



Soluble guanylyl cyclase mediates noncanonical nitric oxide signaling by nitrosothiol transfer under oxidative stress

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ABSTRACT

Soluble guanylyl cyclase (GC1) is an α/β heterodimer producing cGMP when stimulated by nitric oxide (NO). The NO-GC1-cGMP pathway is essential for cardiovascular homeostasis but is disrupted by oxidative stress, which causes GC1 desensitization to NO by heme oxidation and S-nitrosation (SNO) of specific cysteines. We discovered that under these conditions, GC1- α subunit increases cellular S-nitrosation via transfer of nitrosothiols to other proteins (transnitrosation) in cardiac and smooth muscle cells. One of the GC1 SNO-targets was the oxidized form of Thioredoxin1 (oTrx1), which is unidirectionally transnitrosated by GC1 with α C610 as a SNO-donor. Because oTrx1 itself drives transnitrosation, we sought and identified SNO-proteins targeted by both GC1 and Trx1. We found that transnitrosation of the small GTPase RhoA by SNO-GC1 requires oTrx1 as a nitrosothiol relay, suggesting a SNO-GC1 \rightarrow oTrx1 \rightarrow RhoA cascade. The RhoA signaling pathway, which is antagonized by the canonical NO-cGMP pathway, was alternatively inhibited by GC1- α -dependent S-nitrosation under oxidative conditions. We propose that SNO-GC1, via transnitrosation, mediates adaptive responses triggered by oxidation of the canonical NO-cGMP pathway.

1. Introduction

The NO-sensitive guanylyl cyclase (GC1, previously called sGC) is the major receptor for nitric oxide (NO). GC1 is a heme-containing heterodimer composed of one α and one β subunit. Upon binding of NO to the heme in the β subunit, GC1 catalytic activity is stimulated to produce the second messenger, 3',5'-cyclic guanosine monophosphate (cGMP). Association of GC1 α and β subunits is required for cGMP production. The NO-GC1-cGMP pathway is a key player in numerous physiological events such as vasodilatation, inhibition of platelet aggregation and neurotransmission. Oxidative stress is known to disrupt the NO-GC1-cGMP pathway not only through endothelial dysfunction via reduction of NO availability, but also via oxidative reactions involving the thiols and heme of GC1, which make it unresponsive to NO stimulation (for review see Refs. [1–3]). The latter, called NO resistance, is an obstacle to the therapeutic use of NO generators/donors in cardiovascular pathologies, yet explains the remarkable clinical efficiency of small molecule stimulators of GC1 in treating various forms of

pulmonary hypertension and heart failure [4].

We previously showed that NO-stimulated activity of GC1 is inhibited by the thiol oxidation, S-nitrosation, a post-translational modification (PTM) that adds a NO moiety to specific cysteines (Cys) and alters properties (location, activity, interaction) of the S-nitrosated proteins [5]. GC1 is readily and specifically S-nitrosated *in vivo* in an Angiotensin II (Ang II)-induced hypertensive rodent model and during development of nitrate tolerance [6,7], resulting in GC1 desensitization to NO stimulation. Our recent study in neonatal cardiomyocytes (NCM) identified additional modified S-nitrosated Cys (SNO-Cys) in GC1 [8]. While mutation of some of these Cys indicates a role in desensitization to NO stimulation, the functions of other SNO-Cys were unknown.

Transnitrosation is the direct transfer, by protein-protein interaction, of an NO moiety from a thiol on one protein to a target thiol on another protein, enabling a high degree of target specificity of this PTM [5,9]. The list of proteins with transnitrosation activity with physiological relevance is still small [10–12] and includes Thioredoxin 1 (Trx1). Trx1 is a key regulator of redox thiol in cells via reduction of disulfide bonds

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and removal of SNO (denitrosation) of target proteins in a reducing environment [13]. Conversely, in an oxidative environment, we showed that Trx1 becomes oxidized (oTrx1), is S-nitrosated at Cys73 (SNO-oTrx1) and gains the ability to catalyze the transfer of its SNO to target proteins [14]. One of the best-characterized transnitrosation reactions of Trx1 is the transfer of its S-nitrosothiol to caspase-3 [15,16].

While conducting the previous study identifying S-nitrosation sites of GC1 in cardiomyocytes [8], we observed that levels of S-nitrosated proteins increased with over-expression of GC1 in those cells, in particular under oxidative and nitrosative stress. This was surprising because physiological modulation of S-nitrosation levels has been shown to be independent of cGMP levels, using an inhibitor of GC1 activity such as ODQ [17]. Considering that GC1 is the receptor for NO, is heavily yet specifically S-nitrosated, and is often associated with NO synthases [18, 19], we herein explored the intriguing possibility that GC1 could have a transnitrosating activity, in addition to its more conventional cGMP-forming activity.

2. Material and methods

2.1. Reagents

Purified recombinant human GC1 protein was purchased from Enzo Life Sciences (Farmingdale, NY, USA). All antibodies used in this study were from Abcam (Cambridge, MA, USA) unless otherwise indicated. All chemical reagents, including purified recombinant human Trx1 protein and 8-Br-cGMP, were purchased from Sigma-Aldrich unless otherwise indicated. MS-grade trypsin, streptavidin-agarose beads, Tandem Mass Tag (TMT), 10plex isobaric label reagent and the bicinchoninic acid (BCA) protein assay kit were purchased from Thermo Fisher Scientific (Waltham, MA, USA). HPLC-grade acetonitrile (ACN) and water were obtained from T. J. Baker (Center Valley, PA, USA). N-[6-(Biotinamido) hexyl]-3'-(2'-pyridylidithio)propionamide (Biotin-HPDP), EDTA, HEPES, C₁₈ spin columns, and ammonium bicarbonate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The ICAT avidin enrichment kit was purchased from AB Sciex (Framingham, MA, USA). Enhanced chemiluminescent substrate was purchased from PerkinElmer Life Science (Boston, MA, USA). RhoA G-LISA activation kit was purchased from Cytoskeleton (Denver, CO, USA).

2.2. Vectors & adenoviruses

The shRNA against GC1- α and Trx1 were designed from mouse cDNA for HL-1 cells experiments and from rat cDNA for experiments involving NCM; sequences are available upon request. The various shRNAs were subcloned into the plasmid pDC silencer U6 (kindly provided by Dr. Junichi Sadoshima [20]). The vector constructs were transfected in HL-1 cells to over-express the shRNAs or were used to generate adenoviruses expressing rat shRNA for the infection of NCM, as described in Ref. [20]. The adenovirus constructs encoding for rat GC1- α , GC1- β and GC1- α C609 (in rat amino-acid sequence, Cys is in position 609. C610, the position in mouse and human is used for simplicity along the text), used to infect NCM and A7r5, were described previously [21].

2.3. Cell cultures

The mouse cardiac cell line HL-1 was cultured according to Ref. [22]. NCM were kindly provided by Dr. Junichi Sadoshima. Briefly, NCM were isolated from 1 to 2 days old pups from Wistar rats (Harlan Laboratories, Somerville, USA) by Percoll gradient centrifugation and plated overnight in cardiomyocyte culture media containing 5% horse serum and 100 μ M bromodeoxyuridine (BrdU). Plates were coated with gelatin. The solution from Dulbecco's modified Eagle's medium (DMEM)/F-12 was supplemented with sodium pyruvate, glucose, L-ascorbic acid, bovine serum albumin (BSA), sodium selenite, sodium bicarbonate, and antibiotics. Twelve hours after seeding, the cells were transferred to a

medium without BrdU or horse serum. All animal experimentation followed the protocol approved by the Institutional Animal Care and Use Committee of Rutgers University–New Jersey Medical School.

Rat GC1 was overexpressed in NCM by infecting the cells with adenovirus constructs expressing α , α C609 and β subunits with a multiplicity of infection (MOI) of 5, for 48 h. For GC1- α and Trx1 depletion in HL-1 cells, plasmids were transfected with 5 μ g of shRNA and incubated for 48–72 h at 37 °C. In NCM, depletion of Trx1 was achieved via adenoviral infection with a shRNA against rat Trx1. A7r5 cells were cultured in a 60 mm dish at 80% confluency with DMEM medium supplemented with 5% FBS and penicillin/streptomycin.

2.4. Cell treatment and lysate preparations

HL-1 cells transfected with either the vector or GC1- α shRNA or/and Trx1-shRNA for 72 h were treated with or without 100 nM Ang II for 16 h at 37 °C in 5% CO₂. Cells were lysed in a lysis/blocking Buffer (LB) containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 2% SDS, 0.1 mM neocuproine, 0.2 mM PSMF and 20 mM methanethiosulfonate (MMTS), supplemented with a protease inhibitor cocktail. To assess whether cGMP affects the levels of S-nitrosation, the HL-1 cells transfected with either vector or GC1- α shRNAs were first treated with 100 μ M 8-Bromo-cGMP or solvent for 24 h. Next, the cells were treated with 100 nM Ang II for 12–16 h in the Claycomb' medium still supplemented with 100 μ M 8-Bromo-cGMP. Cells were lysed in LB. NCM cells were treated with 100 μ M DETA-NO for 30min, which induces S-nitrosation via formation of biological dinitrosyliron complexes (DNIC [23]) or with control buffer (potassium phosphate buffer to evaluate basal S-nitrosation) and incubated at 37 °C in 5% CO₂. The cells were then lysed in LB. A7r5 cells were infected with 5 MOI of the adenovirus expressing GFP as a vector control, wild-type GC1- α , or GC1- α C610S mutant and incubated at 37 °C with 5% CO₂. After 48 h incubation, the cells were treated or not with 100 nM Ang II and incubated at 37 °C with 5% CO₂ for 16 h. After a total of 24 h incubation, the cells were harvested for biotin switch assay.

2.5. Biotin switch assays

Biotin switch was performed to identify SNO-proteins using a modified protocol from Ref. [24] and as described in Ref. [14]. Briefly, free thiols of proteins were alkylated with 20 mM MMTS in LB at 50 °C for 30 min in the dark with regular agitation. Excess MMTS was removed by cold acetone precipitation and the protein pellets were collected by centrifuging at 5,000g for 10 min at 4 °C and washed 3 times with cold acetone. The protein pellets were re-suspended and the SNO-Cys were biotinylated in a solution containing 25 mM HEPES, pH 7.7, 1 mM EDTA, 1% SDS in HENS buffer, supplemented with 0.8 mM biotin-HPDP and 10 mM ascorbate (Asc), at room temperature (RT) for 1 h in the dark. Excess biotin-HPDP was removed by cold acetone precipitation. For detection of S-nitrosated proteins, biotinylated proteins were separated by a non-reducing 15% SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-biotin antibodies (1:3,000, Abcam, USA). Negative controls were samples without Asc treatment.

The avidin enrichment of biotinylated proteins was performed following the modified protocol from Ref. [14]. Briefly, biotinylated proteins were precipitated in cold acetone at –20 °C for 1 h, followed by centrifugation (5000 g, 10 min at 4 °C). The pellets were dissolved in a buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, and 0.5% SDS. Protein concentrations were determined by the BCA method and adjusted to 1 μ g/ μ l. One hundred μ g of proteins were diluted with 500 μ l of phosphate buffered saline (PBS) then mixed with 100 μ l of streptavidin-agarose beads. The mixture was incubated for 1 h at RT with regular agitation. The beads were pelleted by centrifuging at 500 g for 10 min and washed five times with 1 ml of PBS, then mixed with an 1 \times SDS-PAGE loading buffer containing 10% β -mercaptoethanol (β -ME) and heated at 100 °C for 5 min. The proteins released from

the beads were separated by a reducing 15% SDS-PAGE. The proteins were then transferred onto a nitrocellulose membrane. The membrane was first blocked with 5% milk blocking buffer and then probed with the corresponding antibodies.

2.6. Transnitrosation assays

Between GC1/SNO-GC1 and Trx1/SNO-Trx1. Fifty μg of recombinant human Trx1 were pretreated with 100 mM DTT or 100 mM H_2O_2 at 37 °C for 30 min to generate the reduced (rTrx1) and oxidized Trx1 (oTrx1), respectively. To remove the excess reagents, the proteins were precipitated with cold acetone at −20 °C for 1 h, and the pellets were generated by centrifugation at 5000 g for 10 min at 4 °C, followed by 3 washes with cold acetone. Ten μg of recombinant GC1 or oTrx1 proteins were pretreated with 100 μM S-nitrosoglutathione (GSNO) at 37 °C for 30 min in the dark to S-nitrosate the proteins. S-nitrosated GC1 (SNO-GC1) and Trx1 (SNO-Trx1) were precipitated with cold acetone at −20 °C for 1 h and the pellets were washed 3 times with cold acetone to remove the excess GSNO. The pellets were resuspended in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, pH 7.4. The various combinations of untreated and GSNO-treated proteins were mixed (1 μg GC1 vs. 5 μg Trx1) and incubated for 30 min at 37 °C in the dark; the reactions were stopped by cold acetone precipitation and the samples were analyzed by the biotin switch assay followed by either Western blotting or in-gel trypsin digestion, extraction and desalting for MS analyses. MS analysis of oTrx1 treated with GSNO confirmed that cysteine 73 was the S-nitrosated cysteine under these conditions, as reported previously (not shown) [14].

Between GC1/SNO-GC1, Trx1/SNO-Trx1 and RhoA. SNO-GC1, oTrx1, and SNO-Trx1 were prepared as mentioned above. Five μg of recombinant human RhoA was treated with 100 μM GSNO at 37 °C for 30 min in the dark. SNO-RhoA was precipitated with cold acetone at −20 °C for 1 h and the pellet was generated by centrifugation at 5000 g for 10 min at 4 °C. The pellet was washed 3 times with cold acetone to remove the excess GSNO, and resuspended in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, pH 7.4. The transnitrosation reaction was performed in 50 μl of a buffer containing 50 mM Tris-HCl, 1 mM EDTA, 0.1 mM neocuproine, pH 7.5. Four independent reactions were performed. One μg of SNO-GC1 was mixed with either 0.4 μg of oTrx1 (1), or 0.8 μg of RhoA (2), or 0.4 μg of oTrx1 plus 0.8 μg of RhoA (3). Separately, 0.4 μg of SNO-oTrx1 was mixed with 0.8 μg of RhoA (4). The mixtures were incubated at 37 °C for 30 min in the dark. The transnitrosation reactions were stopped by cold acetone precipitation. The biotin switch was performed to derivatize SNO-proteins into biotinylated proteins. The biotinylated proteins were enriched with streptavidin beads and eluted with 1X Laemmli buffer supplemented with β -ME. The eluted proteins were run on a 12% reducing gel and visualized by Western blotting.

2.7. Transnitrosation of HL-1 proteins by SNO-GC1

SNO-GC1 was produced by incubating 5 μg of recombinant human GC1 α/β heterodimer with 500 μM GSNO at 37 °C for 30 min in the dark. The excess GSNO was removed by cold acetone precipitation and the pellet was washed 3 times with cold acetone, then resuspended in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, pH 7.4. The HL-1 cells were harvested, washed with cold PBS, and lysed with LB. The protein concentration of HL-1 cell lysate was measured with the BCA assay. For *in vitro* transnitrosation of the HL-1 cell proteins, 200 μg of HL-1 proteins were mixed with 5 μg of SNO-GC1 and incubated at 37 °C for 30 min in the dark. The negative controls were HL-1 lysates without GC1 addition or with addition of un-nitrosated GC1. Biotin switch assay, followed by Western blot probed with an anti-biotin antibody, was used to detect S-nitrosated proteins. SNO-proteins were further identified via Avidin enrichment coupled with LC/MS/MS.

2.8. RhoA activity assay

The RhoA activity was measured using a RhoA G-LISA activation assay kit following the manufacturer's instruction (Cytoskeleton, Inc. USA). Briefly, the HL-1 cells were transfected for 24 h with either the vector or shRNA to knockdown GC1- α , and then starved in a serum-free medium for 24 h; HL-1 cells were then treated with or without 100 nM Ang II in a serum-free medium for 24 h at 37 °C in a CO_2 incubator. The cells were treated with either DMSO or 100 $\mu\text{g}/\text{ml}$ calpeptin for 30 min at 37 °C, then collected and lysed with the lysis buffer provided in the kit. Following the supplier instruction, RhoA activity was determined as a function of the HRP absorbance signal at 490 nm (each sample was measured in duplicate).

2.9. In-solution digestion and TMT labeling

Protein pellets were dissolved in 300 μl of 100 mM triethylammonium bicarbonate (TEAB), at pH 8.5. Two hundred micrograms of proteins were digested at a protein/trypsin weight ratio of 20:1, and at 37 °C overnight. The digested peptides were labeled with each of the TMT 10-plex reagent according to the manufacturer's instruction. After labeling, the ten peptide samples were combined and desalted using C_{18} cartridges and fractionated via high-pH RPLC separation. Peptides were separated with a 105-min binary gradient (Solvent A: 10 mM ammonium formate and Solvent B: 10 mM ammonium formate and 85% ACN) using a XBridge BEH 130 C_{18} column (4.6 X 250 mm, 5 μm , Waters USA) on ACQUITY UPLC system (Waters USA). Next, the samples were used for affinity enrichment of biotinylated peptides to study the transnitrosation changes among the peptides.

2.10. Identification of biotinylated-peptides by LC/MS/MS

The peptides were desalted using AB Sciex ICAT Cation Exchange Buffer kit and the biotinylated peptides were enriched using the AB Sciex ICAT Affinity Buffer pack, following the manufacturer's instruction. The enriched peptides were desalted with Pierce C_{18} spin columns, dried in a SpeedVac and re-suspended in 10 μl of mobile phase A (2% ACN and 0.1% formic acid in H_2O). Analysis of peptides was carried out via LC/MS/MS analysis on an LTQ-Orbitrap Velos Pro mass spectrometer (MS, Thermo Fisher Scientific), coupled with an Ultimate 3000 Chromatography System. The peptides were first trapped on a C_{18} pre-column (Pepmap C_{18} , 5 mm \times 300 μm , Dionex) at 2% mobile phase B (85% ACN and 0.1% formic acid), at a flow rate of 30 $\mu\text{l}/\text{min}$. The peptides were separated on a 15 cm C_{18} PepMap100 column (75 μm capillary 3 μm , 100 Å, Dionex), using an 85 min gradient from 1% to 50% of mobile phase B, at a flow rate of 250 nl/min . The eluted peptides were introduced into the MS through a Proxeon Nanospray Flex™ ion source. The spray voltage was 2.15 kV and the capillary temperature was 275 °C. The spectra were acquired in a data-dependent mode. Full scans of the MS spectra (from m/z 300–2000) were acquired in an Orbitrap analyzer at a resolution of 60,000 (at m/z 400), with the lock mass option enabled. Ten most intense peptide ions with charge states of 2–4 were sequentially isolated and fragmented using collision-induced dissociation (CID) with a normalized collision energy of 30%. The ion-selection threshold was set at 3000 for the MS/MS analysis.

2.11. Data analysis

Protein database searches were performed with the Proteome Discoverer software. The LC-MS/MS spectra were searched against an UniProt mouse database (downloaded on March 22nd, 2019 with 54,109 entries), or rat database (downloaded on March 20th, 2019 with 29,942 entries) or human database (downloaded on March 1st, 2022 with 78,787 entries) using the Sequest search engine on Proteome Discoverer (V2.3) platform. The search parameters were set as follows: trypsin with 2 missed cleavage sites; precursor mass tolerance was set at

10 ppm and the fragment mass tolerance was set at 0.5 Da. Oxidation of methionine, cysteine biotinylation (Biotin HPDP), methylthiolation (MMTS) and N-terminal TMT modifications were set as variable modifications. Protein and peptide False Discovery Rates were filtered to less than 1%. The SNO motif analysis was performed using the MoMo software ([25], <http://meme-suite.org>) with the following parameters: motif-x as the searching algorithm, motif width is 21 (10 amino acids on each side of the centered SNO-Cys), minimum number of occurrences as 5, and binomial probability threshold for Residue/Position pair is < 0.0001 .

2.12. Statistical analysis

Differences were considered statistically significant based on Student's T-test, with p values < 0.05 , $n = 3$ for all LC/MS/MS experiments. Data are expressed as means \pm S.E. For the densitometry analysis, statistical analysis was performed using a two-tailed unpaired Student's t -test with Excel (data were expressed as means \pm SEM). Statistical analysis for Rho-A activity measurements in HL-1 cells was performed by two-way ANOVA followed by Tuckey posthoc test with $p < 0.05$ and $n = 5$.

3. Results

3.1. GC1 modulates cellular S-nitrosation (SNO) via specific transnitrosation

We recently showed that GC1 is S-nitrosated at multiple Cysteines (Cys) in neonatal cardiomyocytes (NCM) treated with S-nitrosating agents [8]. Some of the S-nitrosated Cys (SNO-Cys) of GC1 are involved in desensitization to NO stimulation of GC1 activity [2,7,26] but the function of the other SNO-Cys remains unknown and their impact on cellular SNO has yet to be explored. We serendipitously observed that overexpression of GC1 in NCM increases SNO cellular levels (not shown). Thus, our first goal was to determine whether GC1 has the ability to modulate S-nitrosation in cells. Considering the critical role of S-nitrosation in cardiac health and disease [27,28], we used a mouse cardiac cell line (HL-1 [22]), which expresses a functional GC1 (producing detectable cGMP levels in response to NO stimulation but not under basal conditions) to assess GC1-dependent S-nitrosation. We used two complementary approaches by assessing a) the decreased levels of S-nitrosation in HL-1 cells depleted for the α subunit of GC1 and b) the increased levels of S-nitrosation of HL-1 protein lysates mixed with SNO-GC1. We then applied nitroso-proteomics to identify, quantify and compare the SNO-GC1-dependent targets from both approaches.

In the (a) approach, we used shRNA against mouse GC1- α and the physiologically relevant oxidant Ang II, which induces GC1 S-nitrosation *in vivo* and *in vitro* [6]. We chose to deplete the α subunit of GC1 because it contains a higher number of reactive Cys than the β subunit [2]. We verified that Ang II treatment increases cellular S-nitrosation levels, and the efficient depletion of GC1- α shRNA knockdown (Supplementary Fig. 1). We then assayed the levels of S-nitrosation in lysates of Ang II-treated HL-1 cells not depleted for GC1- α subunit (control), depleted for GC1- α (shGC1- α) and depleted for GC1- α but complemented with 100 μ M 8-Bromo-cGMP. The latter condition aimed to determine whether S-nitrosation was affected by production of cGMP or lack thereof. S-nitrosation was detected by biotin switch assays of the cell lysates followed by Western blotting with anti-biotin antibodies. Fig. 1a indicated that GC1- α is involved in Ang II-induced nitrosation and that addition of 8-Br-cGMP could not restore cellular S-nitrosation to the level of Ang II-treated HL-1 cells not depleted for GC1- α . Densitometry analysis indicated a significant SNO decrease in both GC1- α -depleted HL-1 cells treated or not with cGMP compared to control cells (Fig 1b; $n = 4$). This needed to be confirmed by showing that the opposite was true, i.e., exposure of HL-1 cell lysates to SNO-GC1 leads to increased SNO-proteins.

In the (b) approach, purified GC1 α/β heterodimer was S-nitrosated with S-nitrosoglutathione (GSNO) then mixed with lysates of HL-1 cells to determine whether SNO-GC1 can transnitrosate target proteins. S-nitrosation levels were assayed as above in HL-1 lysates with no added GC1 (Ctrl), with untreated GC1 and with GC1 treated with GSNO (SNO-GC1) (Fig. 1c). Western blots probed with anti-biotin displayed a drastic increase in S-nitrosation when SNO-GC1 was added to the cell lysates, compared to controls. These results indicated that SNO-GC1 could catalyze transnitrosation of proteins.

3.2. Proteomics analyses of GC1 SNO-targets in cardiac cells

We conducted a comprehensive proteomics analysis to quantify and identify GC1 transnitrosation targets in three groups of Ang II-treated HL-1 cells: (1) not depleted for GC1- α and (2–3) depleted for GC1- α \pm 8-Br-cGMP (as in Fig. 1a). The tryptic peptides derived from the biotinylated proteins were labeled with 10-plex tandem mass tag (TMT) reagents prior to avidin enrichment, and were analyzed by LC/MS/MS (workflow and method of the experiment are described in Supplementary Fig. 2). Three hundred and forty-one proteins had significant decreased S-nitrosation in GC1- α depleted HL-1 cells compared to the non-depleted control (TMT ratio < 0.8 , $p < 0.05$, $n = 3$). The proteins group identified from the MS/MS analysis, including their SNO-Cys sites, are referred as group A (Dataset1 Table S1).

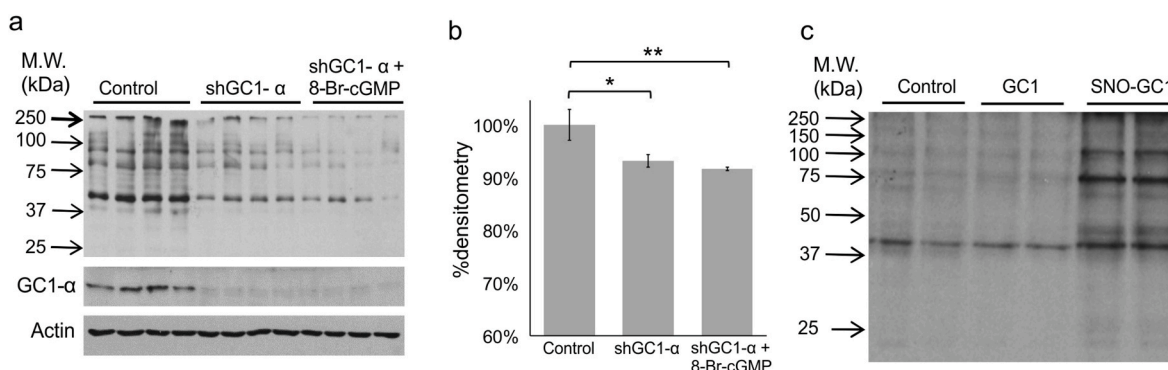


Fig. 1. Biotin switch assays show that S-nitrosation levels in HL-1 cardiac cells are modulated by GC1 expression, independently of cGMP. (a) Western blot (WB) of biotin switch assays of lysates from AngII-induced S-nitrosation in HL-1 cells (control), in HL-1 cells depleted for GC1 α subunit (shGC1- α) and GC1- α -depleted cells pretreated with 8-Bromo-cGMP. Lower panel confirms effective depletion of GC1- α levels using shRNA; β -actin is a loading control. WB were probed with anti-biotin, anti-actin or anti-GC1- α antibodies; $n = 2$ independent experiments. (b) Graph of densitometry analysis from WB; $n = 4$; Student's T-test compared to control; *, $p < 0.05$; **, $p < 0.01$; data are means \pm SEM. (c) WB of biotin switch assays probed with anti-biotin from HL-1 lysates with no GC1 added (control) or mixed with untreated GC1 (GC1) or mixed with GSNO-treated GC1 (SNO-GC1) $n = 2$.

Likewise, biotinylated samples obtained from the HL1 lysates mixed with or without SNO-GC1 α/β (similar to Fig. 1c) were avidin purified and analyzed by LC/MS/MS. Using a label-free quantification method, 1886 biotinylated peptides in over 1000 proteins were reproducibly identified with more than two-fold increase in S-nitrosation compared to the control ($n = 2$). The proteins group identified from the MS/MS analysis are referred as Group B and includes the identified SNO-peptides (DataSet1 Table S2).

The HL-1 proteins which are S-nitrosated targets of GC1 from Group A (decreased SNO with depletion of GC1- α) and from Group B (increased SNO in the presence of SNO-GC1) were compared to build a Venn diagram (Fig. 2a). One hundred and ninety-seven overlapping proteins between these two groups were identified as potential SNO-targets of GC1, as well as their common SNO-Cys sites (highlighted in DataSet1 Table S3). Bioinformatics analysis of these 197 proteins using the MoMo software [25], identified four potential consensus SNO-motifs, which may be important for regulating GC1 binding or transnitrosation (Fig. 2b and Supplementary Fig. 3). Among the 197 proteins, some belong to apoptotic pathways, glycolytic processes, cell redox homeostasis and interestingly, 24 SNO-proteins are involved in calcium signaling, muscle contraction regulation and cytoskeleton dynamics (Supplementary Table 1), pathways that are linked to the canonical NO-GC1-cGMP pathway [29]. Among them was the small GTPase RhoA, part of the

RhoA/ROCK muscle contraction pathway, which is antagonized by the NO-GC1-cGMP pathway which prevents muscle contraction and induces muscle relaxation [30,31].

3.3. SNO-GC1 transnitrosates RhoA and inhibits its activity

To establish whether GC1 transnitrosation activity has a cellular function, we investigated further S-nitrosation of RhoA in our cell system, as RhoA activity is inhibited by S-nitrosation [32,33]. We analyzed in HL-1 cells depleted for GC1- α whether RhoA S-nitrosation was decreased and correlated with increased RhoA activity under oxidative stress. HL-1 cells were transfected with shRNA against mouse GC1- α or empty vector as control, then treated with vehicle or Ang II (100 nM, 16 h), as above. S-nitrosation was assessed by a WB of biotin/avidin switch assay of the cell lysates probed for $\alpha 1$, $\beta 1$, and RhoA. Fig. 3a indicated that Ang II treatment leads, as expected, to increased S-nitrosation of GC1- α , GC1- β , and RhoA in HL-1 cells (lane 1 vs. lane 2), yet the depletion of GC1- α (lanes 3, 4) mostly abolishes S-nitrosation of RhoA (lane 4 vs. lane 2) and decreases SNO-GC1- β to some extent. The drastic decrease of SNO-RhoA in GC1- α -depleted cardiac cells validates the proteomics results of Fig. 2 and confirms that S-nitrosation levels of RhoA depend on GC1- α expression. We next measured RhoA activity in the lysates of HL-1 cells depleted or not for GC1- α and treated with vehicle or AngII, as above. As an additional control, we used calpeptin, which positively regulates RhoA in cells (via inhibition of a tyrosine phosphatase [34]). Fig. 3b shows that compared to basal RhoA activity (DMSO), calpeptin significantly increased RhoA activity in both GC1- α -depleted and control HL-1 cells. When the non-depleted HL-1 cells were treated with Ang II to induce S-nitrosation, the calpeptin-induced RhoA activity was blunted compared to control HL-1 cells (DMSO), as expected if RhoA is S-nitrosated. In sharp contrast, Ang II-treated GC1- α -depleted cells displayed a significant increase in calpeptin-induced RhoA activity compared to their DMSO control and also compared to calpeptin + Ang II-treated non-depleted cells. Hence, the decreased S-nitrosation of RhoA in HL-1 cells depleted for GC1- α correlates with an increase in RhoA activity under Ang II-induced oxidative stress, suggesting that GC1- α transnitrosates RhoA, and in turn potentially inhibits RhoA activity. As such, transnitrosation could be another means by which GC1 antagonizes RhoA signaling pathway, in particular under nitrosative stress/oxidative stress when GC1 is desensitized to NO stimulation via S-nitrosation.

3.4. SNO-GC1 transnitrosates oxidized Trx1 in cells and in vitro

From the proteomics studies, GC1 SNO-targets that are known to interact directly with GC1 were identified and included Hsp90 β [18,35], CCT η [36], HSP70 [37] and Trx1 [21]. Trx1 was of particular interest because it is a known regulator of thiol redox in cells with the ability, in addition to its reductase activity, to modulate both denitrosation and transnitrosation [13]. In fact, Cys73 is S-nitrosated in oxidized Trx1 (oTrx1) and has the ability to transfer the SNO group to other targets [14]. Thus, we further investigated GC1's ability to catalyze directly S-nitrosation of Trx1 and assessed whether GC1's modulation of SNO in cells could be mediated and amplified by transnitrosation of Trx1.

To validate the proteomics identification of Trx1 as a transnitrosation target of GC1- α , we conducted a biotin-avidin assay that assayed S-nitrosation levels of Trx1 in HL-1 cells depleted for GC1- α , under basal or oxidative conditions (100 nM Ang II, 16 h). HL-1 cells were transfected with shRNA against mouse GC1- α , or empty vector as above. Fig. 4a indicated that S-nitrosation of GC1- α and Trx1 increases with Ang II treatment, as expected ("SNO" lane 2 vs. lane 1) and that shRNA-depletion of GC1- α was efficient (lane 3 and 4, upper panel). The depletion did not affect Trx1 expression but drastically decreased S-nitrosation of Trx1 (SNO-Trx1 panel, lanes 3–4), confirming that GC1 has the ability to transnitrosate Trx1 in cardiac cells. To establish whether the SNO transfer was direct through a GC1/Trx1 complex or

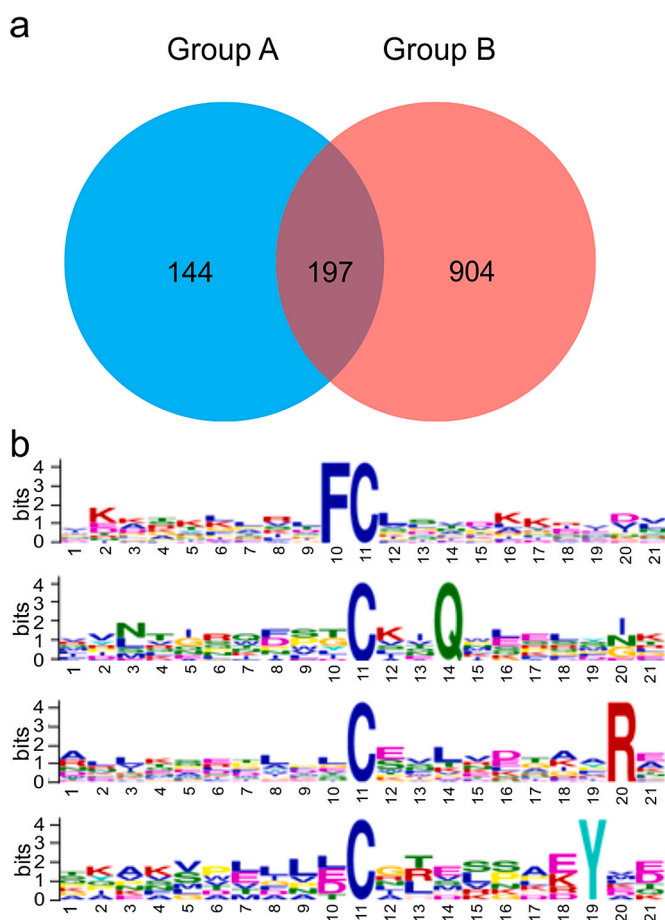


Fig. 2. Mass Spectrometry identification of GC1-dependent SNO targets and potential SNO-GC1 specific motifs. (a) Venn diagram from overlapping proteins with decreased SNO in HL-1 depleted for GC1- α (group A, 341 proteins) and HL-1 SNO-proteins with increased SNO when exposed to SNO-GC1 (group B, 1101 proteins) indicates that 197 proteins are GC1-dependent SNO targets. (b) Determination of GC1-specific SNO consensus motifs using Motif X analysis with the following parameters: motif width: 21; minimum occurrences for residue/position pair: 5; binomial probability threshold for Residue/Position pair: 0.0001.

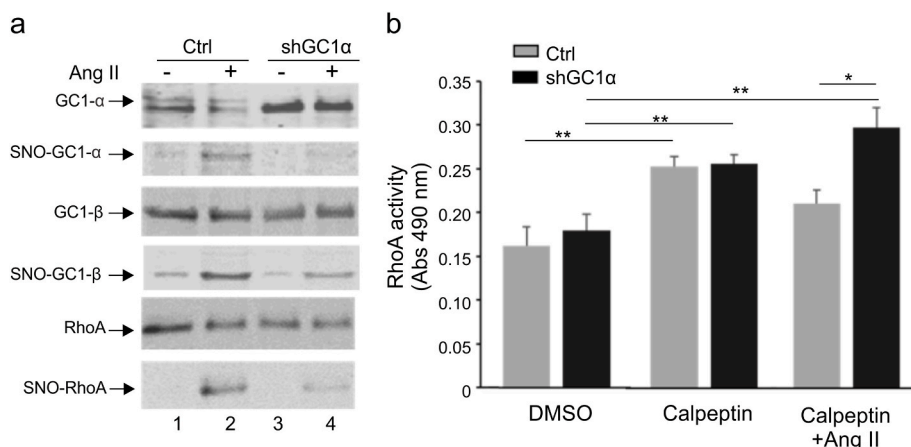


Fig. 3. Cardiac cells depleted for GC1- α have decreased S-nitrosated RhoA and increased RhoA activity under oxidative stress. (a) Representative WB of a biotin/avidin assay measuring S-nitrosation of GC1- α , GC1- β and RhoA in HL-1 cells depleted for GC1- α (shGC1- α , lane 3 and 4) or not (Ctrl, lane 1 and 2) and treated with Ang II (lane 2,4) or vehicle (lane 1,3). $n = 2$ independent experiments. (b) RhoA activity was measured in cells depleted for GC1- α (■) or not (□) following treatment with calpeptin and calpeptin + AngII (DMSO was the vehicle control). $n = 5$, *: $p < 0.05$, **: $p < 0.01$; data are expressed as means \pm SEM.

dependent on other proteins, we used purified Trx1 and GC1 α/β heterodimer. As we previously showed that only the oxidized form of Trx1 (oTrx1) is S-nitrosated [14], we first oxidized Trx1 then treated oTrx1 or GC1 with GSNO to generate SNO-oTrx1 and SNO-GC1, which were mixed with untreated GC1 or untreated oTrx1 or reduced Trx1 (rTrx1). The transfer of the SNO groups between the molecules was assayed by biotin switch assay. Fig. 4b confirmed our observation in cells and showed that SNO-GC1 has the ability to transnitrosate oTrx1 *directly* (lane 2 compared to lane 1 with a clear decrease in SNO-GC1 bands intensity and appearance of SNO-Trx1 bands) but the opposite was not true, i.e. SNO-oTrx1 could not transnitrosate GC1 (lane 5 compared to lane 4). Of note, SNO-oTrx1 was detected as both dimer and monomer under the non-reducing electrophoretic conditions. Moreover, GC1 transnitrosation of Trx1 could only be seen with oTrx1 as this transnitrosation did not occur with rTrx1 (lane 3). Interestingly, a denitrosation activity of rTrx1 toward SNO-GC1 was not observed when rTrx1 was mixed with SNO-GC1 (lane 3 vs. lane 1, SNO-GC1 bands intensity remains the same). We next conducted a LC/MS/MS analysis to identify the Cys that were involved in the transnitrosation reaction between SNO-GC1 and oTrx1 (Fig. 4c). As above, GC1 was treated with GSNO, then mixed with oTrx1, and the relative LC/MS ion intensities of NEM-labeled (free SH) vs. biotin-labeled (SNO) peptides were determined. Left panel of Fig. 4c indicated that the SNO-GC1 peptide containing α C610 has the highest levels of SNO (biotinylation $>65\%$); in addition, α C79 and β C174 have a lower but discernible level of S-nitrosation ($\sim 40\%$). After mixing with oTrx1, α C610 showed a large drop in biotinylation/S-nitrosation, as did α C79 and β C174 (right panel, Fig. 4c). These results indicate that α C610, and to a lesser extent α C79 and β C174 are involved in the GC1 \rightarrow oTrx1 transnitrosation. The mapping by MS/MS of SNO- α C610 is shown in the lower panel of Fig. 4c; MS analysis of SNO-oTrx1 identified Cys73 as the recipient of the transfer (not shown). We next determined whether GC1- α C610 was essential to GC1 transnitrosation activity and verified whether the GC1- α subunit alone, in the absence of the GC1- β subunit hence a functional GC1, could support the transnitrosation reaction. Adenoviruses expressing, GFP, GC1- α , or a mutant for which C610 was replaced with a serine (GC1- α C610S) were used to infect A7r5 smooth muscle cells, which do not express detectable GC1, as previously described [21]. Biotin/avidin assays of Fig. 4d indicated that under Ang II treatment, S-nitrosation was increased in control (GFP-infected) cells and further increased in cells infected with the GC1- α WT subunit but this Ang II-induced increase was blunted in cells infected with the mutant GC1- α C610S and was similar to GFP-infected cells (upper panel). The expression of GC1- α WT and mutant was similar, yet the SNO levels of GC1- α C610S were remarkably reduced compared to GC1- α WT (Fig. 4d, lower panel). Conversely, the elevated levels of SNO-Trx1 and also SNO-RhoA seen in α WT-infected A7r5 cells treated with Ang II were remarkably decreased in the cells

infected with the mutant and comparable to the levels seen in GFP-infected cells (Fig. 4d, lower panels; the expression levels of Trx1 and RhoA remain similar under the different conditions). These results confirm that cellular levels of SNO-Trx1 (and SNO-RhoA) are dependent on SNO-GC1 under oxidative stress, do not require a functional cGMP-forming GC1 heterodimer, and identify C610 of GC1- α as involved in the transnitrosation activity *in vitro* and in cells. Because SNO-Trx1 is itself a nitrosothiol relay, we sought to identify SNO-targets of Trx1 that are also S-nitrosated by GC1. These common SNO targets could reflect, in cell types expressing GC1, a potential SNO-GC1 \rightarrow oTrx1 \rightarrow protein transnitrosation cascade, which may regulate the specificities of S-nitrosation for select proteins.

3.5. Several SNO-targets of Trx1 are also SNO-GC1 targets

We conducted a proteomics analysis to quantify and identify transnitrosation targets in neonatal cardiomyocytes (NCM) overexpressing GC1 and depleted or not for Trx1. This study was done under basal and nitrosative conditions using DETA-NO (100 μ M, 30 min). GC1 was overexpressed by infection with adenoviruses and Trx1 was depleted by infection with a shTrx1-RNA (NCM infected with scramble shRNA were used for negative control). The workflow and description of the experiment to identify and quantify SNO-peptides by LC/MS/MS as a function of Trx1 absence or presence, using a 10-plex TMT quantitative proteomics approach, is provided in Supplementary Fig. 4. In NCM overexpressing GC1 and depleted for Trx1 (compared to NCM not depleted for Trx1), proteomics analysis identified significant decrease of SNO-peptides in 85 and 74 proteins, under basal (DataSet1 Table S4, $n = 2$) and nitrosative (DataSet1 Table S5, $n = 3$) conditions, respectively (TMT ratio <0.8 , $p < 0.05$). Interestingly, several Cys of the GC1- α subunit (but not the β subunit) displayed increased S-nitrosation when Trx1 is depleted and, strikingly, S-nitrosation of GC1- α C610 was increased 665-fold under these conditions (Supplementary Table 2). We next searched for SNO-targets that are common to both GC1 (Groups A and B, Fig. 2a) and Trx1 (group C, DETA-NO conditions) under oxidative/nitrosative stress and identified 30 proteins for which S-nitrosation is dependent on both GC1 and Trx1 expression (Fig. 5, Venn diagram); the identity of the SNO peptides in the 30 GC1/Trx1-targeted proteins are indicated in Supplementary Table 3. RhoA was one of the identified proteins targeted by both GC1 and Trx1, with one SNO-RhoA peptide targeted by both GC1 and Trx1 (HFC¹⁰⁷PNVPIILVGNGK), while the other identified SNO-RhoA peptide was specific for GC1 (IGAFGY-MEC¹⁵⁹SAK). To validate our proteomics findings, we conducted a biotin/avidin assays using lysates of HL-1 cells depleted for GC1- α , Trx1 or both, and treated with Ang II or untreated (Supplementary Fig. 5). We observed a decrease in overall S-nitrosation for cells depleted for GC1- α or Trx1 that was more pronounced when both GC1- α and Trx1 were

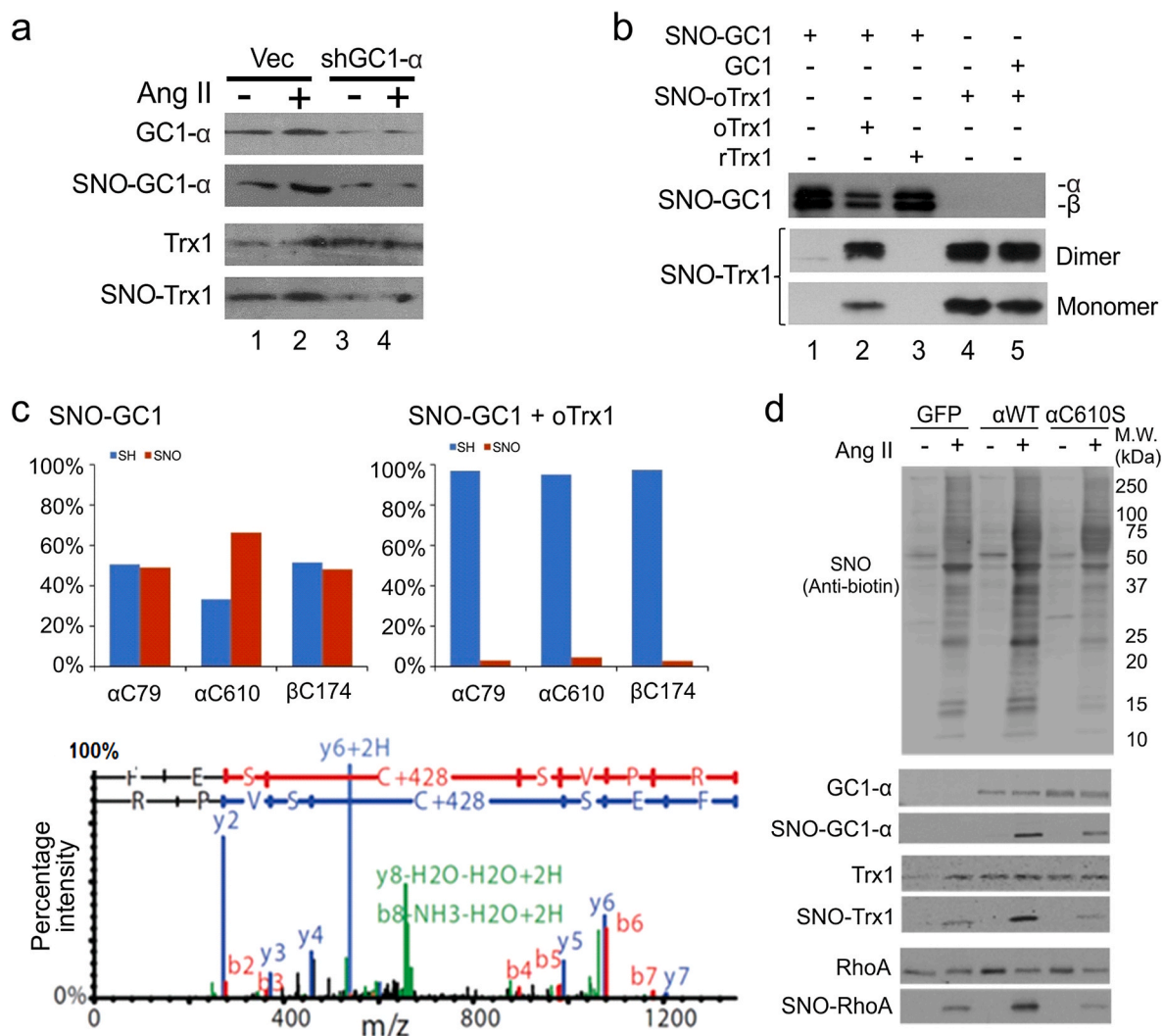


Fig. 4. GC1 transnitrosates Trx1 in cells and in a purified system. (a) Representative WB of a biotin/avidin assay measuring SNO in HL-1 cells depleted for GC1-α (shGC1-α, lane 3 and 4) or not (vec, lane 1 and 2) and treated with Ang II (lane 2,4) or solvent (lane 1,3). n = 2; vec = empty vector; (b) Representative WB (probed with anti-biotin, non-reducing gel) of a biotin switch assay of purified proteins indicating transfer of SNO groups between SNO-GC1 (lane 1) and oTrx1 (both monomer and dimer, lane 2). No SNO transfer took place between SNO-GC1 (lane 1) and rTrx1 (lane 3) or between SNO-oTrx1 (lane 4) and GC1 (lane 5). (c) MS identification of the SNO-Cys of GC1 involved in the SNO transfer. Relative quantification (expressed as percentage) of free thiol (SH) and biotinylation/S-nitrosation (SNO) of GSNO-treated GC1 prior to mixing with oTrx1 (left panel) and after mixing with oTrx1 (right panel). Bottom panel: mapping of SNO-αC610 by LC/MS/MS (mass of Cys +428 amu). SNO-αC610 was identified by the y-(red) and b-(blue) ions. (d) Representative WB of a biotin/avidin assay measuring global SNO and specific SNO-[GC1-α, Trx1 and RhoA] in Ang II-treated (+) or not (-) A7r5 cells infected with adenoviruses expressing GFP, GC1-αWT or αC610S. Upper panel was probed with anti-biotin (non-reducing gel). WB of input and avidin enrichment (lower panels) to detect expression and S-nitrosation of GC1-α, Trx1 and RhoA for WT, mutant and control GFP. n = 2 independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

depleted. Conversely, depletion of GC1-α or Trx1 alone or together, drastically decreased SNO-RhoA levels. These biotin/avidin assays confirmed that S-nitrosation of RhoA requires both GC1-α and Trx1 in HL-1 cells. As we know that GC1-α transnitrosates Trx1 in cells and in a purified system, we hypothesize that a transnitrosation cascade SNO-GC1→oTrx1→RhoA could be at work.

3.6. oTrx1 is a required nitrosothiol relay from GC1 to RhoA in vitro

Our proteomics analysis and avidin assays of oxidized HL-1 cells depleted for GC1-α and Trx1 did not allow us to determine whether GC1 transnitrosates directly RhoA or/and requires Trx1. As we established that SNO-GC1 transnitrosates oTrx1, we investigated GC1-dependent S-nitrosation of RhoA in the absence or presence of oTrx1 in a purified system. After S-nitrosation of GC1, SNO-GC1 was mixed with oTrx1 or RhoA separately, or with oTrx1 and RhoA together. SNO-oTrx1 was also

prepared and was mixed with RhoA alone. S-nitrosation levels under the various combinations were assayed by biotin/avidin assay (Fig. 6). Fig. 6a (left panel) indicated the inputs used in the mixes, while Fig. 6b (right panel) showed the resulting S-nitrosation for each sample and combination. In the right panel, lane 1 confirmed that SNO-GC1 transnitrosates oTrx1, lane 2 indicates that RhoA is S-nitrosated when mixed with SNO-GC1 and oTrx1; in addition, SNO-GC1 and SNO-Trx1 were not detected in lane 2 suggesting that GC1 and oTrx1 have transferred the majority of their nitrosothiols. Remarkably, mixing SNO-GC1 and RhoA alone (lane 3) does not produce any detectable SNO-RhoA and consequently the SNO-GC1 signal is not decreased, suggesting that SNO-GC1 cannot directly transnitrosate RhoA. On the other hand, mixing SNO-Trx1 and RhoA (lane 4) results in S-nitrosation of RhoA while the SNO-Trx1 intensity is greatly reduced (compared to lane 6, SNO-control) confirming a transnitrosation reaction from SNO-Trx1 to RhoA. Overall, this biotin/avidin assay in a purified system suggested that S-nitrosation

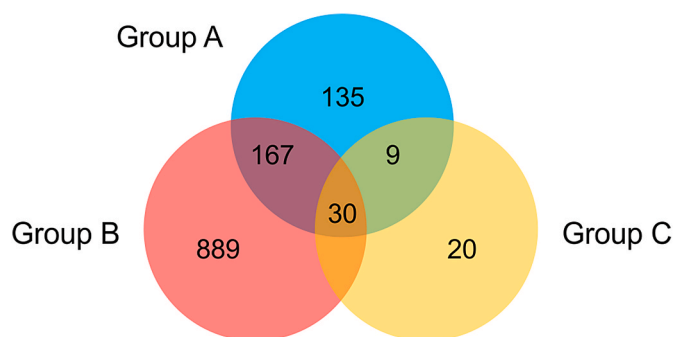


Fig. 5. SNO-targets dependent on both GC1- α and Trx1 expression under oxidative/nitrosative stress. Venn diagram indicating that 30 proteins are SNO targets of both GC1 and Trx1; group A and B are GC1-dependent SNO targets as in Fig. 2a. Group C are Trx1-dependent SNO targets under nitrosative conditions.

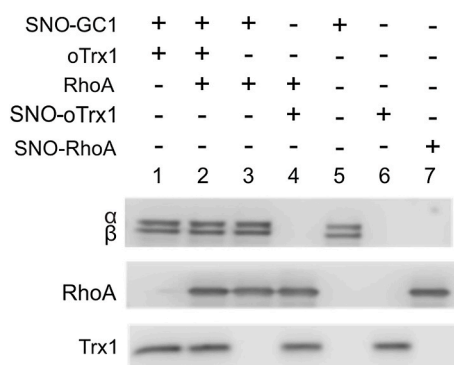
of RhoA by GC1 requires oTrx1 as an intermediate, i.e. oTrx1 is a nitrosothiol relay for the SNO-GC1 initiated transnitrosation cascade.

4. Discussion

Our study shows that GC1, the receptor and effector of NO signaling via its cGMP-forming activity, also bears the “moonlighting” function of modulating the redox/nitrosothiol balance of cells by extensively and specifically transferring S-nitroso groups (SNO) to the cysteines (Cys) of other proteins (transnitrosation). The paradigm emerging from our current study is that GC1 supports two pathways of NO signaling by driving the NO-cGMP pathway under homeostatic conditions, while initiating transnitrosation cascades under oxidative conditions. This dual function of GC1 is apparently amplified by its ability to interact with the oxido-reductase Trx1, a major regulator of cellular thiol redox state. We previously showed in a cellular system that under reducing conditions GC1 forms a complex with reduced Trx1 (rTrx1), which in turn enhances its NO-stimulated GC1 activity, potentially by preventing the formation of SNO-GC1 [21]. Conversely, our current study indicates that under oxidative conditions, GC1 forms another type of complex with oxidized Trx1 (oTrx1) to propagate nitrosative NO signaling via this distinct transnitrosation mechanism. The redox state of the cells is critical in the initiation of these two divergent pathways; in a reduced state, Trx1 denitrosates proteins because the dithiols active site of Trx1 has free thiols ($C^{32-SH}XXC^{35-SH}$) while in an oxidized state, the dithiol site is oxidized to a disulfide allowing oTrx1 to be S-nitrosated at C73 and to transnitrosate proteins [14,38]. Under this same oxidized state, GC1 is desensitized to NO stimulation because of heme oxidation and S-nitrosation (thiol oxidation) of its Cys.

Using biochemical approaches, we demonstrated that cellular S-nitrosation increases with GC1- α subunit (GC1- α) overexpression and decreases with GC1- α depletion in cardiac and smooth muscle cells under Ang II-induced oxidative stress. Our proteomics analysis showed that under oxidative conditions, GC1- α modulates the S-nitrosation of approximately 200 proteins in HL-1 cardiac cells. This is probably an underestimate because the knockdown of GC1- α was not complete, but it would mean that GC1 (or GC1- α) has direct interaction with over 200 proteins, seemingly a high number of specific interactions. This does not have to be the case if oTrx1 is a nitrosothiol relay between GC1 and its SNO-targets. In fact, oTrx1 has a well-established transnitrosation activity, is ubiquitously expressed and is an oxido-reductase with a wide spectrum of potential targets [13]. As Trx1 was one of the 200 identified SNO-GC1 targets, we used purified GC1, Trx1 and their S-nitrosated forms to show that GC1, once S-nitrosated, transfers its S-nitrosothiol to the oxidized form of Trx1 but not its reduced form, supporting the idea that the redox state of the cells is a factor in triggering this transnitrosation cascade. MS identification confirmed that C73 of oTrx1 was the unique SNO-recipient, as previously reported [13–15] and that GC1- α C610 was a major SNO donor to oTrx1. The role of GC1- α C610 was supported by a mutational analysis in A7r5 SMC, which also showed that the α subunit alone is necessary and sufficient to execute the transnitrosation reaction. This was an important observation for two reasons; a) in the canonical NO-stimulated cGMP-forming activity, GC1 is only functional as an α and β subunit heterodimer, and b) GC1 inactivation by S-nitrosothiols/heme oxidation leads to partial heterodimer disruption with accumulation of isolated SNO-subunits [39], supporting the concept that GC1 transnitrosation activity is triggered by oxidative stress following desensitization of GC1. This observation does not rule out that SNO-GC1 heterodimers are still available to conduct transnitrosation in cells. Likewise, whether their specificity for SNO-targets is different from isolated SNO-subunits (α and potentially β) is currently under investigation and will require to assay *in vitro* transnitrosation reaction with separately purified α and β subunits. The critical involvement of α C610 in transnitrosation activity was further confirmed by the MS observation that in cardiac cells depleted of Trx1, the SNO levels of α C610 were increased by more than 600-fold, suggesting that Trx1 is by far the predominant recipient of SNO from GC1 and that without its primary target, SNO- α C610 accumulates. Interestingly, our previous study in cells and *in vitro* under reducing conditions [21] indicated that α C610 was involved in the interaction between GC1 and Trx1, probably via a mixed disulfide with the $C^{32-SH}XXC^{35-SH}$ Trx1 active site and we speculated that rTrx1 protected GC1 from S-nitrosation, hence desensitization to NO stimulation, in cells. Here, we did not observe denitrosation of SNO-GC1 by Trx1 in the purified experimental system, but it is possible that the extensive S-nitrosation of multiple Cys of purified GC1 by GSNO precludes the formation of a complex with rTrx1.

a. Input



b. Biotin/avidin assay

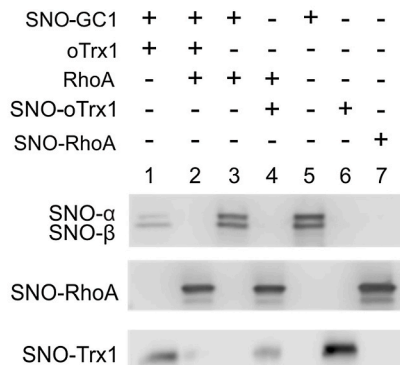


Fig. 6. *In vitro* confirmation of a SNO-GC1→oTrx1→RhoA transnitrosation cascade. (a) WB of the purified proteins (input, S-nitrosated or not) used in the various mixes to conduct the biotin/avidin assays. (b) WB of the biotin/avidin assay to detect S-nitrosation between GC1, oTrx1 and RhoA. Lane 1 indicates that SNO-GC1 transnitrosates oTrx1. Lane 2 shows that SNO-GC1 transnitrosates RhoA in the presence of oTrx1. Lane 3 shows that SNO-GC1 cannot directly transnitrosate RhoA. Lane 4 shows that SNO-oTrx1 transnitrosates RhoA. Lane 5, 6 and 7 are controls of S-nitrosation with GSNO of GC1, oTrx1 and RhoA, respectively. Both WB were probed with anti- α , anti- β , anti-RhoA, and anti-Trx1 and electrophoresis was conducted under reducing conditions (hence Trx1 only shows as a monomer). n = 2 independent experiments.

Interestingly, we did not find any evidence in cells or *in vitro* of a SNO transfer from Trx1 to GC1 whether it was under reduced or oxidative/nitrosative conditions. This unilateral SNO-transfer has previously been observed, for example from Caspase-3 to XIAP but not vice-versa [40]. We speculate that the non-reciprocal SNO transfer could be due to a structurally favorable interaction between SNO-GC1 and oTrx1, interaction that does not occur between GC1 and SNO-oTrx1. As S-nitrosation of Cys is expected to induce conformational changes [9], once SNO-GC1 and oTrx1 have interacted, and the SNO-transfer occurred from GC1- α C610-SNO to Trx1-C73SH, then GC1- α C610SH and Trx1-C73-SNO could dissociate. Of note, α C610 appears to fulfill most conditions for a reactive cysteine in a protein-interactive region as the recently published structure of GC1 [41] shows that it is located at an exposed surface of the heterodimer, in a short loop separating an α helix and β strand, and forming an hydrogen bond with K606 and in proximity of acidic residues (including E608) [5,42].

Our proteomics analysis in oxidized cells depleted of GC1- α also revealed that several targets of GC1-mediated transnitrosation were functionally related to the canonical NO-cGMP signaling pathway. We explored the intriguing possibility that GC1-transnitrosation activity could reflect a rescue mechanism that compensates for the oxidative-induced disruption of the NO-stimulated cGMP-forming activity. RhoA was one of these targets and we used it as a case study because a) it was S-nitrosated by GC1 under Ang II-induced stress, b) in a reducing environment, the RhoA-Rock signaling pathway is antagonized by the NO-cGMP pathway to modulate contraction/relaxation [29–31], and c) RhoA activity is inhibited by S-nitrosation [32,33,43]. The increased activity and decreased S-nitrosation of RhoA in the HL-1 cells depleted for GC1- α and treated with Ang II, suggest that GC1- α -dependent S-nitrosation of RhoA would lead to its inhibition under oxidative stress. We propose that the physiological/pathological relevance of GC1 transnitrosation activity is, in some cases, to compensate for the oxidation-dependent loss of the NO-stimulated GC1 activity.

The ability of oxidized Trx1 to itself drive transnitrosation prompted us to conduct proteomics analysis of Trx1-depleted neonatal cardiomyocytes under nitrosative stress. We identified SNO-targets of Trx1 that are also SNO-GC1 targets, among them was RhoA. One RhoA SNO-Cys peptide (C107) was targeted by both GC1- α and Trx1. This raised two possibilities: a) GC1 and Trx1 could independently S-nitrosate RhoA at Cys107 and/or b) S-nitrosation of C107 depends on a SNO-GC1/oTrx1 complex. The possibility of GC1 directly S-nitrosating C107 of RhoA was excluded by our transnitrosation assay in a purified system revealing that GC1 cannot directly S-nitrosate RhoA unless oTrx1 is present. In addition, the fact that SNO-C107 of RhoA is a common target of oTrx1 and of the oTrx1/GC1 complex suggests that a function of oTrx1 is to impart a specificity for GC1-dependent S-nitrosation of C107, with the caveat that our transnitrosation assay involved the full SNO-GC1 (α/β heterodimer) while cellular transnitrosation activity and specificity could involve the interaction of oTrx1 with the α subunit alone. Mutational analysis of RhoA had suggested that C16 and C20 of the redox active site and C159, but not C107, were involved in SNO-dependent inhibition of RhoA activity [32,33]. However, C107 was recently shown to be located in an allosteric pocket and to be targeted by covalent small inhibitors leading to RhoA inactivation [44], providing a means by which GC1-dependent S-nitrosation of C107 could affect RhoA activity. The other GC1/Trx1 common SNO-targets (including RhoA SNO-C159) will require future research to determine the specificity and extent of transnitrosation cascades mediated by a SNO-GC1/oTrx1 complex or SNO-GC1 alone, and whether their physiological relevance is to provide an adaptive response to oxidative stress. Our evidence of unilateral transfer of SNO from GC1 to Trx1 to RhoA and the likelihood of other targets (yet to be validated) is consistent with the idea of signaling by specific S-nitrosothiol relays, as proposed by others [5]. To our knowledge, only one tri-component S-nitrosation cascade, which propagates aberrant S-nitrosation in neurodegenerative diseases, has been identified to date [45]. While stable and specific S-nitrosation of

proteins is mostly achieved by transnitrosation, the number of proteins with transnitrosation activity remains strikingly small [11]. To reconcile this small number with the multiple (patho)physiological events modulated by transnitrosation, one can think of an amplification process, which could be achieved by existence of transnitrosating units (two of them have been described [10,12]). Another means is by global modification of the thiol-redox state of cells, as was shown recently with transnitrosation-induced repression of an S-nitrosoglutathione reductase [46] and as we show in the current study with SNO-GC1 transnitrosation of oTrx1 which becomes “transnitrosation competent” due to its own oxidized state.

5. Conclusion

We proposed that GC1 mediates NO signaling by a dual cGMP-forming and transnitrosating activity as a function of the thiol redox state of the cells. The fact that GC1 catalyzes S-nitrosation of oTrx1 and initiates transnitrosation cascades such as the SNO-GC1 \rightarrow oTrx1 \rightarrow RhoA could have clinical relevance. Previous studies have shown that the α and β subunits of GC1 exhibit differential expression and cellular localization in several cell types and, pathologically, increased expression of GC1- α correlates with increased proliferation in different tumor cell types including endometrial cancer cells [47] and advanced prostate cancer [48], independently of cGMP levels. The upregulation of Trx1 in carcinogenesis is well documented but poorly understood as Trx1 could provide anti-oxidant defense against tumor development in an oxidized milieu, yet it also seems to be a key factor in supporting highly invasive, aggressive tumors and with upregulation of S-nitrosation [49–51]. In fact, oTrx1 was shown to promote cancer cells proliferation by inhibiting caspase-induced apoptosis via transnitrosation [16]. Together with the well-known cardioprotective effect of Trx1-dependent S-nitrosation [52], these observations underlie the importance of the identification of SNO-GC1 as a major and specific source of SNO-oTrx1, and could open new avenues of clinical research.

Data sharing

Mass Spectrometry data for protein S-nitrosylation have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXPXD033105 and 10.6019/PXD033105.

Tables of S-nitrosated proteins derived from the MS analysis are included in this manuscript as Dataset1 Tables S1 to S5.

Declaration of competing interest

No competing interest to declare.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2022.102425>.

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