by incubation with DNIC-G in the presence of 2 mM GSH. Reactivity of two bands in the sGC lane further suggests that both subunits become *S*-nitrosated by DNIC-G.

Discussion

It is well established that DNICs cause vasodilation through the NO/cGMP pathway (Mülsch et al., 1991; Alencar et al., 2003). Although nitrosation of cellular thiols and homolytic cleavage of the resulting *S*-nitrosothiols is considered as major pathway of NO release from these complexes, we observed fairly rapid release of NO upon decomposition of DNICs in PBS in the absence of thiols. This finding agrees well with a previous study showing that DNICs with either cysteine or GSH as ligands release NO in buffer solution with rates depending on the nature of the ligand (Alencar et al., 2003). Thus, it is very likely that these compounds activate sGC through the release of NO, which binds to the regulatory ferrous heme group bound to His105 of the β_1 subunit of the enzyme (Friebe and Koesling, 2003). The mechanism of NO release is unknown but may involve intramolecular electron transfer from iron to the NO^+ moiety in a reaction similar to homolytic cleavage of *S*-nitrosothiols catalyzed by trace metal ions (Gorren et al., 1996). Clarification of this issue was beyond the aim of the present study.

As expected, purified sGC was stimulated by low concentrations (up to approximately 1 μM) of the DNICs. However, the effects of both compounds were biphasic with pronounced inactivation of sGC occurring at approximately 10-fold higher DNIC concentrations. The biphasic effects became most clearly apparent in the presence of the NO sensitizer YC-1 (Friebe et al., 1996), which potentiated sGC stimulation but did not affect the sensitivity of the enzyme to DNIC-triggered inactivation. In addition, the results obtained with YC-1 indicate that sGC stimulation is mediated by NO binding to the heme, whereas enzyme inactivation is caused by another reaction. There is good reason to conclude that sGC inactivation was due to *S*-nitrosation of cysteine residues essential for catalytic activity. First, the enzyme was protected against inactivation by GSH, which is well known to outcompete *S*-nitrosation of proteins. Second, the activity of DNIC-treated sGC was slowly restored by excess GSH, presumably reflecting the expected transfer of protein-bound NO^+ equivalents to the low molecular weight thiol (Scharfstein et al., 1994). Third, the nitrosating agent GSNO also caused inactivation of DEA/NO-stimulated sGC that was prevented by GSH. Finally, sGC inactivation was accompanied by stoichiometric *S*-nitrosation quantified by a modification of the Saville assay and demonstrated by the biotin switch assay (Fig. 8).

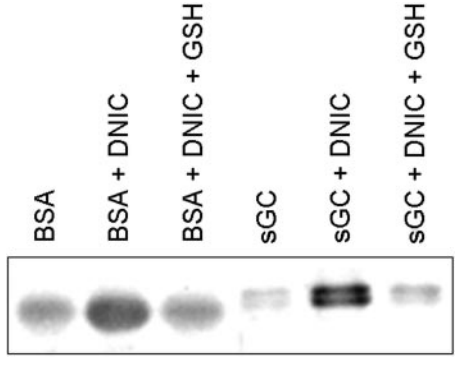

Fig. 8. *S*-Nitrosation of sGC visualized by the biotin switch assay. BSA (0.1 mg) and sGC (7.4 μg) were incubated in a final volume of 0.1 ml of 50 mM Tris buffer, pH 7.4, at 37°C for 3 min in the dark with and without 20 μM DNIC-G in the absence and presence of 2 mM GSH. Samples were analyzed for *S*-nitrosated proteins with the biotin switch assay using the *S*-nitrosylated protein detection kit from Cayman. The Western plot shown is representative of three.

Our data suggest that one cysteine in each subunit becomes nitrosated upon exposure of sGC to DNICs and agree well with a previous study reporting on GSNO-triggered nitrosation of Cys243 and Cys122 in the α and β subunits, respectively (Sayed et al., 2007). However, there is major discrepancy between that study and our findings, and this difference could be biologically relevant. Although Sayed et al. (2007) reported that nitrosation of sGC resulted in desensitization of the enzyme to NO without affecting basal cGMP formation, our data, in particular the assays performed in the presence of YC-1, indicate complete inactivation of basal enzyme activity by DNICs. There are several possible explanations for this discrepancy. First, Sayed et al. (2007) performed the activity assays with desalted cytosolic fractions of cultured cells, which exhibited approximately 20-fold lower specific sGC activities than the pure enzyme. Obviously, a variety of unknown processes, including transnitrosation of other proteins, may have interfered with sGC inactivation in cell lysates. Second, Sayed et al. (2007) used *S*-nitrosopenicillamin as an NO donor for sGC stimulation even though this drug, similarly to GSNO, is a well established nitrosating agent. The use of *S*-nitrosopenicillamin may have hampered the clean distinction between enzyme stimulation and inactivation and may at least partially explain the only 5-fold stimulation of cGMP formation in the NO controls presented by Sayed et al. (2007) compared with the 150-fold activation of purified sGC by DEA/NO that we observed in our study. Finally, protein *S*-nitrosation by GSNO, and possibly also *S*-nitrosocysteine, may be significantly less efficient than the reaction triggered by DNICs; in our hands, exposure of sGC to GSNO, even at a concentration as high as 1 mM, caused only approximately 50% enzyme inhibition. Because Sayed et al. (2007) did not quantify the amount of sGC that became nitrosated by GSNO or *S*-nitrosocysteine under their experimental conditions, it cannot be excluded that the apparent lack of full enzyme inactivation resulted from only partial nitrosation of the protein.

In a recent article, the same authors reported that sGC becomes *S*-nitrosated by the antianginal drug GTN and suggested that this effect may contribute to the development of vascular nitrate tolerance (Sayed et al., 2008). It is well known that sGC becomes desensitized upon exposure to high concentrations of GTN, but this effect is presumably due to slow oxidation of enzyme-bound heme and has been questioned seriously as a pharmacologically relevant mechanism of nitrate tolerance (Mayer and Beretta, 2008). Contrasting the proposal of Sayed et al. (2008), we observed no inhibition of cGMP formation by NO-stimulated sGC exposed to 0.3 mM GTN, suggesting that GTN, unlike established nitrosating agents, does not significantly inactivate purified sGC. Inactivation of sGC through heme oxidation was not expected to occur in our experimental setup because NO binding protects enzyme-bound ferrous heme iron from oxidation (Schrammel et al., 1996).

Because DNICs are formed endogenously upon activation of NO synthases, nitrosative inactivation of sGC has to be considered as biologically relevant mechanism of vascular dysfunction, even though high cellular GSH levels may be protective under normal physiological conditions. The published GSH levels in vascular tissue vary considerably (Brault et al., 2007; Monti et al., 2007), making it difficult to judge the concentration of the free thiol in blood vessels. However, there is ample evidence for significant depletion of reduced GSH in a variety of pathologies involving oxidative stress (Griffith, 1999), and our data revealed increasing protection of sGC over a fairly wide range of GSH concentrations (Fig. 4). Thus, even relatively small changes in the cellular redox state may significantly affect vascular NO/cGMP signaling. Further studies are warranted to clarify the contribution of nitrosative sGC inactivation to vascular dysfunction in inflammatory disorders associated with oxidative stress.

Acknowledgments

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