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Redox Modulation of Endothelial Nitric Oxide Synthase by Glutaredoxin-1 through Reversible Oxidative Post-Translational Modification

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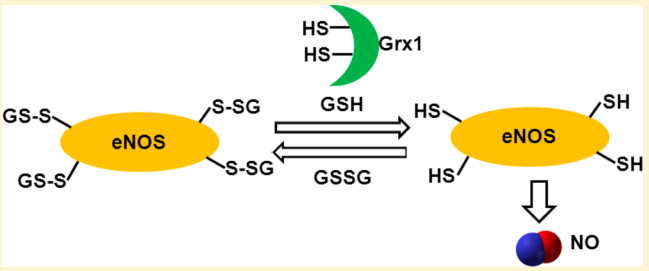
ABSTRACT: S-Glutathionylation is a redox-regulated modification that uncouples endothelial nitric oxide synthase (eNOS), switching its function from nitric oxide (NO) synthesis to $^{\bullet}O_2^-$ generation, and serves to regulate vascular function. While *in vitro* or *in vivo* eNOS S-glutathionylation with modification of Cys689 and Cys908 of its reductase domain is triggered by high levels of glutathione disulfide (GSSG) or oxidative thiyl radical formation, it remains unclear how this process may be reversed. Glutaredoxin-1 (Grx1), a cytosolic and glutathione-dependent enzyme, can reverse protein S-glutathionylation; however, its role in regulating eNOS S-glutathionylation remains unknown. We demonstrate that Grx1 in the presence of glutathione (GSH) (1 mM) reverses GSSG-mediated eNOS S-glutathionylation with restoration of NO synthase activity. Because Grx1 also catalyzes protein S-glutathionylation with an increased [GSSG]/[GSH] ratio, we measured its effect on eNOS S-glutathionylation when the [GSSG]/[GSH] ratio was >0.2, which can occur in cells and tissues under oxidative stress, and observed an increased level of eNOS S-glutathionylation with a marked decrease in eNOS activity without uncoupling. This eNOS S-glutathionylation was reversed with a decrease in the [GSSG]/[GSH] ratio to <0.1. Liquid chromatography and tandem mass spectrometry identified a new site of eNOS S-glutathionylation by Grx1 at Cys382, on the surface of the oxygenase domain, without modification of Cys689 or Cys908, each of which is buried within the reductase. Furthermore, Grx1 was demonstrated to be a protein partner of eNOS *in vitro* and in normal endothelial cells, supporting its role in eNOS redox regulation. In endothelial cells, Grx1 inhibition or gene silencing increased the level of eNOS S-glutathionylation and decreased the level of cellular NO generation. Thus, Grx1 can exert an important role in the redox regulation of eNOS in cells.



Endothelial nitric oxide synthase (eNOS) is responsible for the enzymatic production of nitric oxide (NO) at the expense of nicotinamide adenine dinucleotide phosphate (NADPH).[1−3] NO is a critical small molecule generated within the endothelium, involved in regulating vascular tone, vascular growth, platelet aggregation, and modulation of inflammation.[1,2,4] In many cardiovascular diseases, a decrease in the level of bioavailable NO is a hallmark of endothelial dysfunction, and a common mechanism underlying this dysfunction is the overproduction of reactive oxygen species (ROS) in the vasculature.[5−7] Under conditions of oxidative stress, it has been demonstrated that eNOS can become uncoupled, resulting in further ROS generation. Oxidation of the NOS cofactor, tetrahydrobiopterin (BH_4), an increase in the number of cellular methylarginines, and eNOS S-glutathionylation all have been demonstrated to increase the level of eNOS uncoupling, with secondary eNOS-derived ROS generation contributing to many cardiovascular diseases.[8−15]

eNOS S-glutathionylation was first observed to occur in endothelial cells under oxidative stress and in vessels of hypertensive rats.[14] Cells treated with PABA/NO can also exhibit this post-translational modification of eNOS.[15] We have previously shown that eNOS S-glutathionylation at two specific sites of the reductase domain (Cys689 and Cys908) triggers a marked increase in the level of generation of superoxide ($^{\bullet}O_2^-$) from the isolated enzyme, as well as from eNOS in endothelial cells and intact vessels. More recently, studies have further supported the importance of eNOS S-glutathionylation, as this modification was observed in endothelial cells treated with nitroglycerin,[16] and streptozotocin-induced hyperglycemia in rats or that with isosorbide 5-mononitrate treatment.[17] Together, these results demonstrate that eNOS S-glutathionylation is a critical modification that can redox-modulate its function under cellular oxidative stress, which occurs in a broad range of cardiovascular diseases.

Similar to protein phosphorylation, protein S-glutathionylation is a reversible post-translational modification. It can redox-regulate protein function through the formation of a mixed-disulfide bond between active cysteine residues and glutathione. This modification has been shown to regulate protein function

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through either activation or inactivation under oxidative stress.[18,19] Moreover, the formation of this mixed-disulfide bond provides a protective effect that can prevent the further irreversible oxidation of active protein thiols with an increase in the level of ROS generation.[20−22]

Because protein S-glutathionylation is a reversible modification, the reduction of this mixed-disulfide bond, a process termed deglutathionylation, is expected when the intracellular redox state is normalized after oxidative stress. Grx1, a cytosolic oxidoreductase, has been demonstrated to specifically and efficiently reduce protein mixed-disulfide bonds with deglutathionylation at the expense of reduced glutathione (GSH).[23,24] This Grx1-catalyzed deglutathionylation process plays an important role in redox regulation and signaling under oxidative stress, which occurs in cardiovascular disease, neurodegenerative disease, and cancer.[23,24] Several studies have demonstrated that Grx1 not only effectively reduces this thiol modification but also can glutathionylate target proteins through Grx-catalyzed disulfide exchange or the formation of a stabilized Grx-glutathionyl disulfide anion radical.[25−29] The dynamic steady state of this Grx1-catalyzed thiol modification depends on the cellular ratio of GSSG to GSH.[30] This dual role of Grx1 makes it an important player in redox modulation of proteins with active thiols and in signal transduction.

In this study, we demonstrate that eNOS S-glutathionylation of cysteine residues in the reductase domain can be reversed by Grx1 in the presence of GSH. However, when the level of oxidant stress is increased with a corresponding increase in the level of GSSG, the reaction by Grx1 switches from deglutathionylation to glutathionylation and oxidatively modifies eNOS with S-glutathionylation and inactivation of NO production. Mass spectrometry identifies a new site of this modification at Cys382 on the surface of the eNOS oxygenase domain. We further demonstrate that inhibition of Grx1 or *grx1* gene silencing leads to eNOS dysfunction in endothelial cells through oxidative modification and S-glutathionylation of eNOS. Thus, Grx1 has an important role in regulating eNOS function, catalyzing deglutathionylation of eNOS under conditions of normal thiol redox balance, while with a shift to oxidation of the glutathione pool, it induces glutathionylation at a unique site on the oxygenase, downregulating eNOS activity without uncoupling, providing a unique mechanism of eNOS redox regulation.

MATERIALS AND METHODS

Materials. Anti-NOS3 (C-20) HRP and anti-NOS3 (C-20) agarose conjugate (AC) antibodies were both obtained from Santa Cruz (Santa Cruz, CA). Anti-Grx1 was from Abcam (Cambridge, MA). Anti-GSH was from ViroGen (Watertown, MA). NADPH, L-Arg, calmodulin, hemoglobin, *N*-ethylmaleimide (NEM), GSH, GSSG, HEPES, and Tris were from Sigma-Aldrich (St. Louis, MO). Secondary anti-rabbit and anti-mouse IgG-HRP antibodies were purchased from GE Life Sciences (Piscataway, NJ).

Protein and Heme Concentration Determination. The protein concentration of purified eNOS was determined by the Bradford assay from Bio-Rad (Hercules, CA) using a bovine serum albumin standard. The heme content of the purified eNOS was determined by the pyridine hemochromogen assay as previously described.[31,32]

eNOS S-Glutathionylation by GSSG. Purified eNOS (1 μg/μL) was incubated with 2 mM GSSG at room temperature (RT) for 20 min, within which time both Cys689 and Cys908 were S-glutathionylated.[14] After incubation, the excess GSSG was removed when the sample was passed through a 5 mL HiTrap desalting column from GE Life Sciences (Piscataway, NJ) and concentrated or through buffer exchange using a 50 kDa cutoff centricon from Millipore (Billerica, MA).

Deglutathionylation of S-Glutathionylated eNOS. The deglutathionylation of eNOS was tested in a reaction in which S-glutathionylated eNOS was incubated with either 1 mM GSH or 1 mM GSH and 1 μM Grx1 at RT for 10 min. For immunoblotting analysis, the reaction was quenched by adding NEM to a final concentration of 10 mM at specified time points. For NO activity measurements, the reaction aliquot was directly incubated with all cofactors in a given volume for 1 min before the reaction was started.[33]

Measurement of NOS Activity. The production of NO from purified eNOS was measured by the conversion of ferrous oxyhemoglobin to ferric methemoglobin at RT. The initial rate of NO production was determined from the slope of $\Delta A_{401-411}$ ($\Delta\varepsilon_{401-411}$ = 38600 M^{-1} cm^{-1}) using an Agilent (Santa Clara, CA) 8453 UV−visible spectrophotometer.[33] The reaction mixture contained 10 μg of CaM, 100 μM BH_4, 100 μM L-Arg, 200 μM NADPH, 200 μM $CaCl_2$, and 10 μM oxyhemoglobin in 50 mM Tris-HCl (pH 7.4) in a total volume of 500 μL. Normally, 2−5 μg of eNOS was used, and the reaction was initiated by the addition of 10 μL of 10 mM NADPH. For FAD and FMN supplementation, 4 μM FAD and FMN were incubated with glutathionylated or deglutathionylated eNOS for 5 min before the NOS activity measurement. The activity of untreated eNOS is 85−100 nmol mg^{-1} min^{-1} at RT.

Immunoblotting for eNOS or eNOS S-Glutathionylation. The procedure for immunoblotting for eNOS and eNOS S-glutathionylation was followed as previously described.[14,32]

Site-Directed Mutagenesis of Cys689 of eNOS to Ala. The single mutation of C689 of eNOS to Ala was generated to determine the effect of this specific cysteine residue on flavin binding. The wild-type eNOS gene was mutated at position 689 to Ala using the QuikChange mutagenesis kit from Stratagene (Santa Clara, CA). The sequences of primers for the C689 to Ala mutation were 5′ GGCGACGAGCTGGCCGGCCAGGAGG 3′ and 5′ CCTCCTGGCCGGCCAGCTCGTCGCC 3′.

Flavin Adenine Dinucleotide (FAD) and Reduced Flavin Mononucleotide (FMN) Measurement Using High-Performance Liquid Chromatography (HPLC). The FAD and FMN content of eNOS or S-glutathionylated eNOS was determined using HPLC.[32,34] The purified eNOS or S-glutathionylated eNOS (100 μg in 500 μL) was first boiled for 10 min to release FAD and FMN from the protein. Protein was then removed by filtration through a Microcon-3 (Millipore); 50 μL of the FAD- and FMN-containing filtrates was separated on a Shimadzu (Columbia, MD) LC-2010AT HPLC system with a C18 reversed-phase column (Alltima Reversed-Phase C18 HPLC Columns, 4.6 mm × 150 mm). After injection, buffer A [5 mM ammonium acetate (pH 6.0) and 7% methanol] with a flow rate of 0.5 mL/min was used for 2 min. Then, a linear gradient was developed to 70% methanol using buffer B [5 mM ammonium acetate (pH 6.0) and 70% methanol] over 13 min. A Shimadzu RF-10A XL fluorescence detector with the excitation wavelength set to 460 nm and the emission wavelength set to 530 nm was used to detect FAD and FMN. FAD and FMN were completely separated with elution times of 13.0 and 14.5 min, respectively. The peak area

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was used to calculate the content of FAD and FMN compared to the content of FAD and FMN standards.

Site-Directed Mutagenesis of Cys689 and Cys908 of eNOS to Ala. A double mutant of eNOS (C689 and C908 to Ala) was generated to test the effect and importance of these two specific cysteine residues in maintaining the structure and function of the eNOS reductase domain. The wild-type eNOS gene was first mutated (C908 to Ala), followed by another single mutant (C689 to Ala) to form a eNOS double mutant. The sequences of primers for the C908 to Ala mutation were 5′ GAAGTGGTTCCGCGCCCCCACGCTGCTG 3′ and 5′ CAGCAGCGTGGGGGCGCGGAACCACTTC 3′. The sequences of primers for the C689 to Ala mutation were 5′ GGCGACGAGCTGGCCGGCCAGGAGG 3′ and 5′ CCTCCTGGCCGGCCAGCTCGTCGCC 3′. The QuikChange mutagenesis kit from Stratagene was used for this site-directed mutagenesis of eNOS.[14,32]

Flavin Fluorescence Measurement. Fluorescence spectra of eNOS flavins were measured using a SpexFluoroMax fluorescence spectrometer to determine the flavin content of eNOS. The total amount of protein used for the measurement was 50 μg for both the wild type and the double mutant in 2 mL of phosphate-buffered saline (PBS). The excitation spectrum of eNOS flavin was determined with an emission wavelength of 530 nm. The emission spectrum was determined with an excitation wavelength of 449 nm.

Overexpression and Purification of Grx1. The Grx1 bacterial overexpression plasmid was a gift from J. J. Mieyal (Case Western Reserve University, Cleveland, OH). The procedure for Grx1 overexpression and purification was followed as previously described. The activity of Grx1 was determined using a glutathione reductase-coupled reaction with Cys-SG from Toronto Research Chemicals Inc. (Toronto, ON) as a substrate.[35]

eNOS S-Glutathionylation by Grx1. eNOS S-glutathionylation by Grx1 was conducted in a reaction mixture containing 5 μg of eNOS in 50 mM Tris (pH 7.4) with different GSSG/GSH ratios in a final volume of 20 μL. The concentration of GSSG or GSH ranged from 0.1 to 1 mM. The reaction was initiated by addition of Grx1 to a final concentration of 1 μM at RT for 10 min. For mass spectrometric and immunoblotting analysis, NEM was added to a final concentration of 10 mM to quench the reaction. To measure eNOS activity, the oxyhemoglobin assay was conducted as previously described.[33]

Measurement of $^{\bullet}O_2^-$ Generation by EPR Spin-Trapping. EPR spin-trapping measurements of the production of the oxygen radical from eNOS (2 μg) or free FAD and FMN (1 μM) were performed as previously described.[32]

Mass Spectrometric Analysis of eNOS S-Glutathionylation by Grx1. The S-glutathionylated eNOS was subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) on a 4 to 20% gradient polyacrylamide gel. Protein bands on the gel were then stained with Coomassie Blue. The band containing S-glutathionylated eNOS, which was confirmed by immunoblotting against the anti-GSH antibody, was cut and digested in-gel with trypsin, chymotrypsin, or trypsin and chymotrypsin before the mass spectrometric measurement. The S-glutathionylation of eNOS was assessed with capillary liquid chromatography tandem mass spectrometry (Nano-LC−MS/MS). The detailed parameters used in the MS measurements have been described in our previous study.[14,32] Sequence information from MS/MS data was processed with Mascot Distiller, by using standard data processing parameters. Database searches were performed with Mascot (Matrix Science).

Site-Directed Mutagenesis of Cys382 of eNOS to Ala. The single mutation of C382 of eNOS to Ala was generated to test the effect of this specific cysteine residue in the resistance to the Grx1-directed eNOS S-glutathionylation. C382 of the wild-type eNOS gene was mutated to Ala using the QuikChange mutagenesis kit from Stratagene. The sequences of primers for the C382 to Ala mutation were 5′ GAGGATGTGGCTGTCGCCATGGACCTGGATAC 3′ and 5′ GTATCCAGGTCCATGGCGACAGCCACATCCTC 3′.[14,32]

Cellular and *in Vitro* Analysis of eNOS−Grx1 Interaction. To assess the eNOS−Grx1 interaction in endothelial cells, the co-immunoprecipitation of eNOS and Grx1 in bovine aortic endothelial cells (BAECs) was performed using the anti-eNOS agarose conjugate (Santa Cruz) or the Grx1 antibody with protein A/G. The control experiment was performed using protein A/G beads only. The co-immunoprecipitation products were analyzed using immunoblotting against eNOS and Grx1. To assess the eNOS−Grx1 interaction *in vitro*, the purified eNOS with a His tag was first incubated with Ni-NTA magnetic beads from Invitrogen (Carlsbad, CA) followed by incubation with different concentrations of Grx1 at 4 °C for 1 h before they were washed three times with HEPES buffer (50 mM, pH 7.4, 150 mM NaCl) containing 5 mM imidazole to eliminate the nonspecific binding. In the negative control, different concentrations of Grx1 were directly incubated with only Ni-NTA magnetic beads for 1 h at 4 °C and washed three times with HEPES buffer (50 mM, pH 7.4, 150 mM NaCl) containing 5 mM imidazole. Next, the coprecipitated product was eluted with HEPES buffer (50 mM, pH 7.4, 150 mM NaCl) containing 250 mM imidazole, followed by electrophoresis via 4 to 20% SDS−PAGE separation and immunostaining with anti-Grx1 and anti-eNOS.

***Ex Vivo* eNOS S-Glutathionylation by Grx1 and *ex Vivo* Analysis of the eNOS−Grx1 Interaction.** For each experiment, one T-75 flask of BAECs was used to assess eNOS S-glutathionylation *ex vivo*. First, cells were lysed in 500 μL of 1× TBS buffer [50 mM Tris-HCl (pH 7.6) and 150 mM NaCl] with 1% NP-40 and 0.5% sodium deoxycholate. In the control experiment, the supernatant was treated with either 0.5 or 1 mM GSSG at RT for 10 min. In the reaction with Grx1, the supernatant was treated with either 0.5 or 1 mM GSSG and 1 μM of Grx1 at RT for 10 min. All reactions were quenched by addition of NEM to a final concentration of 10 mM. After the reaction, immunoprecipitation of eNOS was performed, followed by electrophoresis via 4 to 20% gradient SDS−PAGE and immunostaining with anti-GSH and anti-eNOS to determine the level of eNOS S-glutathionylation. The eNOS co-immunoprecipitated product was also separated via 4 to 20% SDS−PAGE and immunostained with anti-Grx1 to further analyze the eNOS−Grx1 interaction *ex vivo*.

Inhibition of Grx1 by Cd^{2+} in BAECs. The maintenance of the BAEC culture was the same as that previously described.[14,32] Cd^{2+} (200 μM) with 10% FBS complete medium was added to cells for 3 h to inhibit Grx1 activity in endothelial cells.[36] eNOS activity from BAECs was measured using EPR NO spin-trapping with Fe-*N*-methyl-D-glucamine dithiocarbamate (Fe^{2+}-MGD) after treatment. To determine the level of eNOS S-glutathionylation and multimerization through interdisulfide bond formation, BAECs were first lysed in RIPA buffer [50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1%

NP-40, 0.5% sodium deoxycholate, and 0.1% SDS] containing 10 mM NEM followed by either immunostaining for eNOS or immunoprecipitation of eNOS and then immunostained for protein S-glutathionylation with the GSH antibody.[14,32]

Bovine Aortic Endothelial Cell *grx1* Gene Silencing. *grx1* gene silencing in BAECs was used to confirm the effect of Grx1 on eNOS function. The sense siRNA strand was 5′ CUGUUGACACGGCUAAAGCUU 3′ and the antisense siRNA strand 3′ UUGACAACUGUGCCGAUUUCG 5′. These siRNAs were custom synthesized by Thermo Scientific (Rockford, IL). DharmaFECT Transfection Reagent from Thermo Scientific was used to deliver *grx1* siRNAs to BAECs. After 48 h, Grx1 immunoblotting was used to determine the Grx1 knockdown efficiency. NOS activity was measured using EPR with Fe^{2+}-MGD as a spin-trap.[14]

EPR Spin-Trapping Measurements of NO Production. Spin-trapping measurements of NO from BAECs were performed with a Bruker EMX spectrometer with Fe^{2+}-MGD as the spin-trap.[14,32] Spin-trapping experiments were performed on cells grown in six-well plates (10^6 cells per well). Before EPR spin-trapping measurements, control cells, cells treated with 200 μM Cd^{2+}, and cells treated with *grx1* siRNA were washed twice with PBS. Next, 0.8 mL of PBS containing glucose (1 g/L), $CaCl_2$, $MgCl_2$, the NO spin-trap Fe^{2+}-MGD (0.5 mM Fe^{2+} and 5.0 mM MGD), and a calcium ionophore (1 μM) was added to each well, and the plates were incubated for 20 min at 37 °C in a humidified environment containing 5% CO_2 and 95% O_2. After incubation, the medium from each well was removed, and the trapped NO in the supernatants was quantified by EPR. Spectra recorded from these cellular preparations were obtained with the following parameters: microwave power of 20 mW, modulation amplitude of 4.0 G, and modulation frequency of 100 kHz.

RESULTS

Deglutathionylation of S-Glutathionylated eNOS by Grx1 and GSH. As reported previously, in the presence of elevated levels of GSSG, eNOS S-glutathionylation occurs with modification of Cys689 and Cys908. When eNOS is incubated with 2 mM GSSG for 20 min, ~50−75% modification occurs with loss of eNOS activity and uncoupling with loss of NO synthesis and an increase in the level of $^{\bullet}O_2^-$ production.[14] Consistent with our prior reports, following incubation of eNOS with 2 mM GSSG for 20 min and gel filtration to remove the GSSG, immunoblotting showed prominent eNOS S-glutathionylation with an ~55% loss of eNOS activity, as measured by the rate of conversion of oxyhemoglobin to methemoglobin.

We next studied the effect of GSH and Grx1 on reversing eNOS S-glutathionylation. While in the presence of reduced GSH (1 mM) alone, no deglutathionylation of eNOS was seen even after incubation for 20 min (Figure 1A), with addition of both Grx1 (1 μM) and GSH (1 mM), a marked decrease in the level of eNOS S-glutathionylation was seen after incubation for only 5 min. After reaction for 10 min, eNOS S-glutathionylation was almost totally reversed. In tandem with this, the oxyhemoglobin assay demonstrated a partial rescue of eNOS activity with an increase in basal activity from ~45 to 70% (Figure 1B,C). Because S-glutathionylation of Cys689 and Cys908 within the eNOS reductase has been reported to increase the solvent accessibility of FAD and FMN, we hypothesized that it could weaken their binding, resulting in partial loss and removal upon gel filtration. Therefore,

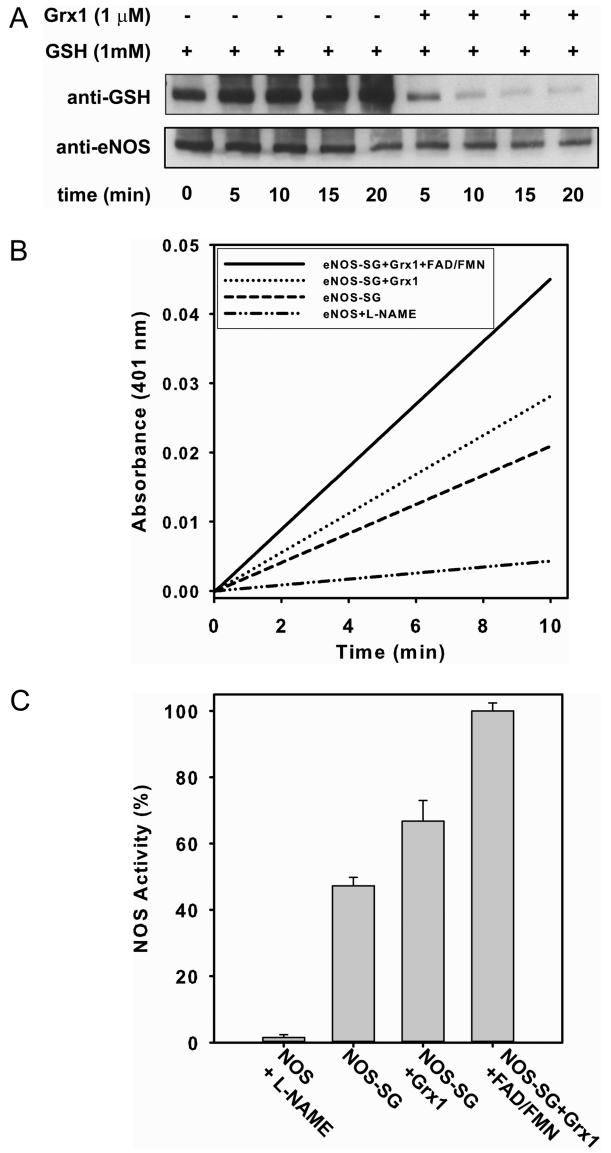

Figure 1. Deglutathionylation of S-glutathionylated eNOS requires Grx1. (A) Immunoblotting of eNOS deglutathionylation. The top panel shows immunoblotting against the GSH antibody. The bottom panel shows immunoblotting against the eNOS antibody. Grx1 is required for the reverse reaction. No eNOS deglutathionylation was seen in the absence of Grx1. (B and C) NOS activity determined by the oxyhemoglobin assay. (B) Kinetic traces of the absorbance change at 401 nm. The NOS activity was inhibited when 2 mM L-NAME was present. (C) Reconstituted eNOS with 4 μM FAD and FMN after deglutathionylation by Grx1 and GSH was required to fully restore the activity to that without modification. The activity of untreated eNOS is 100 nmol mg^{-1} min^{-1} at RT. Data are expressed as means ± the standard error of the mean (n = 3).

additional measurements were performed with addition of FAD and FMN (4 μM each), and the activity was observed to fully recover to 100% of basal values, identical to that of the unmodified enzyme. In contrast, supplementation of both FAD and FMN alone without GSH and Grx1 had no significant effect.

eNOS S-Glutathionylation Weakens eNOS Flavin Binding. As described above, incubation of eNOS in the presence of GSSG induces S-glutathionylation of two critical cysteine residues (Cys689 and Cys908) located in the eNOS

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reductase domain as previously identified by mass spectroscopy with confirmation by site-directed mutagenesis. Molecular modeling has shown that these cysteines are near the binding sites for both FAD and FMN cofactors, and this led to the hypothesis that these modifications may weaken flavin binding. Therefore, HPLC measurements were performed to determine the level of eNOS-bound flavins before and after GSSG-mediated S-glutathionylation. The excess GSSG and free or weakly bound FAD and FMN were removed through concentration and buffer exchange using a 50 kDa molecular mass cutoff Centricon from Millipore. The HPLC results (Figure 2A,B) indicated that the levels of both eNOS-bound FAD and FMN after GSSG treatment and buffer exchange decreased to 45 ± 1 and 47 ± 1%, respectively, compared to that of untreated eNOS. Thus, S-glutathionylation weakened the binding of FAD and FMN to eNOS. When Cys908 was mutated to Ala in our previous study,[32] the mutation of this residue slightly affected eNOS flavin binding (by ~20%). To further determine the role of these residues in the effect of eNOS flavin binding, the levels of eNOS-bound FAD and FMN of the C689A mutant measured by HPLC decreased to 20.5 ± 1.3 and 31.4 ± 4.4%, respectively, compared to that of wild-type eNOS. These results suggested that C689 and C908 are both required for flavin binding. The results are expressed means ± the standard error of the mean (SEM) (n = 3).

Consistent with the prior reports,[14] we observe that while eNOS activity and the level of NO generation are decreased by GSSG-mediated S-glutathionylation, the level of $^{\bullet}O_2^-$ generation is increased (Figure 3E). The fact that this $^{\bullet}O_2^-$ generation cannot be inhibited by the oxygenase binding inhibitor L-NAME indicates that this S-glutathionylation of eNOS at Cys689 and Cys908 opens the reductase, providing access of O_2 to the reduced flavins with resultant $^{\bullet}O_2^-$ production.[14]

Mutation of Cys689 and Cys908 of eNOS to Ala Weakens eNOS Flavin Binding. To further test the role of Cys698 and Cys908 in flavin binding, flavin fluorescence was used to investigate the effect of the eNOS C689A/C908A double mutant on eNOS flavin binding. Wild-type eNOS exhibited the intrinsic flavin fluorescence, but an only very weak fluorescent signal was seen with the eNOS Cys double mutant (Figure 2C), indicating that these cysteines have an important role in facilitating or stabilizing FAD and FMN binding. Interestingly, as reported previously, upon mutation of Cys689 and Cys908 to serine, which has polarity and hydrogen bonding similar to those of cysteine, eNOS function and coupling are normal, with no decline in NOS activity indicating no loss of FAD or FMN.[14]

With an Increase in the GSSG/GSH Ratio, Grx1 Enhances eNOS S-Glutathionylation and Inhibits eNOS Activity. When the GSSG/GSH ratio was 0.1 in the absence or presence of Grx1, no or only a low level of eNOS S-glutathionylation was detected on immunoblotting. However, in the presence of Grx1 when the GSSG/GSH ratio was increased to 0.2, the level of eNOS S-glutathionylation was increased, and with a further increase in the GSSG/GSH ratio to 0.5 or 1, S-glutathionylation was dramatically enhanced (Figure 3A).

To determine if Grx1 was required for eNOS S-glutathionylation, studies were performed with low concentrations of GSSG and GSH (0.1 or 0.05 mM) in the absence of Grx1, and no eNOS S-glutathionylation was seen on immunoblotting (Figure 3A,B). However, when 1 μM Grx1 was present, the level of eNOS S-glutathionylation was greatly increased. Upon addition of 1 mM GSH, this oxidative modification was fully reversed within 10 min (Figure 3B).

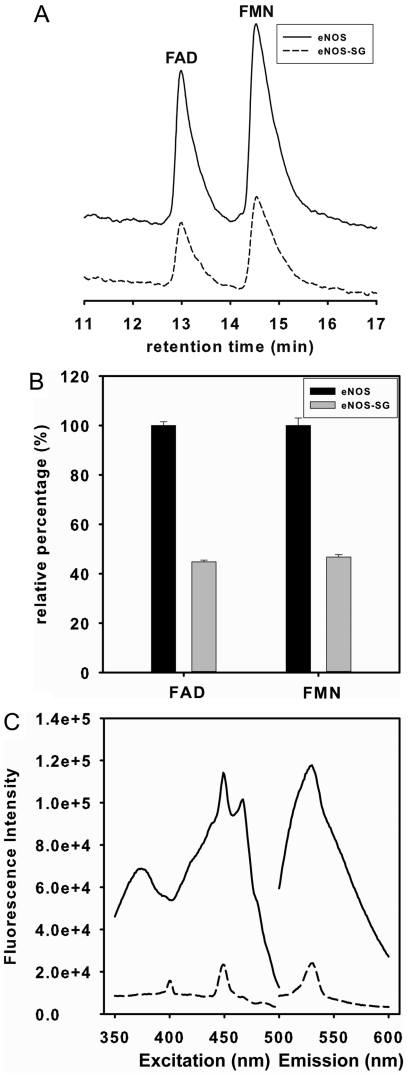

Figure 2. eNOS S-glutathionylation by GSSG or eNOS double mutation (Cys to Ala) induces flavin dissociation. Purified eNOS was first incubated with 2 mM GSSG to induce protein S-glutathionylation followed by removal of GSSG via chromatography and sample concentration by ultrafiltration. The flavin content of the enzyme was determined using HPLC with a fluorescent detector with an excitation wavelength of 460 nm and an emission wavelength of 530 nm before and after treatment. (A) The solid line shows data for untreated eNOS, and the dashed line shows data for GSSG-treated eNOS. The retention time of FAD is 13.0 min, and the retention time of FMN is 14.5 min. (C) The FAD and FMN content of eNOS for the GSSG-treated enzyme decreased compared to that of untreated eNOS. All experiments were performed in at least triplicate. (C) Fluorescence measurement of flavins. Wild-type eNOS (—) exhibited the intrinsic flavin fluorescence, but no fluorescent signal from the eNOS Cys double mutant was seen (---). The excitation spectrum of eNOS flavin was determined with an emission wavelength of 530 nm. The emission spectrum was determined with an excitation wavelength of 449 nm.

Further experiments were performed to measure the effects of Grx1-mediated S-glutathionylation on eNOS activity as assayed by the oxyhemoglobin to methemoglobin conversion assay. Following a 10 min incubation of eNOS in the presence of Grx1 (1 μM) in the presence of 0.1 mM GSSG and 0.1 mM

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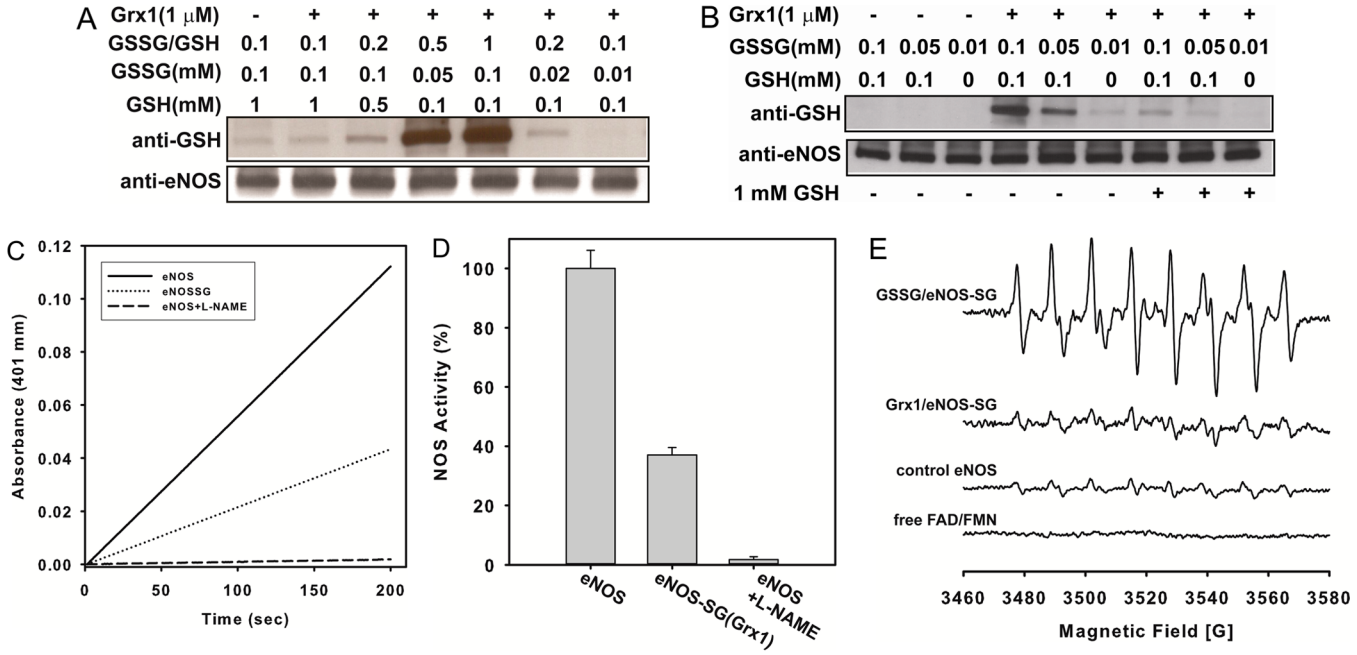

Figure 3. Grx1 enhances eNOS S-glutathionylation with an increased GSSG/GSH ratio and inhibits enzyme activity. (A) The top panel shows the immunoblotting against the GSH antibody and the bottom panel the immunoblotting against the eNOS antibody for the loading control. Grx1 increased the level of eNOS S-glutathionylation when the GSSG/GSH ratio was >0.2. (B) The top panel shows the immunoblotting against the GSH antibody and the bottom panel the immunoblotting against the eNOS antibody for the loading control. When the concentrations of GSH and GSSG were both low, Grx1 was required for eNOS S-glutathionylation. This process was reversible when 1 mM GSH was added. (C and D) eNOS was S-glutathionylated by Grx1 in the presence of 0.1 mM GSSG and 0.1 mM GSH. (C) Kinetic traces of the absorbance change at 401 nm. (D) Activity of control eNOS and S-glutathionylated eNOS by Grx1 (*$P < 0.01$ vs respective controls). Data are expressed as means ± SEM ($n = 3$). The activity of untreated eNOS is 85 nmol mg^{-1} min^{-1} at RT. (E) EPR spin-trapping measurements of oxygen radical generation. No increase in the level of $^{\bullet}O_2^-$ production was seen for this Grx1-modified eNOS compared to control eNOS. eNOS treated with 2 mM GSSG gave rise to a strong $^{\bullet}O_2^-$ signal. No generation of $^{\bullet}O_2^-$ from free FAD and FMN (1 μM) and NADPH (200 μM) was seen.

GSH, NOS activity was ~65% decreased (Figure 3C,D). No increase in the level of $^{\bullet}O_2^-$ production was seen from this Grx1-modified eNOS using EPR DEPMPO $^{\bullet}O_2^-$ spin-trapping (Figure 3E), indicating that this decrease in activity was not associated with uncoupling.

Mass Spectrometric Analysis of eNOS S-Glutathionylation by Grx1. Because the sites of Grx1-mediated S-glutathionylation may be different from that caused by GSSG, mass spectroscopy analysis was performed to identify the specific sites of eNOS S-glutathionylation by Grx1. eNOS (~2 μg in 20 μL) was S-glutathionylated in the presence of 0.1 mM GSSG, 0.1 mM GSH, and 1 μM Grx1 at RT for 10 min. The reaction mixture was then subjected to 4 to 20% gradient SDS–PAGE separation under nonreducing conditions. The band corresponding to S-glutathionylated eNOS was cut and digested with trypsin, chymotrypsin, or both, and then the digested peptide fragments were analyzed by LC–MS/MS. The percentage of sequence coverage determined by LC–MS/MS was 85%.

With the addition of one molecule of glutathione, the molecular mass of the glutathione-modified peptide fragment will increase by 305 Da compared to that of the unmodified peptide fragment. The peptides with a mass difference of 305 Da were identified by LC–MS, and the sequence of the modified peptides was further determined by MS/MS and analyzed by Mascot. From mass determination, Cys382 was identified as the major site of eNOS S-glutathionylation by Grx1 from both trypsin and chymotrypsin digestions. The percentage of this modification at Cys382 was >50%.

S-Glutathionylated Cys382 from fragment $_{373}$YNILEDVAV-CMDLDTR$_{388}$ [amino acids 373–388 (Figure 4A)] was assessed. To further validate the exact location of the glutathione-modified amino acid(s), the MS/MS spectrum of the tryptic and chymotryptic fragment ion at m/z 725.6597^{3+} was obtained as shown in Figure 4A. Under the conditions of the low-energy collision-induced dissociation, both y and b product ions were observed, corresponding to cleavages along the peptide backbone. The y series ions result from C-terminal peptide cleavages, while the b series ions result from cleavages at the N-terminus. In the spectrum of the tryptic and chymotryptic peptide ion, the molecular mass difference between fragment ions y_6 and y_7 was observed with a mass shift of 305 Da compared to the native fragment ions, allowing unequivocal assignment of the glutathionylated adduct to the Cys382 residue of the tryptic and chymotryptic peptides. Neither Cys689 nor Cys908 was S-glutathionylated by Grx1.

Molecular Modeling of eNOS Oxygenase and Reductase Domains. The published crystal structure of the human eNOS oxygenase domain (PDB entry 3NOS) was used to generate a three-dimensional image. PyMOL was used to visualize the location of Cys382 in the oxygenase domain (Figure 4B). From the three-dimensional structure of the eNOS oxygenase domain, it is clear that Cys382 is solvent accessible and available for Grx1-catalyzed eNOS S-glutathionylation. In contrast from modeling of the eNOS reductase (Figure 4C), we can see that neither Cys689 nor Cys908 is fully solvent-exposed, with Cys908 more deeply buried in the protein than Cys689. Thus, these sites would not appear to be susceptible to Grx1 binding and Grx1-mediated S-glutathiony-

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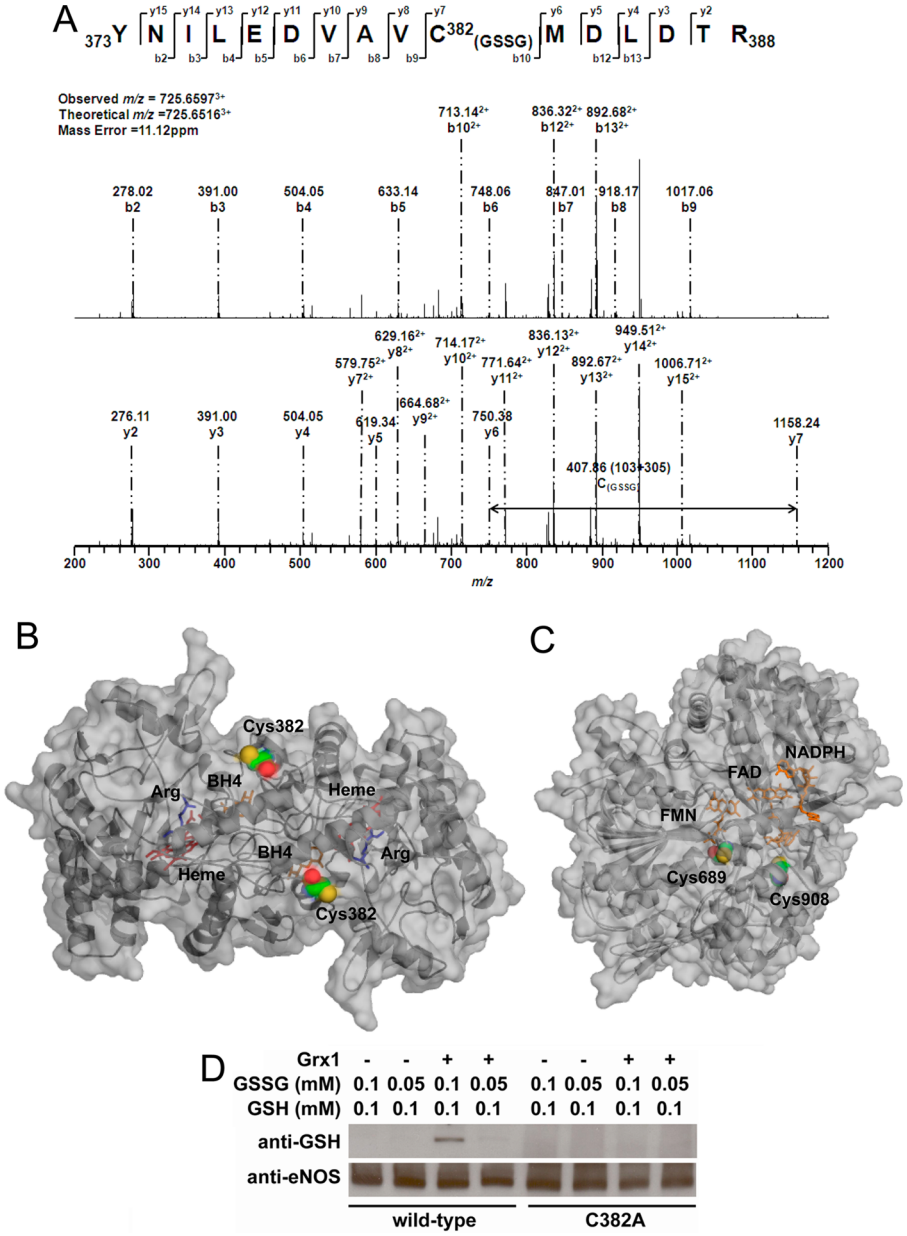

Figure 4. Mass spectrometry and molecular modeling reveal C382 is the site of eNOS S-glutathionylation by Grx1. (A) LC−MS/MS analysis of eNOS S-glutathionylation by Grx1. Cys382 was identified as the specific site for S-glutathionylation by Grx1 in the presence of 0.1 mM GSSG and 0.1 mM GSH. The molecular mass difference between fragment ions y_6 and y_7 demonstrated a mass shift of 305 Da compared to the native fragment ions, allowing the unequivocal assignment of the GSH adduct to Cys382. (B and C) Molecular modeling of the eNOS oxygenase domain and reductase domain. The three-dimensional structure of the human eNOS oxygenase domain (PDB entry 3NOS) (B) and the reductase domain (C) was visualized using PyMOL (Schrödinger, LLC, Portland, OR). The eNOS oxygenase domain is shown in dimeric form. Heme (red) is shown as sticks, L-arginine (blue) as sticks, and BH_4 (magenta) as sticks. The three-dimensional structure of the human eNOS reductase domain was generated by homology modeling referencing the reductase domain of rat neuronal NOS (PDB entry 1F20) using Swiss molecular modeling as previously described.[14] The eNOS reductase domain is shown in monomeric form. FMN, FAD, and NADPH (orange) are shown as sticks. Cys residues are shown as spheres: yellow for S, cyan for C, blue for N, and red for O. (D) The eNOS C382A mutant resists Grx1-induced S-glutathionylation. In the absence of Grx1 with a GSSG/GSH ratio of 1 or 0.5, no eNOS S-glutathionylation was seen in the wild type or the C382A mutant. However, in the presence of Grx1, GSSG, and GSH, only wild-type eNOS exhibited Grx1-catalyzed S-glutathionylation; however, the C382A mutant resisted Grx1-induced S-glutathionylation.

lation. However, following S-glutathionylation by GSSG, these sites may become more solvent-exposed and accessible to Grx1-mediated deglutathionylation. It is worth noting that Cys689 is very close to the C-terminal tail of the enzyme, which acts as one of the two CaM-modulated regulatory elements. Thus, S-glutathionylation of eNOS at Cys689 can potentially disrupt CaM modulation, possibly affecting uncoupling.

The C382A eNOS Mutant Resists Grx1-Catalyzed eNOS S-Glutathionylation. When both wild-type eNOS and the eNOS C382A mutant were incubated (10 min) with GSSG (0.1 or 0.05 mM) and GSH (0.1 mM) alone, no eNOS S-glutathionylation was seen (Figure 4C). However, when Grx1 was included in the reaction mixture, the Grx1-catalyzed eNOS S-glutathionylation was only seen for wild-type eNOS, with no

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Grx1-catalyzed eNOS S-glutathionylation of the eNOS C382A mutant (Figure 4D).

Grx1 Plays an Important Role in Redox Regulation in Endothelial Cells and Is Associated with eNOS. The co-immunoprecipitation of eNOS and Grx1 in BAECs demonstrated that eNOS and Grx1 can form a native complex in endothelial cells (Figure 5A). Grx1 co-immunoprecipitated with eNOS detected by immunoblotting against the Grx1 antibody, and eNOS co-immunoprecipitated with Grx1 detected by immunoblotting against the eNOS antibody. A postlysate of endothelial cells was used to demonstrate that Grx1 can efficiently redox-regulate eNOS function. When a postlysate was incubated with 1 or 0.5 mM GSSG alone for 10 min at RT, no eNOS S-glutathionylation was detected via GSH immunoblotting after immunoprecipitation with the eNOS antibody. However, when GSSG and Grx1 were both incubated with the postlysate, eNOS S-glutathionylation was detected, and the intensity of the modification was dependent on the concentration of GSSG (Figure 5B). Grx1 co-immunoprecipitated with eNOS detected by immunoblotting against the Grx1 antibody

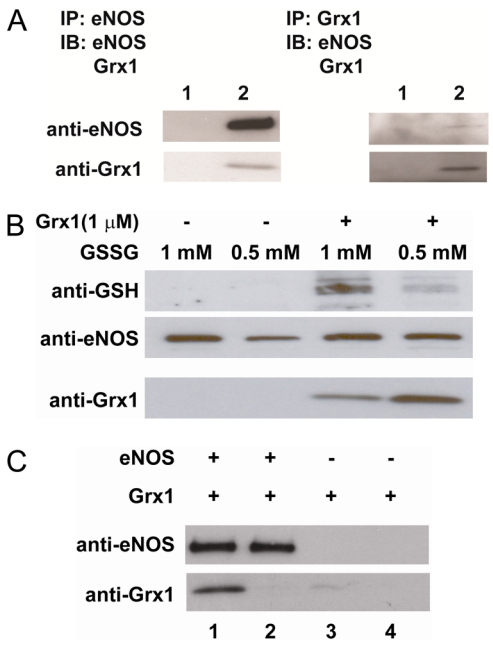

Figure 5. Grx1 is a protein partner of eNOS in endothelial cells and *in vitro*. (A) Co-immunoprecipitation of eNOS and Grx1 from BAECs. In the left panel, lane 1 is the control with protein A/G beads only and lane 2 is immunoprecipitation with the eNOS agarose-conjugated antibody. The top panel shows immunoblotting against eNOS. The bottom panel shows immunoblotting for Grx1. In the right panel, lane 1 is the control with protein A/G beads only and lane 2 is immunoprecipitation with the Grx1 antibody and protein A/G. The top panel shows immunoblotting against eNOS. The bottom panel shows immunoblotting for Grx1. (B) Co-immunoprecipitation of eNOS and Grx1 from an *ex vivo* BAECs postlysate. The top panel shows immunoblotting against GSH. No eNOS S-glutathionylation was seen when the postlysate was incubated with 0.5 or 1 mM GSSG alone for only 10 min. When 1 μM Grx1 was included in the reaction mixture, eNOS S-glutathionylation was enhanced. The middle panel shows immunoblotting for eNOS. The bottom panel shows immunoblotting for Grx1. Grx1 co-immunoprecipitated with eNOS. (C) Coprecipitation of eNOS and Grx1 using His-tagged magnetic beads. The top panel shows immunoblotting for eNOS and the bottom panel immunoblotting for Grx1. Lane 1 shows coprecipitation of 0.2 μM eNOS and 0.4 μM Grx1. Lane 2 shows coprecipitation of 0.2 μM eNOS and 0.04 μM Grx1. Lane 3 shows 0.4 μM Grx1 only. Lane 4 shows 0.04 μM Grx1 only. All experiments were performed in at least triplicate.

Next, the purified His-tagged eNOS was first incubated with magnetic Ni-NTA beads followed by incubation with different concentrations of Grx1. The result of immunoblotting against Grx1 (Figure 5C) clearly indicated that Grx1 is a protein partner that binds to eNOS, consistent with the previous result from the co-immunoprecipitation of eNOS and Grx1 in BAEC experiments.

Inhibition of Glutaredoxin Leads to eNOS Dysfunction with a Loss of NO Generation. Cd^{2+} has been shown to inhibit Grx activity in endothelial cells.[36,37] Inhibition of Grx enzymes by Cd^{2+} (200 μM) in BAECs in complete medium with 10% FBS dramatically decreased the level of NO production by 82 ± 2% as measured by EPR spin-trapping (Figure 6A,B). The decrease in the level of NO production was correlated with an increase in the level of eNOS S-glutathionylation determined from the immunoblotting of protein-bound GSH from immunoprecipitated eNOS, as well as from the formation of interprotein disulfide bonds determined by eNOS immunoblotting via nonreducing SDS–PAGE analysis (Figure 6C). While marked inhibition of eNOS activity was seen in BAECs, only modest (<20%) inhibition of eNOS-mediated NO generation was seen with purified eNOS (10 μg/mL) in matched media. This confirms that Grx1 has an important role in regulating eNOS S-glutathionylation and preserving eNOS activity and NO generation.

***grx1* Gene Silencing in BAECs Leads to NOS Dysfunction.** *grx1* gene silencing with siRNA was performed to further determine the effect of Grx1 on NOS function in endothelial cells. After post-transfection with *grx1* siRNA for 2 days, immunoblotting of Grx1 indicated that the efficiency of Grx1 knockdown was nearly complete (Figure 6D). The NOS activity of control or *grx1* siRNA was measured using NO Fe^{2+}-MGD EPR spin-trapping. The result (Figure 6E) demonstrated that *grx1* gene silencing was associated with NOS dysfunction with a dramatic decrease in the level of NO production in BAECs.

DISCUSSION

Recently, we demonstrated that eNOS can be S-glutathionylated in both hypertensive vessels and cells under oxidative stress, leading to uncoupling of the enzyme.[14] S-Glutathionylation of eNOS has been demonstrated through the direct reaction with GSSG[14] or through the formation of labile intermediate protein radicals.[32] Several studies have also shown that eNOS S-glutathionylation is associated with many cardiovascular diseases and occurs in cells exposed to oxidative stress.[15−17,38] Protein S-glutathionylation, a reversible oxidative modification, plays an important role in redox signaling and can protect against irreversible oxidation of protein thiols in many cardiovascular diseases.[20−22] As such, it is important to identify the mechanism of the reverse process, deglutathionylation of eNOS protein thiols, and this can provide a potential therapeutic target for the treatment of oxidant-induced diseases.

S-Glutathionylation of eNOS has been shown to modulate its structure and function. Thus, it is important to understand the reversible nature of this oxidative modification and whether this reversible thiol modification can redox-regulate eNOS function under oxidative stress as well as during disease progression.

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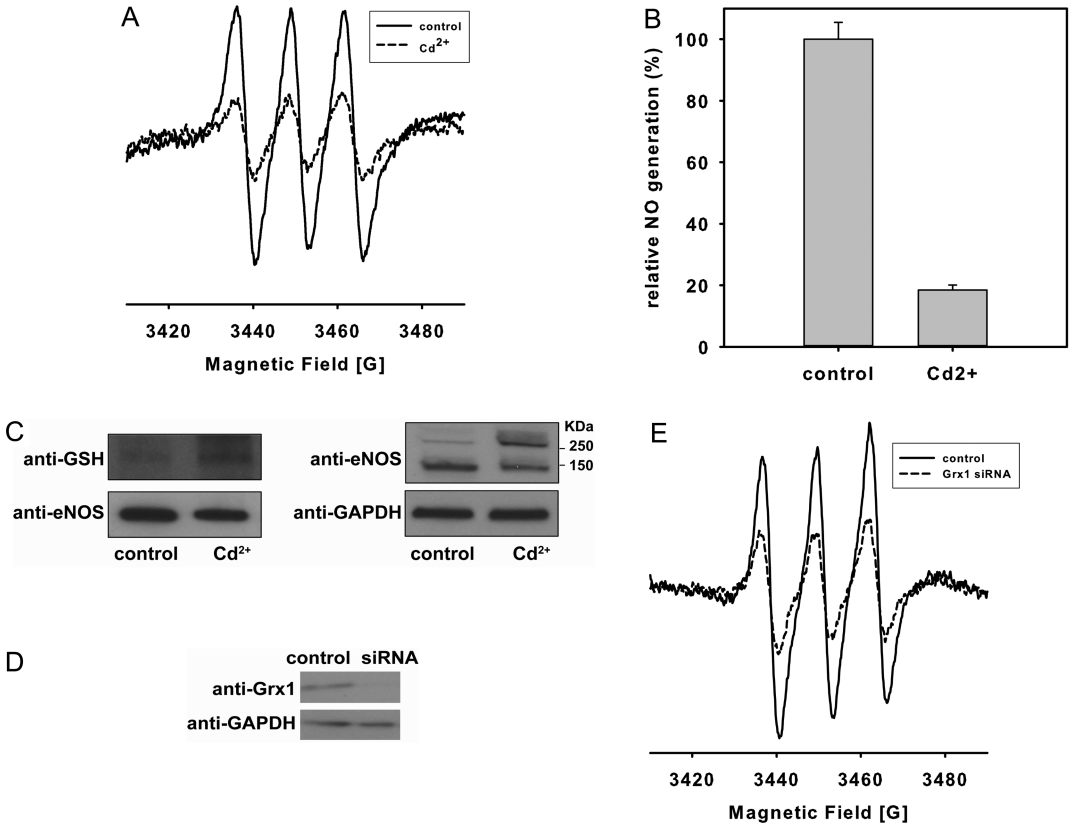

Figure 6. Inhibition of Grx enzymes by Cd^{2+} or *grx1* gene silencing in endothelial cells decreases the level of generation of NO from endothelial cells with a concomitant increase in the level of eNOS oxidative modification. (A and B) Generation of NO from BAECs measured by EPR spin-trapping using Fe^{2+}-MGD. While a strong NO triplet signal was seen in untreated cells, Cd^{2+} treatment dramatically decreased this level of NO generation (*$P < 0.01$ vs respective controls). Data are expressed as means ± SEM ($n = 3$). (C) Immunoblotting of eNOS S-glutathionylation and dimerization. The left panel shows immunoprecipitation of eNOS followed by immunoblotting against the GSH antibody (top) and eNOS antibody (bottom). The right panel shows immunoblotting against the eNOS antibody (top) using nonreducing SDS−PAGE analysis and GAPDH (bottom). All experiments were performed in at least triplicate. (D and E) *grx1* gene silencing in endothelial cells decreases the level of generation of NO from endothelial cells. (D) *grx1* gene silencing. Lane 1 is the control. Lane 2 shows *grx1* siRNA. The top panel shows immunoblotting for Grx1 and the bottom panel immunoblotting for GAPDH as a loading control. After post-transfection with *grx1* siRNA for 2 days, the efficiency of Grx1 knockdown was nearly complete. (E) NOS activity from BAECs measured using EPR with Fe^{2+}-MGD as a spin-trap. *grx1* gene silencing deceases the level of generation of NO from BAECs compared to the control.

This reverse process is termed "deglutathionylation", reduction of a mixed-disulfide bond between GSH and a protein thiol. Grx enzymes, GSH-dependent oxidoreductases, have been implicated in the reduction of mixed-disulfide bonds, especially S-glutathionylated proteins.[23,24]

In this study, we demonstrate that no deglutathionylation occurs when GSH is the only reducing equivalent in the reaction while there is a dramatic decrease in the extent of this oxidative modification when both Grx1 and GSH are present. The reduction of this mixed-disulfide bond (deglutathionylation) by GSH occurs via a bimolecular nucleophilic substitution (an S_N2 reaction).[39] For GSH to be in the correct form for reducing this disulfide bond, the thiol of GSH must be deprotonated. The pK_a of the GSH thiol is approximately 8.5; therefore, most of the GSH is expected to be protonated at physiological pH and not primed for this S_N2 reaction or the charge repulsion between deprotonated GSH and S-glutathionylated eNOS.[40] This explains why eNOS deglutathionylation was not seen with GSH as the only reducing equivalent in the reaction mixture. This further demonstrates that Grx1 is required to overcome the activation energy barrier of this disulfide reduction for this deglutathionylation reaction. Thus, Grx1 is required to effectively reduce this oxidative modification.

We observed that following eNOS S-glutathionylation by GSSG, FAD and FMN binding was weakened so that partial loss was seen. Therefore, addition of FAD and FMN was required to recover the full activity of eNOS. The S-glutathionylated eNOS exhibits a greatly increased level of $^{\bullet}O_2^-$ generation, while comparable levels of free FAD, FMN, and NADPH do not. Thus, S-glutathionylation of eNOS opens the FAD and FMN sites of the eNOS reductase domain to react with O_2 and weakens their binding, so that dissociation with loss of binding to the enzyme can occur in the absence of free FAD and FMN.

Previously, we have identified two critical cysteinyl residues of eNOS involved in S-glutathionylation by GSSG that can uncouple eNOS, leading to an increase in the level of eNOS-derived generation of $^{\bullet}O_2^-$ from the reductase domain. These two highly conserved residues, Cys689 and Cys908 of eNOS, are located at the interface of the FAD and FMN binding domains. Introduction of a bulky and negatively charged molecule (GSH) perturbs the eNOS structure in this region, increasing the solvent accessibility to the FAD and FMN cofactors.[14] Our current HPLC analysis of the FAD and FMN

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content supports the hypothesis that S-glutathionylation of eNOS by GSSG perturbs this interface and weakens the binding of FAD and FMN to the S-glutathionylated eNOS. The importance of Cys689 and Cys908 for flavin binding was further supported by the loss of flavins seen in the eNOS C689A single mutant and C689A/C908A double mutant. Furthermore, the perturbation of the reductase domain induced by GSSG modification allows Grx1 access to these mixed-disulfide bonds (C689/C908), allowing deglutathionylation.

It has been asserted that, under normal physiological conditions, cellular GSSG concentrations may not reach a thermodynamically favorable level that can effectively S-glutathionylate protein thiols to redox-modulate protein function.[41,42] Previously, we have identified an efficient mechanism in which the formation of S-glutathionylated protein thiols of eNOS can go through a labile intermediate protein thiyl radical generated by ROS followed by the reaction with GSH under oxidative stress.[32] It is also important to identify whether there is an efficient enzyme-catalyzed regulation of eNOS S-glutathionylation sensitive to fluctuations in redox state, analogous to phosphorylation by the kinases and dephosphorylation by the phosphatases.

Grx enzymes are GSH-dependent enzymes that can specifically reduce a mixed-disulfide bond from S-glutathionylated proteins generating GSSG as a product. When the level of oxidant stress is increased, the cellular GSSG concentration is also increased. This increase will make the cellular redox potential less negative in favor of S-glutathionylation. A recent study showed Grx can oxidatively modify its target protein when high GSSG concentrations are present.[43]

In this study, we demonstrate that indeed Grx1 can effectively S-glutathionylate eNOS. When Grx1 is included in the reaction mixture, it can overcome the reaction energy barrier and accelerate the formation of eNOS S-glutathionylation. This eNOS S-glutathionylation by Grx1 depends on the GSSG/GSH ratio and the total GSH pool.[39,44,45] Thus, the increase in the level of GSSG is thermodynamically driving this reaction toward S-glutathionylation, and the formation of S-glutathionylated eNOS is more efficient in the presence of Grx1. With the addition of 1 mM GSH, the reaction is favored in the reverse reaction, deglutathionylation, in which Grx1 can effectively reduce this mixed-disulfide bond of eNOS. The discovery of eNOS S-glutathionylation by Grx1 provides an additional pathway for redox modulation of eNOS function under oxidative stress that may be more efficient and sensitive than GSSG acting alone. During ischemia/reperfusion injury, the export of GSSG from cells was reported.[42,46] This can lead to an increase in the GSSG/GSH ratio and a decrease in the size of the total GSH pool. The local GSSG concentration is increased with the burst of $^{\bullet}O_2^-$ generation during reperfusion[47,48] that can dramatically increase the GSSG/GSH ratio locally. Thus, the increase in the GSSG/GSH ratio can provide a driving force shifting the reaction in favor of S-glutathionylation by Grx1.

eNOS S-glutathionylation by Grx1 decreases the level of NO production by the enzyme, but stimulation of eNOS uncoupling is not seen. This is different from what is seen when eNOS is directly S-glutathionylated by GSSG, where S-glutathionylation at Cys689 and Cys908 of the reductase domain leads to the generation of $^{\bullet}O_2^-$ from the enzyme. LC–MS/MS analysis of this Grx1-catalyzed eNOS S-glutathionylation reveals that the primary site of modification is located at Cys382, on the surface of the oxygenase domain (Figure 4B), which is accessible for Grx1 modification. From the three-dimensional structure of the eNOS reductase domain (Figure 4C), the two critical cysteine residues (Cys689 and Cys908) are buried in the interface of this domain and are not accessible for Grx1 oxidative modification. Thus, neither Cys689 nor Cys908 is S-glutathionylated by Grx1 under these conditions.

The identification of eNOS S-glutathionylation at Cys382 provides evidence that this particular modification of the oxygenase domain of eNOS affects only NO production by shutting down its enzymatic function without a concomitant increase in the level of production of $^{\bullet}O_2^-$. The decrease in the level of production of NO from the Grx1-modified enzyme can prevent the formation of the more potent oxidant, peroxynitrite, which could cause further irreversible damage to the enzyme. Mutagenesis of eNOS at this specific residue resists Grx1-catalyzed eNOS S-glutathionylation, indicating that Cys382 is the primary site for Grx1-induced S-glutathionylation. Thus, eNOS S-glutathionylation by Grx1 at Cys382 is sensitive to the fluctuation of the [GSSG]/[GSH] ratio and provides a unique mechanism that may protect this eNOS protein thiol from further oxidation under oxidative stress.

The coprecipitation of Grx1 and eNOS in endothelial cells and in *ex vivo* and *in vitro* experiments further supports the idea that Grx1 has a critical function in the redox modulation of eNOS as a function of the cellular redox state. The Grx1-mediated reduction of S-glutathionylated eNOS as well as its oxidation of eNOS to form protein S-glutathionylated adducts suggests that Grx1 plays a dual role in redox regulation depending on the GSSG/GSH ratio, analogous to the function of kinases and phosphatases in protein phosphorylation and dephosphorylation.

Direct inhibition of Grx enzymes by Cd^{2+} or *grx1* gene silencing in BAECs inactivated NOS through induction of eNOS S-glutathionylation and the formation of interprotein disulfide bonds. It would be expected that S-glutathionylation of eNOS occurs, possibly through disulfide exchange or thiyl radical intermediates, when Grx1 is knocked down or inhibited by Cd^{2+}. This provides evidence that Grx1 is required to maintain eNOS protein thiols in the reduced state supporting proper eNOS coupling in cells. This finding supports our hypothesis that Grx1 is critical in the redox modulation of eNOS function and the maintenance of vascular tone.

In conclusion, Grx1 in the presence of GSH is required for efficient eNOS deglutathionylation and allows restoration of eNOS function. However, under mild to moderate oxidative stress with an increase in the level of GSSG, Grx1 can also S-glutathionylate eNOS with inactivation of NO synthesis without uncoupling, thereby protecting eNOS from further oxidation. Cys382 of the eNOS oxygenase domain is identified as the major site modified by Grx1, in contrast to nonenzymatic S-glutathionylation of eNOS induced by GSSG or radical attack, in which the major modifications are within the reductase domain. Inhibition of Grx enzymes and *grx1* gene silencing in cells lead to endothelial dysfunction with loss of NO production in part because of the increase in the level of eNOS S-glutathionylation and interprotein disulfide bond formation. The identification of the dual role of Grx1 in glutathionylation and deglutathionylation involved in the reversible modulation of eNOS activity as a function of redox state provides a sensitive mechanism of redox regulation of vascular function and signaling during redox stress.

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Notes

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ABBREVIATIONS

BAECs, bovine aortic endothelial cells; CaM, calmodulin; eNOS, endothelial nitric oxide synthase; EPR, electron paramagnetic resonance; Fe^{2+}-MGD, Fe-*N*-methyl-D-glucamine dithiocarbamate; Grx1, glutaredoxin-1; L-NAME, L-N^G-nitro-arginine methyl ester hydrochloride; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; PDB, Protein Data Bank; RT, room temperature; $^{\bullet}O_2^-$, superoxide; BH_4, tetrahydrobiopterin.

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