Guanylyl cyclase sensitivity to nitric oxide is protected by a thiol oxidation–driven interaction with thioredoxin-1

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Nitric oxide (NO) modulates many physiological events through production of cGMP from its receptor, the NO-sensitive guanylyl cyclase (GC1). NO also appears to function in a cGMP-independent manner, via *S*-nitrosation (SNO), a redox-based modification of cysteine thiols. Previously, we have shown that *S*-nitrosated GC1 (SNO-GC1) is desensitized to NO stimulation following prolonged NO exposure or under oxidative/nitrosative stress. In animal models of nitrate tolerance and angiotensin II–induced hypertension, decreased vasodilation in response to NO correlates with GC1 thiol oxidation, but the physiological mechanism that resensitizes GC1 to NO and restores basal activity is unknown. Because GC1 interacts with the oxidoreductase protein-disulfide isomerase, we hypothesized that thioredoxin-1 (Trx1), a cytosolic oxidoreductase, could be involved in restoring GC1 basal activity and NO sensitivity because the Trx/thioredoxin reductase (TrxR) system maintains thiol redox homeostasis. Here, by manipulating activity and levels of the Trx1/TrxR system and by using a Trx1-Trap assay, we demonstrate that Trx1 modulates cGMP synthesis through an association between Trx1 and GC1 via a mixed disulfide. A proximity ligation assay confirmed the endogenous Trx1–GC1 complex in cells. Mutational analysis suggested that Cys[609] in GC1 is involved in the Trx1–GC1 association and modulation of GC1 activity. Functionally, we established that Trx1 protects GC1 from *S*-nitrosocysteine–induced desensitization. A computational model of Trx1–GC1 interaction illustrates a possible mechanism for Trx1 to maintain basal GC1 activity and prevent/rescue GC1 desensitization to NO. The etiology of some oxidative vascular diseases may very well be explained by the dysfunction of the Trx1–GC1 association.

Nitric oxide (NO) is a gaseous signaling molecule that modulates a variety of physiological events, especially in the cardiovascular system. NO is known to function through two different pathways, which are cGMP-dependent and cGMP-independent (1, 2). In the cGMP-dependent pathway, NO stimulates several-hundred-fold the catalytic activity of GC1 (previously soluble guanylyl cyclase; IUPHAR/PBS Guide to Pharmacology (3)) to produce cGMP. The NO–GC1–cGMP pathway plays a critical role in vasorelaxation and inhibition of platelet aggregation and negatively regulates myocardium contractility (1, 4). Catalytically active GC1 is a heterodimer consisting of an α and a β subunit. Each subunit contains three domains: an N-terminal heme–NO/oxygen (HNOX) domain; a "dimerization domain" with a PAS fold and coiled-coil region; and the C-terminal catalytic domain, where cGMP is converted from GTP (5). Following NO stimulation, GC1 undergoes a quick deactivation by an unknown mechanism as well as protracted desensitization that could be triggered by repeated or prolonged NO exposure and/or oxidative stress. This desensitization, which is characterized by GC1 decreased response to NO, affects vascular reactivity. How GC1 recovers from this activity loss and regains sensitivity to NO is key to understanding how vascular basal tone and reactivity are maintained in cardiovascular homeostasis.

In addition to the cGMP-dependent pathway, NO can also signal via *S*-nitrosation, a cGMP-independent pathway. *S*-Nitrosation is a reversible post-translational modification of cysteines, on which a NO moiety is added to the free thiol group. *S*-Nitrosation is proposed to modify protein activity, localization, and interaction (6). We have shown that *S*-nitrosation and other thiol oxidation play a role in the desensitization of the cGMP-dependent pathway (7). In an animal model of nitrate tolerance, we showed that decreased NO-dependent vasorelaxation correlated with enhanced *S*-nitrosation of GC1 and decreased NO-dependent cGMP production (8). Likewise, angiotensin II–induced hypertensive rats contain high levels of vascular SNO[2]-GC1, which was associated with decreased NO-de-

[2] The abbreviations used are: SNO, *S*-nitrosation/S-nitrosated; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; CSNO, nitrosocysteine; DNCB, 1-chloro-2,4-dinitrobenzene; Trx, thioredoxin; TrxR, thioredoxin reductase; HNOX, heme–NO/oxygen; NCM, neonatal cardiomyocyte; MD, molecular dynamics; oTrx, oxidized thioredoxin; PLA, proximity ligation assay; IP, immunoprecipitation; ANOVA, analysis of variance.

pendent vasorelaxation (9). Conversely, another study showed that elevated levels of aldosterone induce *S*-nitrosation and other thiol oxidation of GC1, diminishing its activity (10). All together, these studies indicate that nitrosative/oxidative stress diminishes vascular reactivity through thiol oxidation, including *S*-nitrosation of GC1 (11). Thus, one of the goals of this study is to identify at the molecular and cellular level the mechanism that would restore NO sensitivity of GC1 and preserve vascular reactivity against oxidative/nitrosative stress.

Thioredoxin (Trx), a ubiquitously expressed oxidoreductase, is a major regulator of the cellular oxidative stress and protein thiol redox state (12). Trx1 (the cytosolic form) reduces its client proteins through disulfide exchange between their oxidized Cys and its Cys active site (*e.g.* human Trx1 ^{32}CXXC^{35}), resulting in an inactive and oxidized Trx1 (oTrx1 with a disulfide bond between Cys^{32} and Cys^{35}). Subsequently, oTrx1 can be reduced to the active form by thioredoxin reductase (TrxR), with NADPH as a co-factor. In addition to the disulfide reduction, Trx1 can function as a denitrosylase (*i.e.* removes NO moieties from target proteins) (13–15). The mechanism of Trx1-mediated denitrosation is not fully understood. However, a mechanism similar to disulfide bond reduction has been proposed, in which Cys^{32} interacts with the nitrosated cysteine (SNO-Cys) of a target protein and denitrosates the SNO-Cys via a mixed disulfide and release of nitroxyl (HNO) (14, 16).

Thus, we hypothesized that the reducing activity of cytosolic Trx1 is a potential mechanism by which desensitized/*S*-nitrosated GC1 regains its NO responsiveness (we previously showed that another oxidoreductase protein-disulfide isomerase interacts with GC1, but it is mostly an endoplasmic reticulum resident (17)). As a corollary, nitrosative/oxidative stress could overwhelm and inhibit the constitutive activity of Trx1, affecting the balance between *S*-nitrosated and reduced proteins (12, 14, 16, 18, 19), including GC1. Herein, we describe a previously unknown association between Trx1 and GC1, demonstrate that this interaction takes place via a mixed disulfide and is enhanced by treatment with an *S*-nitrosating agent (*S*-nitrosocysteine (CSNO)), and establish that Trx1 increases GC1 activity and protects it from CSNO-induced desensitization. Our mutational analysis suggests that αCys^{609} of GC1, located at the putative regulatory surface of the catalytic domain, is involved in the interaction with Trx1 and hence in the mechanism of protection. Together, these findings suggest that the Trx1 system could very well maintain vascular reactivity *in vivo* by limiting extensive thiol oxidation of GC1.

Results

We previously showed that GC1 is desensitized to NO stimulation by *S*-nitrosation and other thiol oxidation (7, 9, 10). We reasoned that a mechanism should exist to resensitize GC1 to allow it to be stimulated by subsequent NO signal. Herein we investigated how GC1 response to NO was restored following desensitization. We hypothesized that Trx1, a cytosolic oxidoreductase with denitrosation and reductase activities, could be involved in this process.
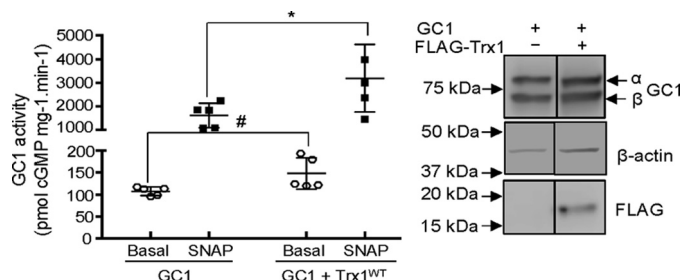
Figure 1. Trx1 enhances GC1 activity. COS-7 cells were transfected with plasmids overexpressing GC1-α and -β subunits with or without FLAG-Trx1^{WT}. The cytosolic fractions of COS-7 cells were assayed for GC1 activity under basal and NO-stimulated conditions. SNAP was used as an NO donor at 100 μM and added to the reaction mix. The NO-stimulated GC1 activity in the presence of Trx1^{WT} overexpression was compared with the GC1 activity without Trx1^{WT} expression. #, $p < 0.05$, by unpaired Student's *t* test; *, $p < 0.05$, two-way ANOVA with Tukey's post hoc test; $n = 5$. Each data point corresponds to two separate transfected wells, pulled together for lysis and with their activity measured in duplicate. *Right*, cell lysates were analyzed by immunoblots to assess the expression of FLAG-Trx1^{WT}, GC1, and β-actin, a loading control. Each sample contained 15 μg of total protein.

Manipulation of Trx1 levels and activity affects GC1 activity

To examine whether Trx1 is involved in modulating GC1 activity, we first transiently expressed in COS-7 cells rat GC1-α and -β subunits with or without co-expression of Trx1 (Trx1 is fused to FLAG; see "Experimental procedures"). Measurements of GC1 activity indicated a significant increase in both basal and NO-stimulated activity when Trx1 was overexpressed (Fig. 1). Western blotting confirmed the expression of Trx1 and GC1 (Fig. 1, *bottom*). Conversely, we observed decreased production of cGMP under basal and NO-stimulated conditions in rat neonatal cardiomyocytes (NCM) depleted for Trx1 (supplemental Fig. S1). These results suggest that Trx1 positively modulates GC1 activity.

To determine whether the redox status of Trx1 impacts GC1 activity and desensitization, we measured GC1 basal and NO-stimulated activity in the absence or presence of 1-chloro-2,4-dinitrobenzene (DNCB), an inhibitor of TrxR, and with or without the addition of CSNO (a nitrosating agent), which induces desensitization of GC1 (7). Inhibition of TrxR is expected to increase the level of oTrx1 (characterized by a Cys^{32}–Cys^{35} disulfide bond in its active site), hence diminishing its reductase and denitrosation activity. A7r5 smooth muscle cells, which lack detectable GC1, were infected with adenoviruses expressing GC α and β subunits (for 48 h, as described previously (9)) and then treated with vehicle or CSNO (100 μM, 30 min) and with vehicle or DNCB (50 μM, 30 min). DNCB did not affect basal activity of GC1 (Fig. 2A, *inset*), yet CSNO activates basal GC activity, as described previously (7). On the other hand, NO-stimulated GC1 activity was significantly decreased by DNCB and even more so by a combination of DNCB and CSNO (Fig. 2A). A biotin switch assay indicated that under these conditions (DNCB + CSNO), GC1 was heavily *S*-nitrosated (Fig. 2B).

All together, these results suggest that Trx1 expression and activity positively impact GC1 activity in cells. We then examined whether the Trx1 effect was dependent on a direct interaction between Trx1 and GC1.

Trx1 and GC1 interact directly in cells

We first demonstrated *in situ* that endogenous Trx1 and GC1 were associated by using a proximity ligation assay (PLA)
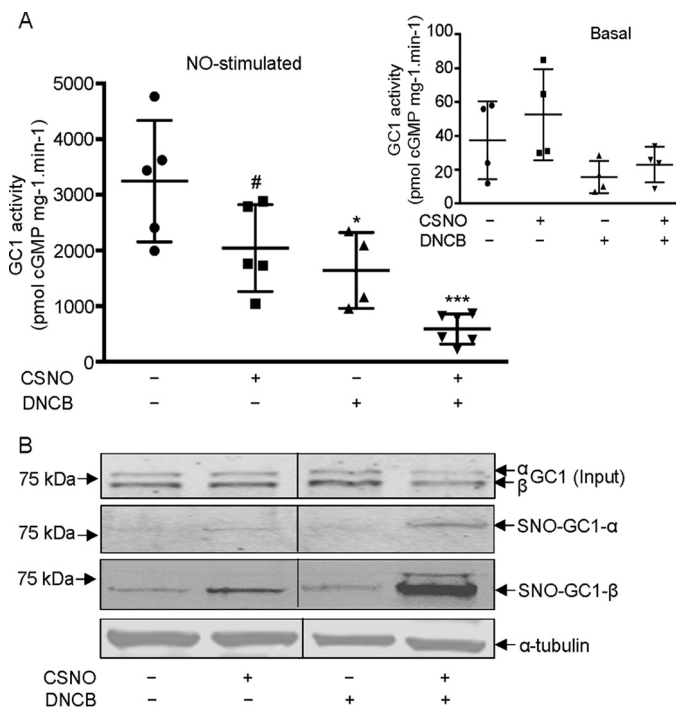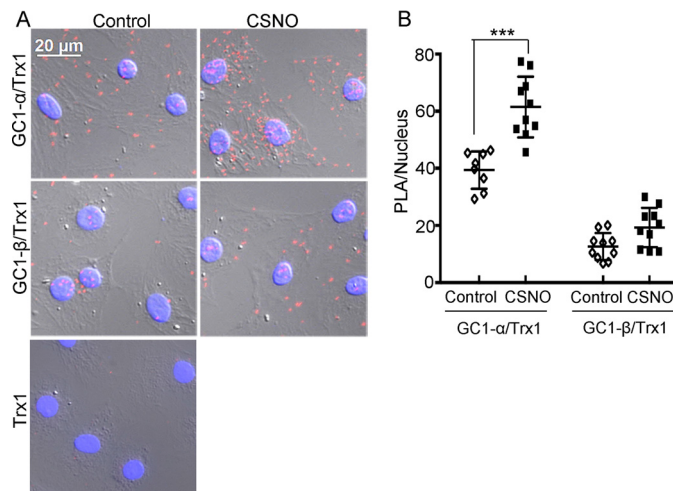
Figure 2. Trx1-mediated regulation of GC activity is redox-dependent. *A*, NO-stimulated and basal (*inset*) GC1 activities were assayed in the lysate of A7r5 cells infected with GC1-α- and GC1-β-expressing adenoviruses, pretreated with CSNO (100 μM, 30 min) or buffer, and then treated or not with DNCB (50 μM, 30 min). Diethylamine (DEA)-NO was added to the reaction mixture and used at 10 μM. #, $p = 0.07$; *, $p < 0.05$; ***, $p < 0.001$ *versus* untreated GC1. $n \geq 4$. One-way ANOVA with Tukey's post hoc test was used. Each data point corresponds to activity measurements done in duplicate of two infected dishes pulled together for lysis. *B*, SNO content of GC1-α and -β subunits were resolved by Western blotting following the biotin switch assay. Lysates prepared from cells were subjected to the same CSNO treatment with or without DNCB as in *A*. Band intensity indicated the extent of protein *S*-nitrosation. α-Tubulin was used for a loading control. The *input* blots indicate that a similar amount of protein was used for analyses. Ethanol and phosphate buffer are the solvent controls for DNCB and CSNO treatment, respectively. *Error bars*, S.D.

Figure 3. Interaction of GC1 with Trx1 *in situ*. *A*, Duolink *in situ* PLA using anti-GC1-α, anti-GC1-β, and anti-Trx1 antibodies in rat NCM (which are amenable to PLA and express detectable levels of endogenous GC1). Interaction between molecules is indicated by a *red* positive reaction. No reaction was detected in the negative control, in which no anti-GC1-α or anti-GC1-β antibodies were added. Nuclei were stained with DAPI in *blue*. Differential interference contrast (*DIC*) was used to visualize cell shape. Magnification was a ×40 objective using a Zeiss 200 microscope. *B*, quantification of PLA reactions from fluorescent microscopy of GC1-α/Trx1 and GC1-β/Trx1 indicates that Trx1 strongly interacts with GC1-α subunit. CSNO treatment significantly enhanced the interaction between GC1-α and Trx1. ***, $p < 0.001$; two-way ANOVA with Tukey's post hoc test was used; $n = 8$–10. *Error bars*, S.D.

(Fig. 3*A*). The assay indicates that Trx1 interacts with both the α and β subunit of GC1, compared with the negative control. This interaction is significantly stronger and more frequent between GC1-α and Trx1 than between GC1-β and Trx1 (Fig. 3*B*), suggesting that Trx1 primarily interacts with the α subunit of the heterodimer. To investigate whether the redox/nitrosative status of cells plays a role in this interaction, we treated the NCM with CSNO (200 μM, 30 min) and repeated the PLA. CSNO treatment significantly promotes the interaction between Trx1 and GC1-α, compared with treatment with control buffer, whereas the Trx1–GC1-β interaction was not significantly enhanced (Fig. 3, *A* and *B*). Because GC1 is *S*-nitrosated by CSNO treatment (7) (Fig. 2*B*), we speculate that *S*-nitrosation of GC1 could facilitate the interaction between Trx1 and GC1; however, we cannot exclude the possibility that *S*-nitrosation of Trx1 could be a factor favoring the interaction as well.

### Trx1 and GC1 interact via a mixed disulfide exchange mechanism

To further characterize the association, we employed a Trx1-Trap mutant, in which the resolving Cys[35] from the Trx1 active site (^{32}CXXC^{35}) is mutated to a serine (Trx1^{C35S}). If Trx1 and its target protein are transiently associated through a disulfide bond, then the target protein will be captured as a mixed disulfide intermediate (20). Trx1, Trx1^{C35S}, or double mutant Trx1^{DM} (Trx1$^{C32S/C35S}$), which should not interact with target proteins, was co-overexpressed with GC1 in COS-7 cells. Trx1 is fused to a FLAG, allowing purification with anti-FLAG antibodies. FLAG-assisted immunoprecipitation of Fig. 4*A* showed that in an exogenous expression system, Trx1 interacts with GC1, confirming the PLA results. GC1 was significantly retained when co-expressed with Trx1^{C35S} (Trx1-Trap), compared with Trx1^{WT} or Trx1^{DM} (Fig. 4*B*), indicating that this association takes place via a disulfide exchange within the active site of Trx1. Of note, using the same Trx1-Trap exogenous expression system, we observed that CSNO treatment enhances GC1 association with Trx1 (supplemental Fig. S2). Thus, both PLA and FLAG immunoprecipitation results indicate a higher level of interaction in an *S*-nitrosating environment, which, we speculate, could be a signal to trigger Trx1-dependent denitrosation of GC1.

Because Trx1 modulates GC1 activity (Fig. 1), we investigated whether the reductase-deficient Trx1 (*e.g.* with a mutated ^{32}CXXC^{35} active site) has an impact on GC1 activity. We conducted the same co-expression as in Fig. 4*A* and measured GC1 activity in cells overexpressing GC1 with Trx1, Trx1^{C35S}, or Trx1^{DM}. Fig. 4*C* showed that the reductase-deficient Trx1 fails to improve significantly NO-stimulated GC1 activity, unlike Trx1^{WT} (no change in basal activity was observed with any combination; not shown). Together, these results indicate that Trx1 and GC1 transiently interact via the Trx1 ^{32}CXXC^{35} active site, and this site is probably involved in the Trx1-dependent modulation of NO-stimulated activity of GC1.
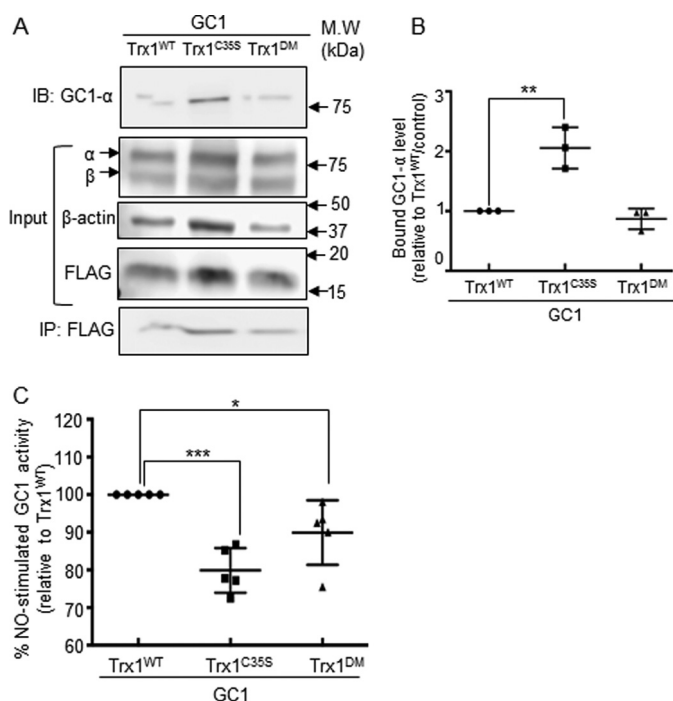
Figure 4. GC1 interacts with the active site of Trx1 (^{32}C*XX*C^{35}). *A*, COS-7 cells were transfected with GC1 and Trx1^{WT}, Trx1^{C35S} (Trap mutant), or Trx1^{DM} (Trx1$^{C32S/C35S}$). The interaction between GC1 and Trx1 was resolved by electrophoresis under reducing conditions and by Western blotting (*IB*) following immunoprecipitation with anti-FLAG. *Input*, starting material; β-actin is a loading control. This blot is representative of three independent experiments. Fifteen micrograms of total protein was used for input of each sample. *M.W.*, molecular weight. *B*, quantification of immunoblot densitometry is shown. **, $p < 0.01$; one-way ANOVA with Bonferroni's post hoc test was used. $n = 3$. The β1 signal is not detectable in the IP elute unless overexposed and as such was not included in the statistical analysis. *C*, cytosolic fractions of COS-7 cells, co-transfected with plasmids encoding GC1-α, GC1-β, FLAG-Trx1, or active site mutants, were assayed for GC1 activity in the presence of SNAP (10 μM). GC1 activity was measured as in Fig. 2*A*. *, $p < 0.05$; ***, $p < 0.001$; one-way ANOVA with Bonferroni's post hoc test was used; $n = 5$. Values are mean ± S.D. (*error bars*).

αCys^{609} is involved in Trx1 interaction with GC1 and modulation of its activity

Recently, we identified, in NCM overexpressing GC1, Cys^{609} as constitutively *S*-nitrosated in the α subunit (21). GC1-αCys^{609} is a potential candidate for interaction with Trx1 because it is a reactive cysteine, which localizes in the α subunit and at the surface of a predicted regulatory interacting region in the catalytic domain (22). Thus, we investigated whether αCys^{609} of GC1 was a target of Trx1 and replaced it with a serine (α^{C609S}). We first measured basal and NO-stimulated activity of the mutant. The NO-stimulated activity of GC1-α^{C609S} was similar to the WT, but the basal activity was significantly reduced by the mutation (basal activity was 108.2 ± 8.8 *versus* 67.5 ± 6.2 pmol·min^{-1}·mg^{-1}). Second, by co-expressing GC1-α^{C609S} with or without Trx1 in COS-7 cells, we showed that the basal and NO-stimulated activity of GC1-α^{C609S} was not increased by Trx1 overexpression (Fig. 5*A*), unlike the Trx1-induced increase of GC1 activity (Fig. 1). Conversely, the Trx1-Trap experiment of Fig. 5*B* shows that GC1-α^{C609S} is not "trapped" by Trx1^{C35S}. Under non-reducing conditions, a GC1–Trx1 complex is detected, as expected, slightly above 150 kDa, but no complex is detected with GC1-α^{C609S} (the same

result was obtained under reducing conditions; supplemental Fig. S3), indicating that αCys^{609} is involved in the interaction between GC1 and Trx1. Together, these results suggest that the inability of Trx1 to modulate GC1-α^{C609S} activity is due to the loss or decreased interaction between Trx1 and the GC1-α^{C609S} mutant.

Trx1 protects GC1 from CSNO-induced desensitization

To this point, we have shown that thiol oxidation of GC1 by CSNO treatment decreases NO-stimulated activity, as is inhibition of TrxR activity, and that Trx1 overexpression enhances NO-stimulated GC1 activity. Thus, we investigated whether one mechanism by which Trx1 regulates GC1 activity is by preventing or reverting CSNO-induced desensitization to NO stimulation. We first co-expressed GC1 or GC1-α^{C609S} and measured GC1 activity under NO-stimulated conditions, following CSNO exposure (Fig. 6) (see "Experimental procedures"). As expected, inhibition of NO-stimulated GC1 activity by CSNO is significant in the WT; on the other hand, CSNO has no effect on NO-stimulated GC1-α^{C609S} activity (Fig. 6, *inset*), suggesting that αCys^{609} could be involved in the desensitization mechanism. We repeated this assay in the presence of Trx1. The *right-hand panel* of Fig. 6 shows that NO-stimulated GC1 activity is not desensitized by CSNO when Trx1 is co-expressed. These results suggest that Trx1 protects GC1 activity from desensitization to NO stimulation. Not surprisingly, because the GC1-α^{C609S} mutant is not sensitive to Trx1 overexpression, there was no alteration of GC1-α^{C609S} activity in the absence or presence of Trx1 regardless of CSNO addition (Fig. 6, *inset*). In addition, we assayed whether the effect of CSNO (in particular at Cys^{609}) induces a more global decreased catalytic activity by measuring GC1 activity in lysates of cells after treatment with CSNO (or buffer), as in Fig. 6, but this time using the heme-dependent stimulator Bay 41-2272 and the heme-independent activator BAY 60-2770 (supplemental Fig. S4). Stimulation by BAY 41 of WT or GC1-α^{C609S} was further increased by pretreatment of the cells with CSNO (suggesting a synergistic effect), whereas BAY 60 activation was also increased but to a lesser extent than BAY 41 stimulation. Thus, the CSNO-induced decrease of GC1 activity appears to be specific to NO stimulation.

Predictive model of a complex between Trx1 and GC1 catalytic domain involving αCys^{609}

The computational model was conducted using GC1 catalytic domain and Trx1 molecular structures (see "Experimental procedures"). Initial docking was achieved by using Trx1-Cys^{32} and GC1-αCys^{609} as interaction sites. This choice was based on the Trx1-Trap assays of GC1 and GC1-α^{C609S} (Fig. 5), suggesting that the two proteins interact via a mixed disulfide exchange involving nucleophilic attack by Trx1-Cys^{32} of GC1-αCys^{609}. This is speculative because we do not know whether the α^{C609S} replacement affects directly or indirectly the interaction and because the model was built with the catalytic domain of GC1 (not the unavailable full-length structure). Complexes with the two Cys residues within a 3.2-Å distance were selected for refinement with molecular dynamics (MD). The model in Fig. 7 represents an average structure over a 20-ns MD simulation of the best docked complex.
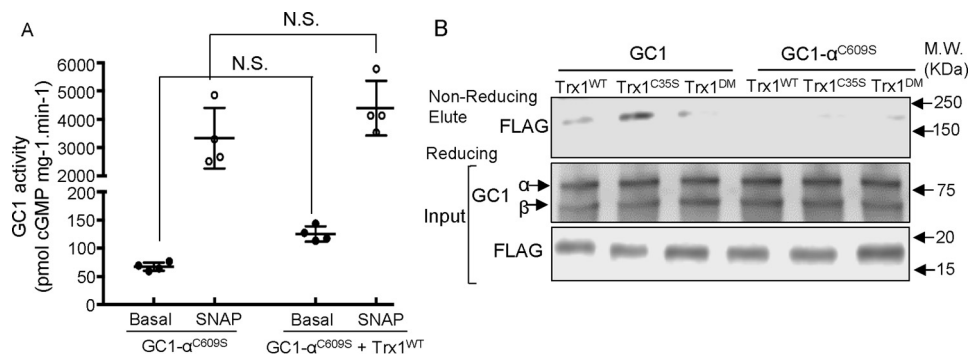
Figure 5. αCys[609] of GC1 is involved in Trx1-mediated modulation and interaction. *A*, GC1 activity under basal and NO-stimulated conditions was measured in COS-7 cells co-transfected with GC1-α[C609S]β with or without Trx1, as described under "Experimental procedures" (10 μM SNAP was added to the reaction mixture for NO-stimulated conditions). Two-way ANOVA with Tukey's post hoc test was used; $n = 4$. Values are expressed as mean ± S.D. (*error bars*). *N.S.*, not significant. *B*, COS-7 cells were transfected with GC1 or GC1-α[C609S] and Trx1[WT], Trx1[C35S], or Trx1[DM]. The cytosol was prepared and immunoprecipitated with anti-FLAG antibodies as described under "Experimental Procedures." A Western blot probed with anti-FLAG antibodies under non-reducing conditions shows that GC1–Trx1[C35S] was pulled down with anti-FLAG as a slightly more than 150-kDa complex (as expected), whereas a GC1-α[C609S]–Trx1[C35S] complex could not be detected. No or little complex could be detected with Trx1[WT] or Trx1[C32S/C35S]. Input of each sample contained 15 μg of total protein. *M.W.*, molecular weight. A Western blot under reducing conditions is shown in supplemental Fig. S3.
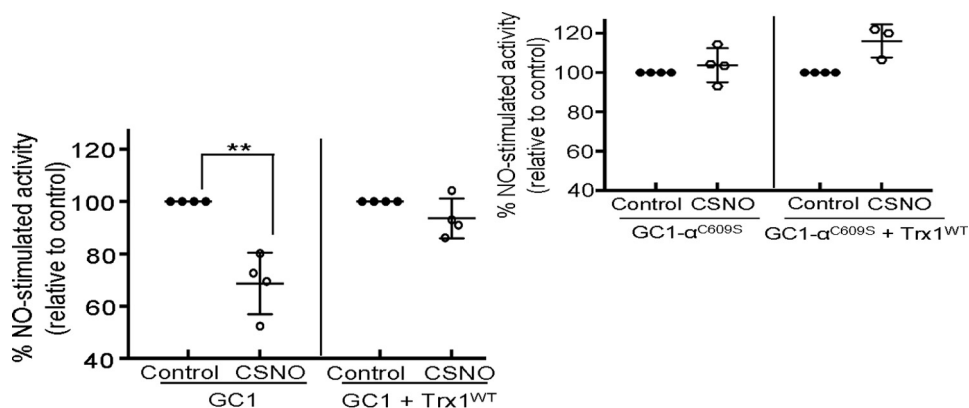
Figure 6. Trx1 protects GC1 from CSNO-induced desensitization. COS-7 cells were co-transfected with plasmids encoding GC1 or GC1-α[C609S] and with or without FLAG-Trx1. Cells were incubated with 200 μM CSNO for 1 h in the dark prior to lysis. NO-stimulated activity (10 μM SNAP) of lysates containing GC1 or GC1-α[C609S] (*inset*) was measured as above. **, $p < 0.01$; two-way ANOVA with Bonferroni's post hoc test was used. Values are mean ± S.D. (*error bars*); $n = 3–4$.

Discussion

The NO–GC–cGMP pathway is a major player in vascular homeostasis, and disruption of this pathway is involved in the etiology of many vascular diseases, including hypertension (23). Understanding the molecular mechanism underlying the regulation of vascular basal tone and reactivity has profound physiological implications. The current *in vitro* study investigated the mechanism by which desensitized GC1 response to NO stimulation is restored or maintained. Our findings revealed that Trx1 could interact with GC1 and protects its activity from desensitization to NO stimulation, potentially by a Trx1-dependent thiol reduction mechanism.

To investigate whether Trx1 regulates GC1 activity, we measured cGMP production by manipulating Trx1 levels in cells. Overexpression of Trx1 significantly enhances GC1 activity, whereas depletion conversely decreases GC1 activity. To determine whether Trx1 activity is necessary to modulate positively GC1 activity, we compromised the reductase activity of Trx1 by mutating the Trx1 [32]CXXC[35] active site and by using DNCB, a TrxR inhibitor that should increase inactive oTrx1. Trx1 mutants, Trx1[C35S] and Trx1[DM] (Trx1[C32S/C35S]), could not significantly enhance NO-stimulated GC1 activity, in contrast to Trx1[WT]. Likewise, inhibition of TrxR activity with DNCB led to

a significant decrease in NO-stimulated GC1 activity. Thus, Trx1 appears to regulate GC1 activity. Also, we previously showed that *S*-nitrosating agents, such as CSNO and other nitrosothiols, inhibit NO-stimulated GC1 activity (7); in the current study, we found that the DNCB inhibitory effect was greatly enhanced by the addition of CSNO. Together with a sharp increase in *S*-nitrosation of GC1 by the DNCB + CSNO combination, these data indicate that desensitization to NO stimulation is probably mediated by a decreased ability of Trx1 to reduce GC1 *S*-nitrosation and other thiol oxidation. Interestingly, it was shown that DNCB reduces vasorelaxation of aortic rings and decreases cGMP levels in response to the NO donor sodium nitroprusside, underlying the patho-physiological relevance of our mechanistic study (25). As shown by others (26), the treatment with CSNO in the 100 μM range probably induces additional thiol oxidations, including sulfenation and disulfide bond, which is also a target of Trx1. These additional thiol oxidations that could affect GC1 activity have yet to be assayed.

Next, we sought to determine whether the observed protection of GC1 from desensitization was due to Trx1 function as a global modulator of cellular nitroso/redox state (*e.g.* to maintain low levels of oxidized proteins) or as a specific reductase of
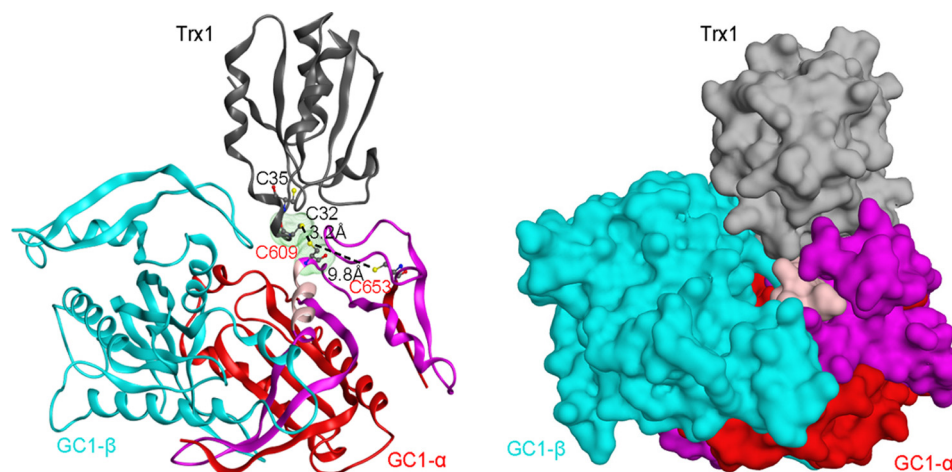
Figure 7. Proposed docking models between Trx1 and the catalytic domain of GC1. The GC1-α subunit is shown in *red*, and the GC1-β subunit is in *cyan*. Predicted interaction regions (as proposed in Ref. 22), residues 573–601 and 610–654, are depicted in *magenta*, and 602–609 is in *pink*. Trx1 is shown in *dark gray*. *A*, Cys^{32} and Cys^{35} of Trx1 active site are indicated, as are Cys^{609} and Cys^{653} in the α subunit. *B*, *surface rendering* of the model. Of note, the docking model predicts a distance of 9.8 Å between αCys^{653} and αCys^{609} of GC1, offering an opportunity of disulfide bonding upon conformational changes.

GC1 via a direct interaction between Trx1 and GC1. Using both PLA in NCM and a Trx1-Trap assay in an exogenous expression system, we demonstrated that Trx1 and GC1 physically associate and that this interaction takes place via a mixed disulfide bond. Intriguingly, via the same assays, we observed that CSNO treatment promotes the interaction between GC1 and Trx1. Moreover, our biochemical study showed that NO-stimulated GC1 activity was not reduced by CSNO treatment, *if Trx1 was overexpressed*, suggesting that Trx-1 protects GC1 from desensitization to NO. Together, these results support our hypothesis that one of the functions of Trx1 is to restore (or maintain) the sensitivity of GC1 to NO, and hence vascular reactivity, by reducing key thiols of GC1. In addition, we speculate that an increased thiol oxidation of GC1 triggers such interaction, because we also observed an increase in GC1–Trx1 complex in rat NCM treated with angiotensin II (PLA; supplemental Fig. S5), a physiologically relevant inducer of oxidative stress implicated in the pathogenesis of hypertension (27, 28). These data would suggest that the initial response to oxidative stress (or following NO exposure) is an increased interaction between GC1 and Trx1, hence allowing GC1 to remain responsive to NO and protecting vascular reactivity.

To further explore a possible mechanism of the interaction via mixed disulfide exchange, we conducted a cysteine mutational analysis of GC1 and concentrated on the α subunit because it is the primary subunit interacting with Trx1, according to our findings. Because there are more than 20 Cys residues in GC1-α (23 Cys for human GC1-α (11)), we eliminated Cys residues that were not predicted to be surface-exposed or thiol-modified. Specifically, we focused on αCys^{609}, because we showed that it is readily *S*-nitrosated (21). Also, from a hydrogen–deuterium exchange mass spectrometry study (22), αCys^{609} is found to reside in the proposed regulatory surface of the catalytic domain (Fig. 7), which could modulate the interaction between the HNOX domain and the catalytic domain. Because the dissociation of these two domains has been proposed as an initial step in the mechanism of activation of GC1 (29), the thiol-redox modulation of αCys^{609} may be key

to this process. Indeed, the mutant GC1-α^{C609S} was resistant to CSNO-induced desensitization to NO, and its activity (basal or NO-stimulated) was not significantly modified by overexpression of Trx1, unlike WT GC1. We also showed that this region in the α catalytic domain can be lysine cross-linked with the β catalytic domain (αLys^{606}–βLys^{539}), a key functional region of the catalytic site (30, 31). To explain the increased GC1 activity with Trx1 overexpression, we speculate that Trx1 potentially competes with the HNOX domain to suppress the inhibitory interdomain interaction in control of GC1 activity. In this view, the mutation GC1-α^{C609S}, which altered Trx1–GC1 interaction could (*a*) decrease the ability of Trx1 to compete with an inhibitory domain and/or (*b*) reduce other *S*-nitrosated Cys residues of GC1 that are responsible for desensitization to NO stimulation. We and others previously showed that Cys^{122} in the β subunit (βCys^{122}) and Cys^{243} in the α subunit (αCys^{243}) are targets of *S*-nitrosation leading to GC1 desensitization to NO stimulation. Conversely, mutating these two Cys residues conferred resistance to desensitization (7), yet the mutation C609S by itself confers resistance to CSNO-induced desensitization. Two possible explanations are (*a*) α^{C609S} mutation precludes *S*-nitrosation of αCys^{243} and βCys^{122} by an unknown mechanism (potentially via Trx1 reductase activity, which is overexpressed in our system); (*b*) αCys^{243} and βCys^{122} are still *S*-nitrosated, but the α^{C609S} mutation prevents the conformational changes induced by SNO-αCys^{243} and/or SNO-βCys^{122} that lead to NO desensitization (32). Which GC1 SNO-Cys residues are reduced by Trx1 or are differently *S*-nitrosated in the α^{C609S} mutant is currently under investigation.

It has been shown that short exposure to NO donor increases GC1 activity via an hsp90-triggered heme insertion in GC1 (33), whereas longer exposure to NO donors decreases active GC1, which, in addition to thiol oxidation, is proposed to be the result of increased apo-GC1 and decreased GC1 heterodimers (34). To determine whether *S*-nitrosation of Cys^{609} causes more general inactivation, COS-7 cells expressing GC1 and GC1-α^{C609S} mutant were treated or not with CSNO, and their lysates activity was assayed in response to BAY 41-2272, which stimu-

lates GC1 with an intact, reduced heme, and BAY 60-2770, which activates GC1 when its heme is oxidized or depleted. Interestingly, BAY 41 synergistically increased WT and mutant activity from cells exposed to CSNO, indicating that BAY 41 stimulation is not blunted by CSNO treatment and that the latter leads to intracellular NO storage in an unknown form. Following CSNO pretreatment, BAY 60 increased GC1 activity of WT and mutant less than 2-fold, which was unexpected because a high concentration of CSNO (200 μM) was shown to induce glutathione depletion and hence a generalized oxidative stress (26), which in turn should impair GC1 activity. This suggests that CSNO treatment does not induce mechanisms of catalytic inactivation other than specific desensitization to NO stimulation in our system. It will be exciting in the future to decipher the mechanisms of CSNO impact on GC1 activity.

As mentioned above, we showed that specific oxidation of Cys thiols in GC1 leads to its desensitization to NO stimulation. In animal models of increased oxidative stress (angiotensin II or aldosterone treatment and during nitrate tolerance (7, 9, 10)), GC1 desensitization and thiol oxidation correlated with decreased vascular reactivity. As such, we propose that dysfunction in the Trx1 system induced by oxidative stress will disturb the dynamic balance between thiol-oxidized and thiol-unmodified GC1, leading to an accumulation of NO-unresponsive GC1, which would contribute, in turn, to the development of oxidative vasculopathies. Therapeutic approaches that would increase Trx1 level may be beneficial for managing diseases associated with GC1 desensitization and resistant to NO donors.

Experimental procedures

Reagents

Unless specifically stated, all biochemical and cell culture reagents were purchased from Sigma-Aldrich and Gibco, respectively. Adenoviruses expressing GC1 and mutants were originally provided by Dr. Papapetropoulos (University of Athens). pCMV5-GC1-α and pCMV5-GC1-β plasmids were used for transient expression of GC1 (35). A cGMP enzyme immunoassay kit (BT-740) was purchased from Alfa Aesar. SDS and all immunoblotting supplies were purchased from Bio-Rad. Sample reducing buffer containing DTT (NP0009) was purchased from Thermo Fisher Scientific. [α-^{32}P]GTP was purchased from PerkinElmer Life Sciences. S-Nitroso-N-acetyl-DL-penicillamine (SNAP; N7927) was purchased from Thermo Fisher Scientific. The Duolink *in situ* red kit mouse/rabbit was purchased from Sigma (DUO92101). Antibodies were obtained from the following companies and used as stated: thioredoxin-1 (Cell Signaling; immunoblotting; 1:1,000), thioredoxin-1 (BD Biosciences; immunofluorescence, 1:200), FLAG (Cell Signaling; immunoblotting; 1:1,000), GC1-α (Sigma; immunoblotting, 1:5,000; immunofluorescence, 1:200), GC1-α (Abcam; immunoblotting, 1:1,000), GC1-β (Cayman; immunoblotting, 1:2,000; immunofluorescence, 1:100), β-actin (Sigma; immunoblotting, 1:8,000), α-tubulin (Millipore; immunoblotting, 1:1,000).

Primary cells

Rat NCM were isolated from 1–2-day-old Wistar rats (Harlan Laboratories, Somerville, NJ) by Percoll gradient centrifugation and plated overnight in cardiomyocyte culture medium, containing 5% horse serum and 100 μM BrdU. The medium from DMEM/F-12 was supplemented with sodium pyruvate, glucose, L-ascorbic acid, BSA, sodium selenite, sodium bicarbonate, and penicillin/streptomycin. Twelve hours after seeding, cells were switched to medium without BrdU or horse serum. All animal experimentation followed the protocol approved by the institutional animal care and use committee of New Jersey Medical School.

Immunoprecipitation and Western blotting

Protein lysates were prepared from COS-7 cells transfected with GC1 or GC1-$\alpha^{C609S}\beta$ and Trx1^{WT}, Trx1^{C35S}, or Trx1$^{C32S/C35S}$ (Trx1^{DM}), respectively. Trx1 was fused with a FLAG tag. For the immunoprecipitation (IP) with Trx1–FLAG, 400 μg of total proteins were precleared with mouse IgG-agarose beads (A0919, Sigma) for 2 h at room temperature and incubated with anti-FLAG beads (M8832, Sigma) overnight at 4 °C. The proteins were eluted from beads with a 2× Laemmli sample buffer and incubated on a heat block at 100 °C for 5 min. The proteins were resolved on 10% SDS-polyacrylamide gels (4568034, Bio-Rad) in the presence or absence of 50 mM DTT for non-reducing or reducing conditions, respectively. Blots were visualized using SuperSignal West ECL system from Pierce followed by LI-COR C-DiGit blot scanner for quantitative analyses.

Biotin switch assay

A7r5 cells were infected with adenovirus expressing GC1-α and -β subunits for 48 h (A7r5 are smooth muscle cells with no detectable GC1). To inhibit TrxR activity, cells were treated with 50 μM DNCB (prepared in ethanol) for 30 min at 37 °C. Then cells were incubated with 100 μM CSNO for 30 min or with control buffer (potassium phosphate buffer: 0.8 M K$_2$HPO$_4$, 0.2 M KH$_2$PO$_4$, pH 7.6) at 37 °C. Protein S-nitrosation was measured by a biotin switch assay as described (9, 36) with minor modifications.

GC1 activity assay

GC1 and Trx1 or mutants were co-expressed in COS-7 cells by transfection for 72 and 48 h, respectively. Cells were incubated with 200 μM CSNO or with control buffer for 1 h at 37 °C in the dark prior to harvest, as described previously (7), and carefully washed three times prior to lysis. Homogenates were obtained by sonication in homogenization buffer (50 mM Tris, 150 mM NaCl, 0.5 mM 3-isobutyl-1-methylxanthine/protease inhibitors), and the cytosolic fraction was used to measure GC1 activity under both basal and NO-stimulated conditions (37). SNAP was used as an NO donor and added to the reaction mixture. The enzymatic reaction was allowed to proceed for 5 min at 30 °C in reaction mixture, containing 100 mM HEPES, pH 8.0, 10 mM MgCl$_2$, and 1 mM GTP (16).

In situ PLA

Isolated rat NCM were incubated with 200 μM CSNO or with potassium phosphate buffer as control at 37 °C for 30 min. Cells

were fixed and permeabilized with 3% paraformaldehyde and 0.2% Triton X-100 for 10 min at room temperature prior to the assay. PLA was performed according to the manufacturer's instructions and as we described previously (16).

Docking model of Trx1 with GC1 catalytic domain

Molecular structures of Trx1 (Protein Data Bank code 1ERT) and catalytic domain of soluble GC (Protein Data Bank code 4NI2) were checked for missing atoms and repaired as needed in MOE2016.08 (Chemical Computing Group, Montreal, Canada) with the structure preparation subroutine. Initial orientation of Trx1 and GC1 complex was achieved by protein–protein docking of Trx1 molecule into GC1 catalytic domain in MOE. Trx1-Cys^{32} and GC1-αCys^{609} were selected as a potential interaction site.

After docking completion, all Trx1–GC1 complexes were evaluated by measuring the distance between Cys^{32} (Trx1) and αCys^{609} (GC1), and those with a distance within 3.2 Å were selected for further refinement with MD. Preparation of the files for MD simulation and the production run were performed with the Amber 2017 molecular dynamics suite (24). Trx1–GC1 protein complexes were solvated in a water box and neutralized with sodium atoms. After initial minimization, heating, and equilibration of the system for 2 ns, the production run for 20 ns was performed for each complex. The distances between Cys^{32} (Trx1) and αCys^{609} (GC1) as well as αCys^{653} (GC1) were closely monitored. The best docking model is presented in Fig. 7 as an average structure over all 20 ns of MD simulation time.

Statistical analysis

p values were calculated by Student's *t* test or one-/two-way analysis of variance (ANOVA). A value of $p < 0.05$ was accepted as significant.

Note added in proof—There were several errors in the version of this article that was published as a Paper in Press on June 28, 2017. Fig. 1 did not indicate the borders between different sections of the same immunoblot. Additionally, the wrong GC1 and actin immunoblots were used. In Fig. 4*A*, the wrong lanes were selected in the input GC1 and IP FLAG immunoblots. Fig. 4*A* contained the wrong input actin and FLAG immunoblots, and supplemental Fig. S3 contained an incorrect actin immunoblot. These errors have now been corrected and do not affect the results or conclusions of this work.

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