Desensitization of soluble guanylyl cyclase, the NO receptor, by S-nitrosylation

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Edited by Solomon H. Snyder, Johns Hopkins University School of Medicine, Baltimore, MD, and approved June 7, 2007 (received for review April 29, 2007)

The molecular mechanism of desensitization of soluble guanylyl cyclase (sGC), the NO receptor, has long remained unresolved. Posttranslational modification and redox state have been postulated to affect sGC sensitivity to NO but evidence has been lacking. We now show that sGC can be S-nitrosylated in primary aortic smooth muscle cells by *S*-nitrosocysteine (CSNO), an S-nitrosylating agent, in human umbilical vein endothelial cells after vascular endothelial growth factor treatment and in isolated aorta after sustained exposure to acetylcholine. Importantly, we show that S-nitrosylation of sGC results in decreased responsiveness to NO characterized by loss of NO-stimulated sGC activity. Desensitization of sGC is concentration- and time-dependent on exposure to CSNO, and sensitivity of sGC to NO can be restored and its S-nitrosylation prevented with cellular increase of thiols. We confirm *in vitro* with semipurified sGC that S-nitrosylation directly causes desensitization, suggesting that other cellular factors are not required. Two potential S-nitrosylated cysteines in the α- and β-subunits of sGC were identified by MS. Replacement of these cysteines, C243 in α and C122 in β, created mutants that were mostly resistant to desensitization. Structural analysis of the region near β-C122 in the homologous *Nostoc* H-NOX crystal structure indicates that this residue is in the vicinity of the heme and its S-nitrosylation could dampen NO activation by affecting the positions of key residues interacting with the heme. This study suggests that S-nitrosylation of sGC is a means by which memory of NO exposure is kept in smooth muscle cells and could be a mechanism of NO tolerance.

cGMP | tolerance | redox | *S*-nitrosothiols

In the cardiovascular system, nitric oxide (NO) is critical for regulation of vascular tone and homeostasis (1, 2). In mammals, the main sensor of NO is the soluble guanylyl cyclase (sGC), a heme-containing heterodimer formed by an α- and a β-subunit. When NO binds to the heme, catalytic activity of sGC increases several hundredfold to produce the second messenger cGMP (3). Despite the importance of the NO–cGMP pathway in the biology and pathology of the cardiovascular and neuronal systems, modulation of sGC is poorly understood (4, 5). In particular, the mechanism of desensitization of sGC has remained unresolved despite 30 years of effort. Desensitization is the transition to a state in which sGC's response to a new NO stimulation is reduced or abolished. This direct effect on sGC differs from the desensitization of the NO–cGMP pathway caused by decrease in cGMP levels [e.g., because of increased phosphodiesterase activity (6)] or decrease in NO availability (7). Desensitization of sGC itself has been reported in various cell types and tissues after exposure to NO (8–11) but its mechanism is unknown. Ser/Thr phosphorylation was proposed to be involved but evidence is still lacking (12), and Tyr phosphorylation was ruled out recently (13). Elucidating the mechanism of sGC desensitization is crucial considering its likely involvement in NO tolerance during the development of oxidative vascular pathophysiologies, atherosclerosis, and pulmonary hypertension.

S-nitrosylation, a NO-dependent posttranslational modification of free-thiol cysteines, alters the function of many proteins, including enzymes (14), and is proposed to be involved in the physiologies and pathophysiologies of the cardiovascular system (15). We reasoned that sGC, as the main, if not only, receptor activated by NO, could be targeted by S-nitrosylation to induce its desensitization, thus constituting an exquisite process of sGC modulation by negative feedback. We tested the hypothesis that sGC desensitization is induced by S-nitrosylation.

Results

sGC Is S-Nitrosylated in Primary Aortic Smooth Muscle Cells (SMC) Treated with *S*-Nitrosocysteine (CSNO). To investigate S-nitrosylation of sGC, we used primary aortic SMC because the function and importance of the NO–cGMP pathway is well defined in this system: vasorelaxation is induced *in vivo* by NO produced by the endothelial cells layer, which diffuses into the underlying SMC where it activates sGC. SMC were treated for 1h with CSNO at 1 mM or L-cysteine (L-Cys; 1 mM) as control. CSNO has been used extensively as an S-nitrosylating agent in intact cells (16). After washout, S-nitrosylation of endogenous sGC in the cytosolic fraction was assessed by the biotin switch assay (17) followed by avidin purification and coimmunoprecipitation using anti-SNO antibodies as described (16, 18). Western blot analysis with anti-sGC antibodies (Fig. 1*A*) showed, with both methods, that cytosolic sGC is S-nitrosylated in cells treated with CSNO but not in cells treated with L-Cys. In addition, the reciprocal immunoprecipitation, using anti-sGC antibodies followed by biotin switch assay and avidin purification showed that only the cytosols from CSNO-treated cells contained S-nitrosylated sGC [supporting information (SI) Fig. 6]. In parallel, we confirmed the capacity of *S*-nitrosoglutathione (GSNO) to S-nitrosylate sGC in cytosols and the specificity of detection of S—NO bond by the biotin switch assay using ascorbate, which can generate thiols from *S*-nitrosothiol but not from other S-oxidized thiols (SI Fig. 7) (19).

sGC Is S-Nitrosylated in Human Umbilical Vein Endothelial Cells (HUVEC) Treated with Vascular Endothelial Growth Factor (VEGF). We demonstrated that sGC S-nitrosylation takes place under more physiological conditions. HUVEC, which express both endothelial NO synthase and sGC were treated with VEGF, which induces NO production (20). One-hour treatment with 10 ng/ml

Author contributions: F.v.d.A. and A.B. designed research; N.S., P.B., and X.M. performed research; and F.v.d.A. and A.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Abbreviations: sGC, soluble guanylyl cyclase; CSNO, *S*-nitrosocysteine; HUVEC, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor; SMC, smooth muscle cells; L-Cys, L-cysteine; GSNO, *S*-nitrosoglutathione; Ach, acetylcholine; SNAP, *S*-nitro-*N*-acetyl-penicillamine; NAC, *N*-acetyl-cysteine; GSH, glutathione.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0703944104/DC1.

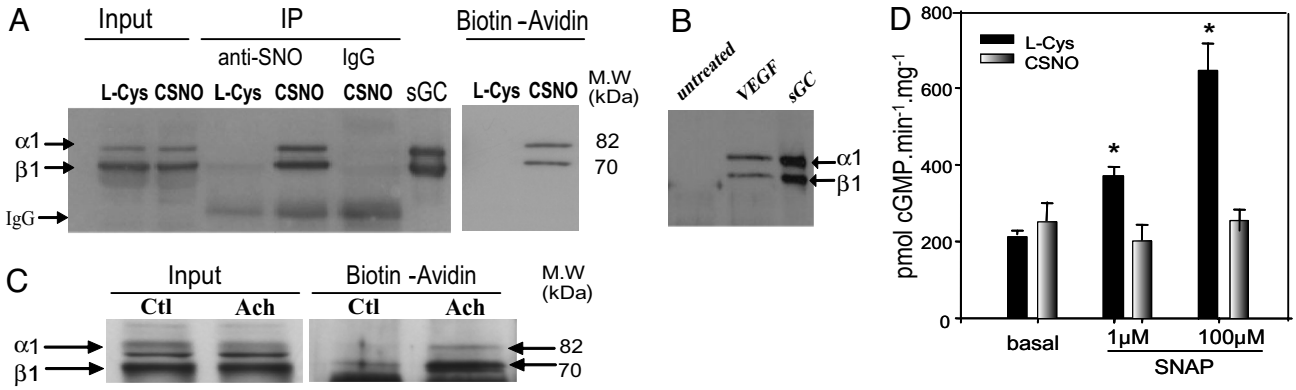

Fig. 1. Endogenous sGC is S-nitrosylated and loses NO responsiveness in primary aortic SMC treated with CSNO. (*A*) (*Left*) Western blot with anti-α and anti-β antibodies showing that sGC from the cytosols of CSNO-treated cells is immunoprecipitated by anti-SNO antibodies whereas no sGC is pulled down from L-Cys-treated cells. (*Right*) Western blot analysis with anti-sGC of a biotin switch assay followed by avidin purification, confirming that sGC is S-nitrosylated by CSNO treatment. Input corresponds to 10% of the 200-μg precleared cytosols. One hundred micrograms of cytosols was used for the biotin/avidin assay. IgG did not immunoprecipitate sGC from CSNO-treated cells, indicating specificity of anti-SNO. Semipurified rat sGC (300 ng) was used as marker: α- and β-subunits are 80 and 72 kDa, respectively. (*B*) The same biotin-avidin assay as in *A* showing that sGC is S-nitrosylated in HUVEC treated with VEGF for 1 h at 10 ng/ml. (*C*) Biotin-avidin assay followed by Western blot with anti-sGC showing that sGC is S-nitrosylated in aorta treated with Ach for 1 h (100 μM). These blots (*A–C*) are each representative of three independent experiments. (*D*) Basal and NO-stimulated sGC activity of the cytosols prepared in *A* showing that the sGC of cytosols from CSNO-treated cells loses responsiveness to NO; basal and NO-stimulated activity (at 1 and 100 μM of the NO-donor SNAP) were not significantly different, in contrast to NO-stimulated activity of cytosols from L-Cys treatment. These experiments were repeated three times with each measurement done in duplicate and expressed as mean $\pm$ SE (*, $P < 0.05$).

of VEGF resulted in S-nitrosylation of sGC, whereas S-nitrosylated sGC could not be detected in untreated cells. In addition to showing that sGC is S-nitrosylated by an endogenous modulator, this result shows that sGC is not "constitutively" S-nitrosylated in these cells (Fig. 1*B*). For further studies only primary aortic SMC were used because the levels of sGC activity are much higher than in HUVEC.

sGC Is S-Nitrosylated in Freshly Isolated Aorta Treated with Acetylcholine (Ach). To determine whether sGC in SMC could be S-nitrosylated from a physiological source of NO, rat aorta were isolated and treated with 100 μM Ach for 1 h (controls were untreated aorta). After removal of the endothelial layer, homogenates were prepared and S-nitrosylation was assayed by a biotin-switch assay coupled to avidin purification. Western blot with anti-sGC (Fig. 1*C*) shows that S-nitrosylated sGC was readily detected in the aorta treated with Ach but not in the untreated aorta (Fig. 1*C Right*), whereas the amount of sGC was similar in untreated and treated samples (Fig. 1*C Left*).

sGC Is Desensitized in SMC Treated with CSNO. The cytosols of SMC prepared above were assayed for basal and NO-stimulated sGC activity [using *S*-nitro-*N*-acetyl-penicillamine (SNAP) at 1 and 100 μM as the NO donor]. Fig. 1*D* shows that cytosols prepared from cells treated with 1 mM CSNO did not respond to NO stimulation, whereas the basal activity was similar to the basal activity of cytosols treated with L-Cys. As expected, the NO-stimulated sGC activity of L-Cys-treated cytosols increases with increasing concentrations of SNAP. This result strongly suggested a correlation between sGC S-nitrosylation and loss of NO sensitivity.

S-Nitrosylation and Desensitization of sGC Are Time- and Concentration-Dependent. To determine the extent of correlation between S-nitrosylation and desensitization, SMC were treated with two different concentrations of CSNO (100 μM and 1 mM) for two periods (15 min and 1 h); L-Cys was used as a control. As shown in Fig. 2*A*, the S-nitrosylation levels of sGC increased as a function of time and concentration of CSNO. One hour of exposure to 100 μM CSNO readily produced sGC S-nitrosylation. The time of exposure could be shortened to 15 min with 1 mM CSNO. Using 1 mM of L-Cys for 1 h did not lead to detectable S-nitrosylation. Importantly, these increased levels of S-nitrosylation were directly paralleled by increased desensitization (shown in Fig. 2*B*). In addition, desensitization of sGC as a function of time and concentration of CSNO was directly assessed in intact cells by measuring the cGMP production in response to SNAP at 100 μM by RIA (for this, the sGC activity *per se* is not measured but the cGMP accumulation during 1h treatment with SNAP). The results were similar, showing that the NO-stimulated cGMP production was reduced as a function of time and CSNO concentration (SI Table 1).

S-Nitrosylation and Desensitization of sGC Can Be Blocked in SMC. S-nitrosylation is a reversible posttranslational modification dependent on the redox state of the cells (21). To assess the specificity and correlation between sGC S-nitrosylation and desensitization, SMC were pretreated with *N*-acetyl-cysteine (NAC), a physiological precursor of glutathione (GSH) (22). Before CSNO and L-Cys treatment of SMC (1 h, 250 μM), 2.5 mM of NAC was applied for 2 h. As shown in Fig. 2*C*, pretreatment with NAC prevented CSNO-induced S-nitrosylation, as the amount of sGC pulled down by anti-SNO in the precleared cytosols was greatly reduced. No sGC was pulled down by anti-SNO after L-Cys or L-Cys + NAC treatment, as expected (Fig. 2*C Left*). In addition, the presence of sGC in the supernatant of the immunoprecipitated cytosols (i.e., non-S-nitrosylated sGC) was verified: higher amounts of sGC were detectable in the L-Cys, L-Cys + NAC, and CSNO + NAC samples than in the CSNO samples, confirming that NAC reduced S-nitrosylation of sGC and suggesting that the proportion of CSNO-dependent S-nitrosylated sGC was high (Fig. 2*C Right*). Importantly, sensitivity to NO of the sGC was restored in the cytosols treated with CSNO if they were pretreated with NAC, compared with cytosols from cells not pretreated with NAC (Fig. 2*D*; 304 $\pm$ 14 vs. 522 $\pm$ 34 pmol/min per mg). Indeed, the NO-stimulated sGC activity was similar in the cytosols from the cells treated with NAC + CSNO and NAC + L-Cys. As above, basal activity was similar under all conditions. The fact that increasing intracellular free thiols can block desensitization of sGC to NO and

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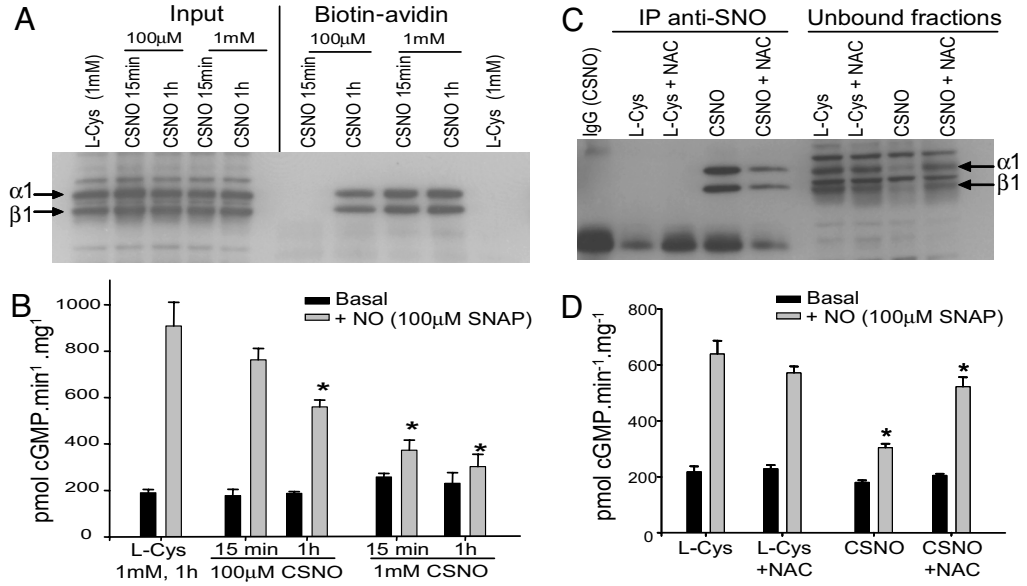

Fig. 2. S-nitrosylation and desensitization of sGC is a function of time and concentration of CSNO and is reduced with NAC pretreatment. (*A*) Western blot with anti-α and anti-β of a biotin-avidin assay showing that S-nitrosylation of sGC in SMC increases with time and concentration of CSNO. The input (10% of the cytosol) indicated similar sGC levels in each sample. (*B*) Basal and NO-stimulated activity of the various cytosols from *A* showed that the decrease in NO-stimulated sGC activity was a function of the concentration and time of CSNO treatment. The small increase in basal activity with 1 mM CNSO was not significant. *, $P < 0.05$ vs. L-Cys. (*C*) (*Left*) Western blot with anti-sGC showing that less sGC is precipitated with anti-SNO from the cytosols of cells treated with NAC and CSNO than from cells treated with CSNO only, whereas none is pulled down with L-Cys or L-Cys + NAC treatment or with IgG. (*Right*) Western blot showing that more sGC is detected in fractions not precipitated by anti-SNO, hence not S-nitrosylated, of cells pretreated with NAC + CSNO than with CSNO alone; α and β are indicated by arrows (the unspecific band between α and β has the same intensity in all samples). Two hundred micrograms of cytosols was used in the experiments and repeated twice with similar results. (*D*) Basal and NO-stimulated sGC activity of treated cytosols from *C*. NO-stimulated activity was inhibited by >50% with 250 μM CSNO but pretreatment with NAC blocks the CSNO-dependent desensitization. Experiments were repeated three times and are expressed as mean ± SEM. *, $P < 0.05$ CSNO vs. CSNO + NAC. The differences between the maximal activities of sGC in Figs. 1*D*, 2*B*, and 2*D* are likely caused by use of different batches of primary aortic SMC; activities are not normalized.

its S-nitrosylation strongly suggests that desensitization of sGC is linked to its S-nitrosylation in cells.

S-Nitrosylation Directly Induces Desensitization of Semipurified sGC. To determine whether S-nitrosylation directly causes desensitization of sGC or other cellular factors are required, we induced S-nitrosylation of semipurified sGC (stored without DTT, GSH, or glycerol). sGC was pretreated with 50 μM GSNO in the dark for 10 min (the S—NO bonds are light sensitive, which probably explains why desensitization of sGC was not observed before, when GSNO was used as a NO donor). After incubation, the sGC/GSNO mix was diluted 100-fold in reaction buffer. and the basal and NO-stimulated sGC activities were measured. Fig. 3*A* shows that GSNO pretreatment compared with GSH reduced by ≈50% the sGC activity stimulated with a saturating concentration of SNAP (100 μM). A biotin switch assay (Fig. 3*B*) confirms that sGC is S-nitrosylated by GSNO but not by GSH. The basal activity of sGC was increased by pretreatment with GSNO (167 ± 13 and 571 ± 59 nmol·min^{-1}·mg^{-1} for GSH and GSNO pretreatment, respectively), as 0.5 μM GSNO is still present in the mix after dilution.

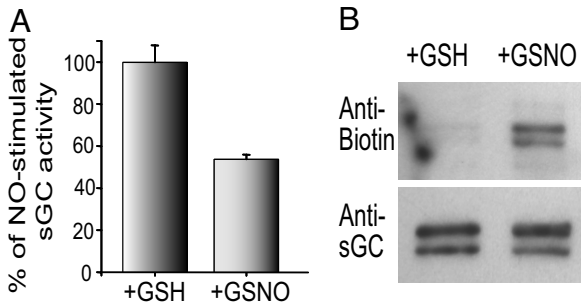

Fig. 3. The NO-stimulated activity of semipurified sGC is reduced by pretreatment with GSNO and correlates with its S-nitrosylation. (*A*) NO-stimulated activity was reduced by ≈50% with 50 μM GSNO pretreatment compared with GSH. Measurements were made after 100-fold dilution of the sGC + GSNO or GSH mixture and in the presence of 100 μM SNAP. This experiment was repeated three times and with two independent preparations of sGC. (*B*) In parallel, sGC was S-nitrosylated by 50 μM GSNO, as shown by a biotin switch assay followed by immunoblot analysis with antibiotin and anti-sGC.

Cys-243 in the α-Subunit (αC243) and Cys-122 in the β-subunit (βC122) of sGC Are S-Nitrosylated, as Determined by MS and Are Involved in sGC Desensitization. Bovine sGC was subjected to the biotin switch assay followed by in-solution tryptic digest. After separation of the peptides by RPLC, potential biotinylated peptides (*m/z* consistent with biotinylation of tryptic peptides of sGC) were identified. Among them, the MS/MS spectra of two peptides with *m/z* 1,232.88 and 1,079.68 Da were acquired and confirmed to correspond to FHQDCR of the α1-subunit and CTDADK of the β1-subunit, which contain C245 (bovine sequence) and C122, respectively (Fig. 4). The two corresponding Cys in rat sGC were replaced by Ala, and the resulting mutants were expressed transiently in COS-7 cells. COS-7 overexpressing WT (α1/β1), α1C243A/β1, and α1/β1C122A were treated with 250 μM CSNO or L-Cys for 1 h. Cytosols were prepared as above and sGC activity was measured in response to NO stimulation (SNAP, 100 μM). As shown in Fig. 5*A*, whereas pretreatment with CSNO decreased NO-stimulated activity of the WT to 46.4 ± 1.5% compared with L-Cys treatment (100%), the NO response of α1C243A/β1 was only slightly decreased (to 84.3 ±

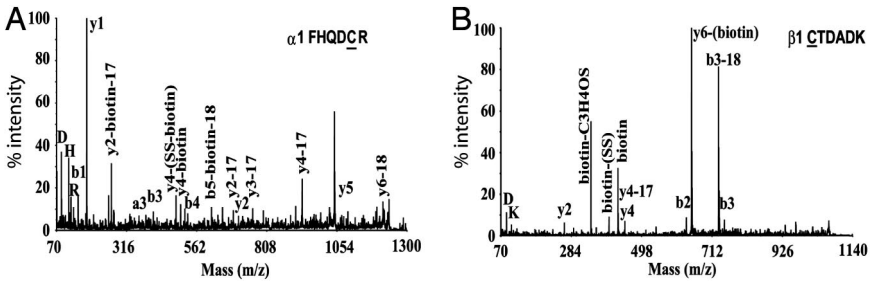

Fig. 4. MALDI-TOF/TOF analysis of S-biotinylated peptides, FHQDCR (α1; *A*) and CTDADK (β1; *B*). The spectra contain most of the y+ and b+ series of ions with the biotin tag and peaks corresponding to C-S and S-S dissociation of disulfide-linked biotin. The spectra confirm the identity of the peptides as S-biotinylated FHQCDR and CTDADK. C̲, biotinylated cysteine.

3.0%), which corresponds to elimination of ≈70% of the desensitization. It is worth noting that mutation α1C243A did not affect NO-stimulated sGC activity *per se*, as the specific activity for the WT and α1C243A/β1 after 1 mM L-Cys treatment were 3,144 ± 46 and 3,025 ± 114 pmol/min per mg, respectively. Mutant α1/β1C122A also lost part of its NO-dependent desensitization; 71.3% ± 4.1% of activity remaining, which corresponds to elimination of ≈45% of the desensitization (its NO-stimulated sGC was ≈3-fold lower: 959.4 ± 107.8 pmol/min per mg). We also assayed the double-mutant α1C243A/β1C122A, which had activity similar to α1/β1C122A and was apparently more sensitive to desensitization (≈65% of NO-stimulated activity remaining; data not shown). Identical results were obtained when, instead of SNAP, diethylamine–NO (100 μM) was used, suggesting that the source of NO for stimulation of sGC did not play a role.

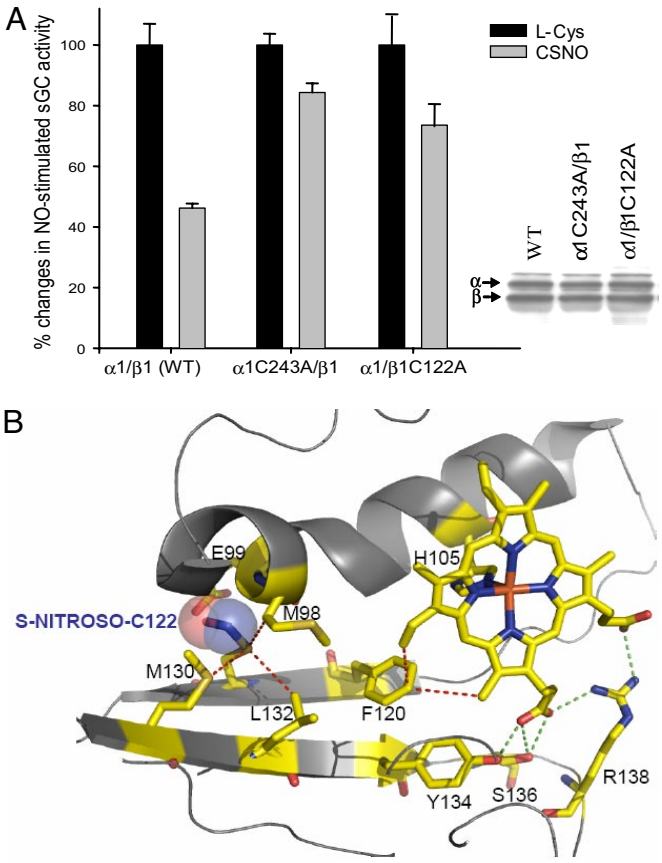

Fig. 5. Mutational analysis of the S-nitrosylated Cys in sGC indicates that α1C243 and β1C122 are involved in sGC desensitization. (*A*) NO-stimulated sGC activity in the cytosols of COS-7 cells overexpressing WT, α1C243/β1, and α1/β1C122 treated with L-Cys or CSNO is less affected by CSNO treatment in the mutants than WT ($P < 0.05$). Experiments were done on two to three independent transfections, repeated two to three times with each measurement done in duplicate. (*Inset*) Western blot showed that WT and mutants were expressed at similar levels. (*B*) Model for the C122 S-nitrosylated form of the H-NOX domain. The heme is shown in stick mode. The helix containing M98 and H105, the strands containing C122 and F120 and M130, L132, and Y134 are shown in cartoon mode, and the remaining residues are in coil representation. The NO group is in ball mode and was modeled according to the S-nitrosylated C62 residue from thioredoxin. The image was generated by using PYMOL (www.pymol.org).

Structural Modeling of the C122 *S*-Nitroso Form of H-NOX Domain. A structural model was generated for the C122 *S*-nitroso form of H-NOX domain from our recently solved apo and liganded form of *Ns* H-NOX (23), which is closely related to the heme binding domain of sGCβ1 (Fig. 5*B*). Residue C122 is conserved in both sGC and *Ns* H-NOX and is located in a conserved hydrophobic region. The C122 in the *Ns* H-NOX is located in the protein interior and its S atom has <1 Å of solvent-accessible areas as calculated with MSCON (24). The buried nature of C122 is consistent with that of the Cys residues that are nitrosylated in tubulin (25) and thioredoxin (26). The buried, nitrosylated C62 in thioredoxin, which is oriented toward the protein interior, thus serves as a prototype for modeling *S*-nitroso-C122 in H-NOX. The model suggests three potential structural changes after *S*-nitroso modification of C122. The S-nitrosylation group increases the length of this side chain by ≈2.5 Å, which would likely cause shifts in either C122 or neighboring residues because the S atom of C122 is already in close van der Waals distance with M98, M130, and L132 (all within 4.1 Å). The latter two residues are adjacent to the Y134xS136xR138 heme propionate interaction sequence, whose position could be altered by nitrosylation of C122, thus affecting signal transduction via space-accommodating shifts in M130 (L in sGCβ1) and L132 (conserved in sGCβ1). The S atom of C122 is also proximate to the CB atom of M98 (L in sGCβ1), which is part of the helix harboring the heme proximal ligand H105. A potential shift of this helix and altered positioning of H105 upon C122 S-nitrosylation could hamper NO activation. Alternatively, C122 itself could be pushed out of the normal position upon S-nitrosylation, which could affect NO activation because C122 is located on the β-strand containing residue F120 (conserved in sGCβ1), which forms ≈4-Å van der Waals interactions with the heme.

Discussion

In the present study, we investigated whether S-nitrosylation of sGC, the receptor for NO, is the molecular mechanism that leads to desensitization/tolerance to NO. We hypothesized that S-nitrosylation of sGC could be responsible, simply because sGC

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is the main receptor for NO and as such could be the primary target of NO signaling negative feedback. To determine whether sGC was S-nitrosylated, we treated primary aortic SMC, which express high levels of sGC, with CSNO, a physiological *S*-nitrosothiol found in plasma and transported into cells via L-Cys transporters (27, 28). Using three methods (immunoprecipitation with anti-SNO or anti-sGC and the biotin switch assay followed by avidin purification), we established that sGC is S-nitrosylated in intact SMC. This was confirmed under more physiological conditions by treating HUVEC that express both endothelial NO-synthase and sGC (at low levels) with VEGF and importantly, by subjecting rat aorta to sustained physiological NO exposure via treatment with Ach. We next demonstrated that CSNO-induced S-nitrosylation correlates with desensitization of sGC in SMC and that desensitization and S-nitrosylation were time- and concentration-dependent and could be prevented by increasing intracellular thiols with NAC. These results suggest that the stability of sGC S-nitrosylation and response to NO depends on the cellular redox state.

S-nitrosylation by GSNO causes directly desensitization *in vitro* with semipurified sGC. To investigate further the mechanism of desensitization, we conducted a MS analysis of sGC S-nitrosylation and identified at least two Cys that were targets of S-nitrosylation *in vitro*. We showed that α1C243 and β1C122 are involved in the desensitization mechanism of sGC in cells, as their replacement conferred resistance to desensitization in COS-7 cells treated with CSNO. Importantly, the replacement of α1C243 with Ala did not affect the NO-stimulated sGC activity but specifically blunted NO desensitization. Interestingly, preliminary study with the double mutant indicated that it was 35% desensitized after CSNO treatment (compared with $\approx$15% and 28% for α1C243A and β1C122A, respectively). One possible explanation for the lack of "additive" effect of both mutations is a compensatory mechanism in which other Cys are S-nitrosylated. Information regarding the structural changes induced by S-nitrosylation is still limited. To our knowledge, the only examples observed by x-ray crystallography are from hemoglobin (29) and thioredoxin (26). Our modeling of the C122 *S*-nitroso form of the heme domain predicts a shift of the helix carrying the His-105 that ligates the heme, or a change in the positioning of F120, which is located on the same β strand as C122 and interacts with the heme, or altering the interaction with L132, which is adjacent to the heme propionate interaction. Each of these modifications would alter NO activation.

β1C122A is widely conserved but α1C243A is conserved only in mammalian species, in which development of desensitization/vascular tolerance is observed. Interestingly, C243 was proposed to be part of a regulatory binding site for BAY 41-2772 (NO-independent activator) (30) but this observation was not supported by site-directed mutagenesis (31). As part of a mutational analysis of conserved Cys residues, Cys-78 and Cys-214 in the β1-subunit were mutated but the lost of NO-stimulated activity of the corresponding mutants was caused by heme depletion (32). It has been suggested that Cys could be involved in modulation of sGC via formation of disulfide bond. Recently, it was shown that treatment of bovine pulmonary arteries with diamide, which oxidizes sulfhydryl groups, inhibited NO-stimulated sGC activity (33), yet the Cys residues potentially involved in this process remain unidentified.

The sustained stimulation of NO production in aortas treated with Ach led to sGC S-nitrosylation, which supports the observation that high NO concentration in the presence of O_2 led to N_2O_3 formation (34), the *in vivo* quintessential S-nitrosylating agent. Moreover, oxidative stress is often associated with the formation of reactive nitrogen species such as peroxynitrite, formed by the reaction of endogenous NO with superoxide, which decreases NO-stimulated sGC activity (35) and N_2O_3 (36). Taken together, our results support the idea that prolonged NO exposure reduces sGC activity caused by redox modulation of thiols (33). Thus, it is tempting to speculate that S-nitrosylation of sGC is the missing link between NO tolerance and oxidative stress in the development of cardiovascular oxidative pathologies.

Among the many mechanisms of receptor desensitization (phosphorylation, internalization, translocation, or protein–protein interaction), this study provides evidence that the mechanism of desensitization of sGC, the NO receptor, can take place via S-nitrosylation. The idea that the NO–cGMP pathway is modulated by S-nitrosylation is indirectly supported by the fact that sGC is not endogenously S-nitrosylated in HUVEC or isolated aorta unless exposed to VEGF or Ach, respectively. Moreover, colocalization of the NO source and its target likely contributes to selective S-nitrosylation (14), and sGC is associated with NO synthase in a multiprotein complex probably involving Hsp90 and PSD95 (37, 38). If S-nitrosylation of sGC leads to desensitization, thereby altering NO-dependent vasodilation (for example), then dysfunction of sGC modulation could constitute a crucial element in the development of cardiovascular diseases, as we have revealed a link between S-nitrosylation of sGC and blunted response to NO.

Methods

Materials. Fetal bovine serum was from Gibco (Carlsbad, CA). All other cell culture reagents were from American Type Culture Collection (Manassas, VA). Other materials are described in *SI Text*.

L-Cys and CSNO Preparation. L-Cys was prepared as a 200 mM solution in 1 M HCl, neutralized with 9 vol of 1 M K_2HPO_4 (pH 7.4). CSNO was prepared by mixing 200 mM L-Cys in 1 M HCl with 200 mM Na-nitrite in the dark for 30 min and neutralized with 9 vol of 1 M K_2HPO_4 (pH 7.4).

Cell Culture and Treatment. Primary rat aortic smooth muscle cells were kindly provided by A. Papapetropoulos (University of Patras, Patras, Greece) and HUVEC cells by W. N. Durán (New Jersey Medical School). RASMC and COS-7 cells were treated with different concentrations of L-Cys and CSNO ranging from 100 μm to 1 mM for 15 min to 1 h. HUVEC were treated with VEGF (10 ng/ml).

Isolation and Ach Treatment of Rat Aorta. Ring segments of thoracic aorta were cleaned and placed in a physiologic buffer (130 mM NaCl/5.6 mM KCl/2 mM $CaCl_2$/1 mM $MgCl_2$/11 mM glucose/10 mM Hepes, pH 7.4) at 37°C and then stimulated with 100 μM Ach for 1 h, followed by denudation of the endothelium. The aortas were then snap-frozen before homogenization in 50 mM Hepes, 150 mM NaCl, 1 mM DTT, 10% glycerol, 1 mM EDTA, and protease inhibitors containing buffer. Soluble fraction was collected by centrifugation. All procedures were approved by the Institutional Animal Care and Use Committee of New Jersey Medical School.

Site-Directed Mutagenesis and Transfection. Templates were cDNAs encoding the α1- and β1-subunits of rat sGC cloned into the mammalian expression vector pCMV5. The α1C243A and β1C122A mutations were introduced by PCR (Quikchange; Stratagene, La Jolla, CA) and checked by DNA sequencing. COS-7 cells were transfected for 48 h with HyFect reagent, according to the supplier's protocol (Denville, South Plainfield NJ).

Cytosol Preparation and Immunoprecipitation. Rat aortic SMC were grown to passages 4–6 in 100-mm dishes and after various treatments were washed twice with ice-cold PBS, scraped off the plate in cold lysis buffer (50 mM Hepes, pH 8.0/1 mM EDTA/150 mM NaCl/protease inhibitors). After sonication, the lysate was centrifuged at 16,000 $\times$ *g* for 10 min at 4°C to collect the soluble

fraction. Cytosols (300 μg) were precleared with Protein A-Sepharose 4B beads and incubated with nonimmune serum or rabbit polyclonal anti-SNO or anti-sGC (α1 and β1) overnight at 4°C. Protein A beads were added to samples for 2 h at 4°C, then pelleted by centrifugation and washed three times with lysis buffer. Proteins were eluted in 1% SDS buffer, resolved on 8% SDS/PAGE, and analyzed by immunoblot with anti-sGC.

S-Nitrosylation (Biotin-Switch) Assay. The Biotin-Switch assay was performed with a NitroGlo Kit following the supplier's protocol (PerkinElmer, Wellesley, MA) on semipurified sGC (1 μg) preincubated with 50–100 μM GSNO for 20 min in dark or on 100 μg of cytosols from CSNO- or L-Cys-treated cells. For avidin purification, biotinylated proteins were diluted with 2 vol of neutralization buffer (20 mM Hepes, pH 8.0/100 mM NaCl/1 mM EDTA/0.5% Triton X-100), and streptavidin–agarose beads were added and incubated for 1 h at room temperature with agitation. Beads were washed five times with the neutralization buffer containing 600 mM NaCl. The biotinylated proteins were eluted by boiling the beads.

sGC Activity Assay. sGC activity was determined by formation of [α^{32}P]cGMP from [α^{32}P]GTP as described (31). Reactions were performed for 5 min at 33°C in a final volume of 100 μl, in 50 mM Hepes, pH 8.0, reaction buffer containing 500 μM GTP, 1 mM DTT, and 5 mM $MgCl_2$. Typically, 40 μg of cytosol or 30 ng of partially purified sGC was used in each assay reaction. Activity was stimulated with the NO-donor SNAP.

Analysis of S-Nitrosylation by MS. Four micrograms of sGC (Alexis Biochemicals, San Diego, CA) were S-nitrosylated with 200 μM GSNO, subjected to biotin switch assay as above, and acetone-precipitated, followed by in-solution trypsin digest in 30 μl of ammonium bicarbonate (pH 8.0) at 37°C for 16 h. The tryptic peptides were desalted by using a C18 ZipTip (Millipore, Billerica, MA) and dried in a speed vac. The peptides were resuspended in 10 μl of solvent A [5% acetonitrile (ACN), 0.1% TFA], and separated by using reverse-phase liquid chromatography (RPLC) (capillary PepMap C18 column, Dionex, Sunnyvale, CA) in a 60-min linear gradient from 10% solvent A to 40% solvent B (95% ACN, 0.1% TFA). The RPLC eluent was mixed with MALDI matrix (7 mg/ml α-cyano-4-hydroxy-cinnamic acid in 60% ACN, 5 mM ammonium monobasic phosphate and internal calibrants, 50 fmol/μl each of GFP and adrenocorticotropic hormone 18-39) in a 1:2 ratio and spotted on a MALDI plate (18 $\times$ 18 array) with a Probot spotting device (Dionex). The peptides were analyzed on a 4700 Proteomics Analyzer tandem mass spectrometer (Applied Biosystems, Framingham, MA). Mass spectra (m/z 800–3,800) were acquired in positive ion reflector mode with internal mass calibration. Spots containing m/z of biotinylated peptides were selected for subsequent MS/MS sequencing analysis in 1-kV mode. These experiments were conducted at the Center for Advanced Proteomics Research, New Jersey Medical School.

Statistical Analysis. Data are expressed as mean $\pm$ SE. For sGC activity, each experiment was done with two to three independent batches of SMC or transfections in COS-7 cells. Coimmunoprecipitation and biotin/avidin assay were repeated at least three times. Comparison of sGC activities between pretreatment with L-Cys and CSNO or between treatment with CSNO and CSNO + NAC were made with Student's t test by using SigmaPlot version 8.0 software (Systat Software, San Jose, CA). $P < 0.05$ was considered statistically significant.

We thank Dr. Tong Liu for technical expertise in MS analyses; Dr. Hong Li, director of the Center for Advanced Proteomics, for critical discussion; and Dr. Andrew Harris for critical reading of the manuscript. This work was supported by National Institutes of Health Grants GM067640 (to A.B.) and HL075329 (to F.v.d.A.) and the American Heart Association (F.v.d.A.).

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