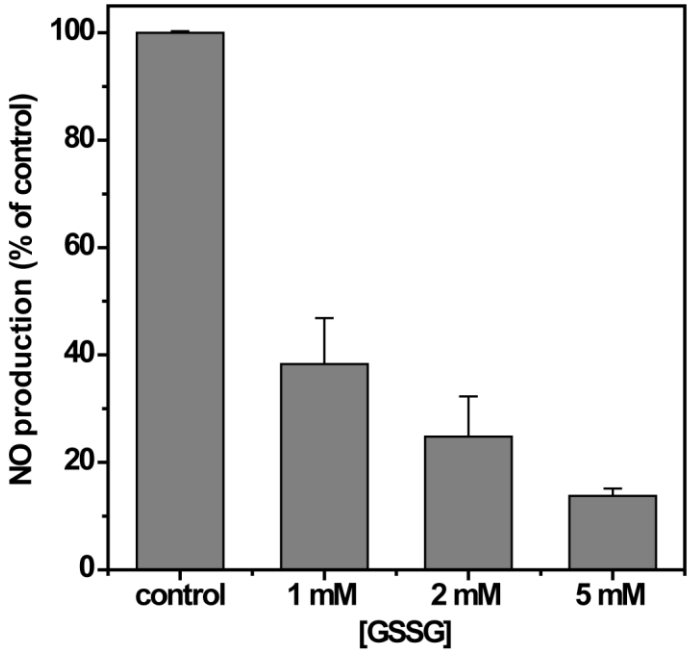
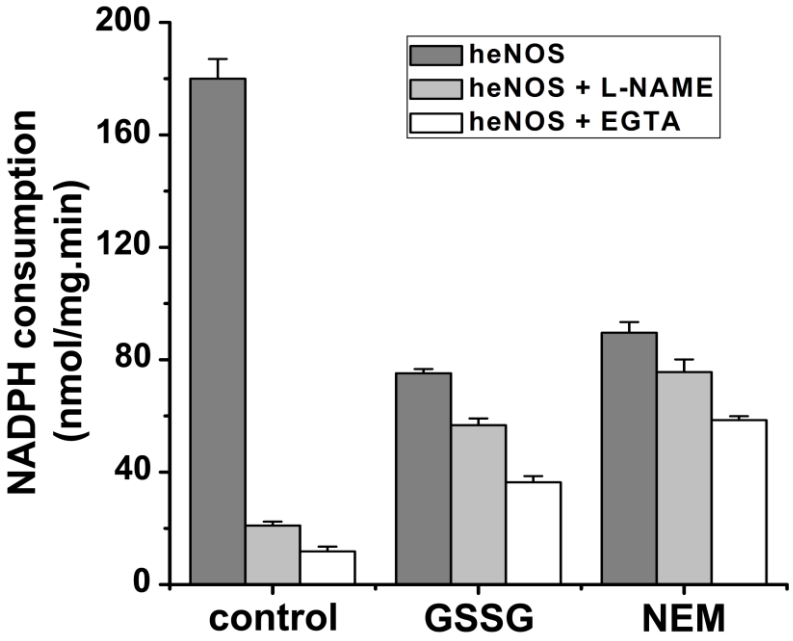
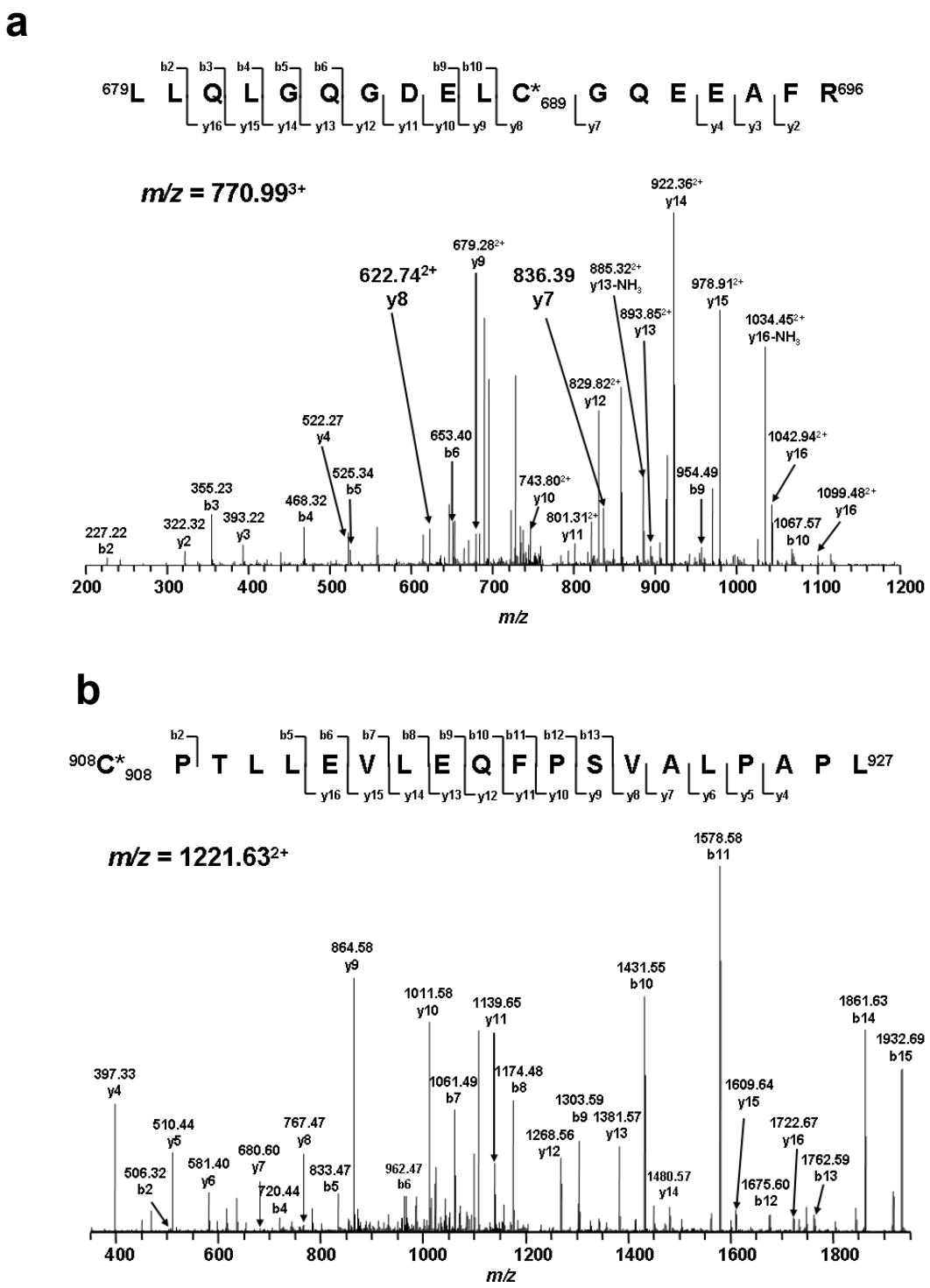
doi:10.1038/nature09229



Supplementary Figure 1. GSSG dose dependence of eNOS activity. 10 μg of heNOS was incubated with the desired concentration (0, 1, 2, and 5 mM) of GSSG at room temperature for 20 min before measuring NO production from eNOS using Fe^{2+}-MGD (0.25 mM Fe^{2+}, 2.5 mM MGD) EPR spin-trapping. S-glutathionylation of heNOS dose-dependently reduced NO generation from the enzyme (n=4, bars show mean ± standard error).

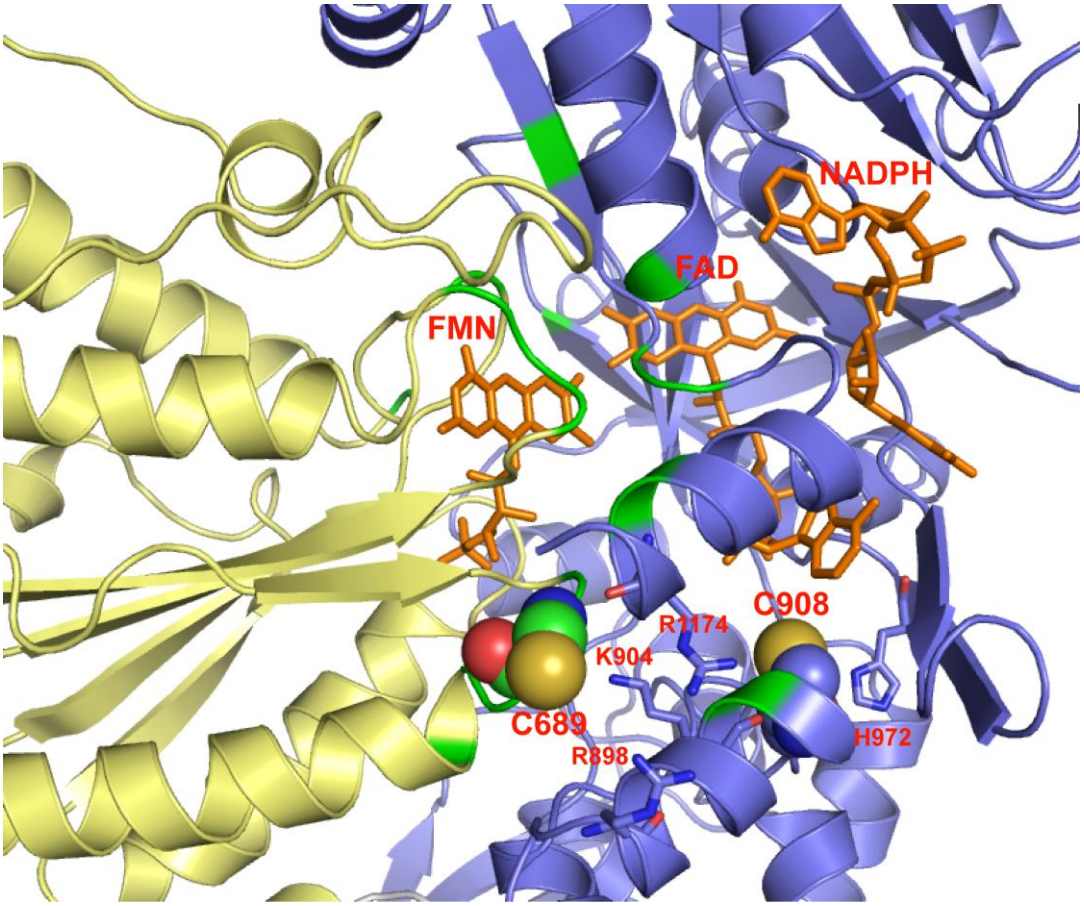


Supplementary Figure 2. Effect of S-glutathionylation and S-alkylation on NADPH consumption. NADPH consumption was measured from control, S-glutathionylated (2 mM GSSG for 20 min), or alkylated (NEM 1 mM for 20 min) heNOS. NADPH consumption from S-glutathionylated or NEM modified heNOS was only partially decreased and, in contrast to NOS activity, this was only partially blocked by L-NAME or EGTA suggesting uncoupling of the enzyme. Data were expressed as mean ± SEM, n=3.

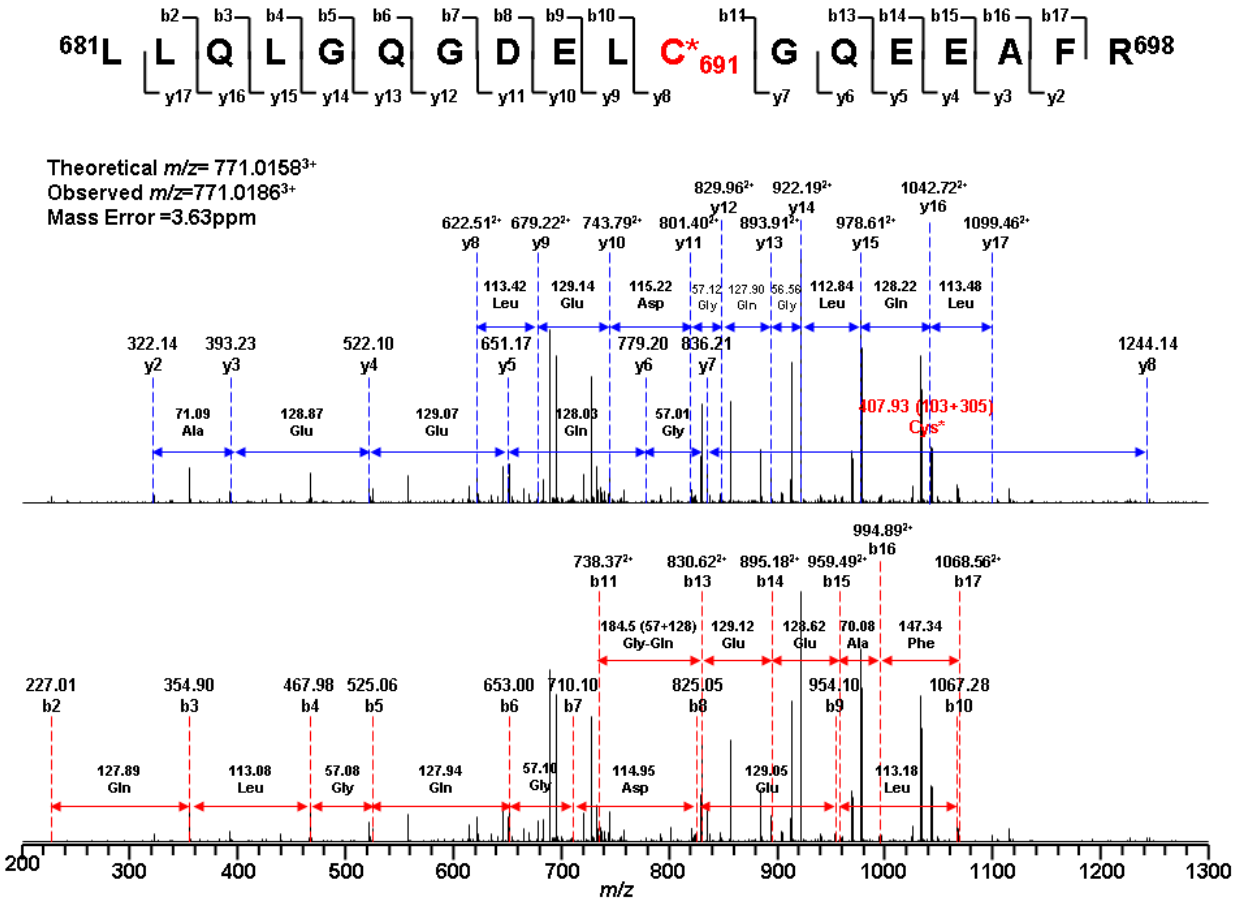


Supplementary Figure 3. Mass spectrometric identification of the sites of cysteine S-glutathionylation. (a) MS/MS spectrum of triply protonated molecular ion, *m/z* = 770.99^{3+} of aa 679-696. S-glutathionylated Cys689 was determined from tryptic fragment

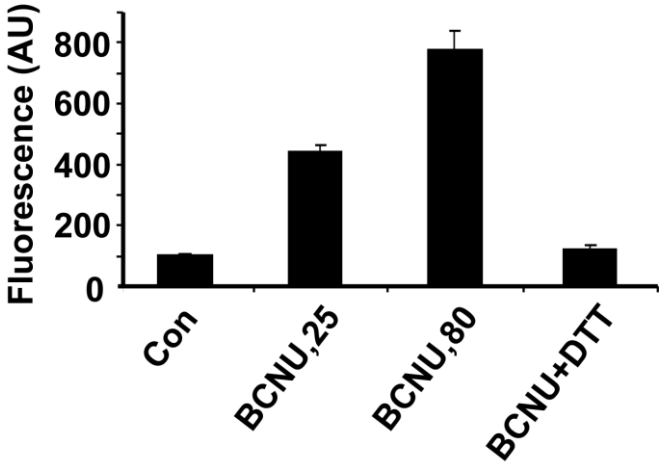
$_{679}$LLQLGQGDELC*$_{689}$GQEEAFR$_{696}$. The original MW for the tryptic fragment LLQLGQGDELCGQEEAFR is 2005.96 while that detected is 2310.97. The mass difference is 305 Da, corresponding to GS. The MS/MS spectrum of the triply protonated tryptic fragment ion at *m/z* 770.99^{3+} was obtained and the MW difference between fragment ions y_8 and y_7 of 305 Da compared to the native fragment ions, allows unequivocal assignment of the glutathionylation site to Cys-689. The sequence-specific ions are labeled as *y* and *b* ions. The amino acid residue involved in glutathionylation is identified with an asterisk. **(b) MS/MS spectrum of doubly protonated molecular ion, *m/z* = 1221.63^{2+} of aa 908-927.** S-glutathionylated Cys908 was determined from tryptic and chymotryptic fragment $_{908}$C*PTLLEVLEQFPSVALPAPL$_{927}$. The original MW for CPTLLEVLEQFPSVALPAPL is 2137.16, while that detected is 2442.26. The mass differences are 305 Da, corresponding to the mass of GS-. The MS/MS spectrum of the doubly protonated chymotrypic fragment ion at *m/z* 1221.63^{2+} exhibits ions including b2 and b5-b13 with a mass shift of 305 Da confirming S-glutathionylation of Cys908. The sequence-specific ions are labeled as *y* and *b* ions.



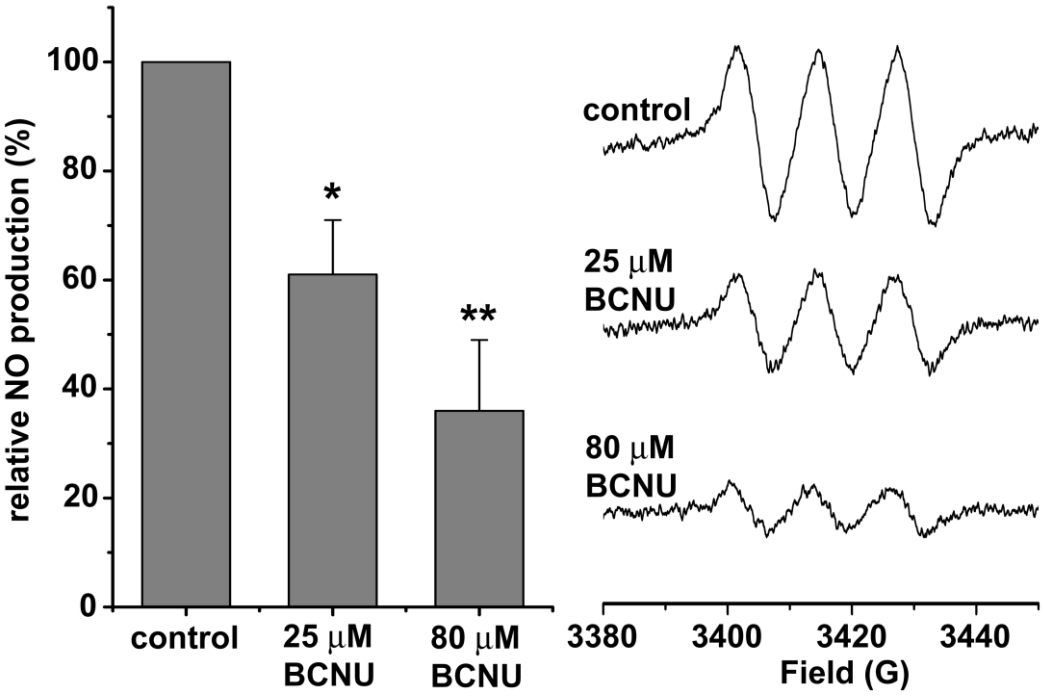
Supplementary Figure 4. Molecular model of the heNOS reductase domain showing sites of S-glutathionylation. Cys689 and Cys908 are both located on the reductase surface surrounded by several positive charge residues. Cys689 is surrounded by Arg898, Lys904, and Arg1174, while Cys908 is in a pocket with His972, Arg1174, and the adenine ring of FAD. Yellow region represents FMN domain, while blue region represents FAD domain. Green region represents residues at the interface of FMN and FAD domains. Cys689 is located at the FMN domain interface and Cys908 is near the FAD domain interface.



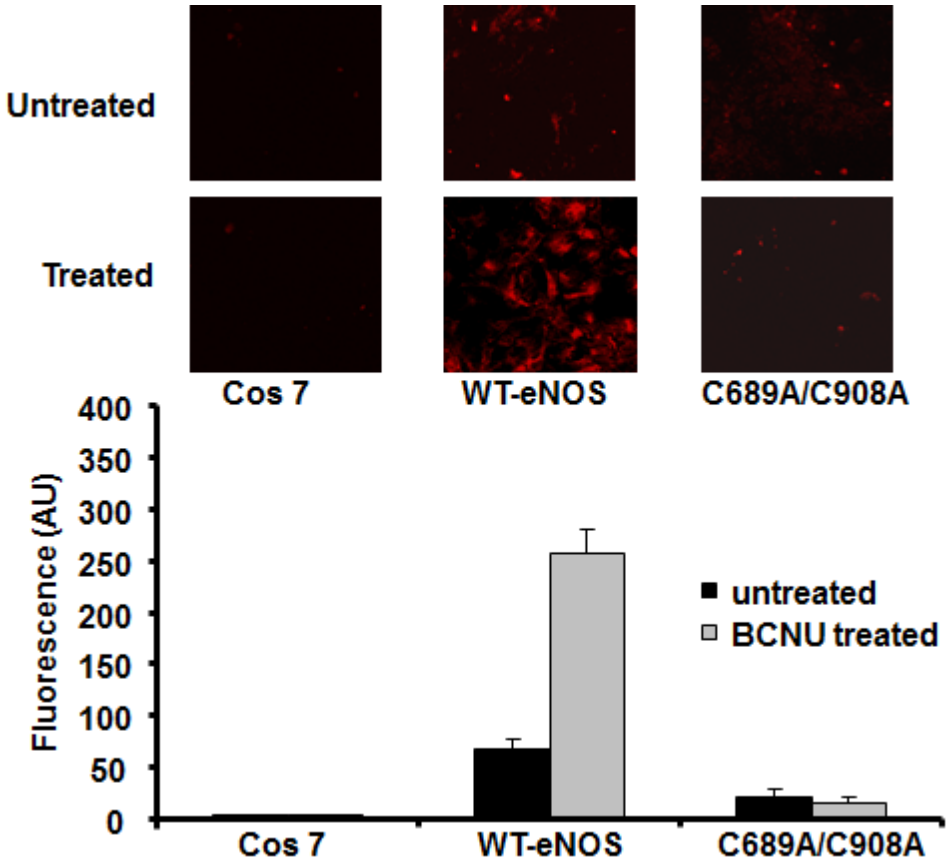
Supplementary Figure 5. Mass spectrometric identification of eNOS S-glutathionylation in BAECs under oxidative stress. To determine if the same cysteine residue of eNOS is glutathionylated in BAECs as that identified from purified eNOS treated with GSSG, BAECs were preincubated with 80 μM BCNU that decreases the ratio of GSH/GSSG and induces eNOS S-glutathionylation. After 4 hours BCNU treatment, cells were homogenized in lysis buffer 50 mM Tris.HCl pH7.4 containing 1% NP-40, 0.1% sodium deoxycholate, 0.1 mM EDTA, and protease inhibitors, followed by immunoprecipitation (IP) using anti-eNOS antibody. The product of immunoprecipitation was separated using 4-20% SDS-PAGE. The band corresponding to eNOS was cut and subjected to mass spectrometric analysis. The detailed procedure is described in the methods section. MS/MS spectrum of triply protonated molecular ion, m/z = 771.0186^{3+} of aa 681-698 (sequences based on bovine sequence, aa 679-696 for human sequence) is shown. S-glutathionylated Cys691 of bovine (Cys689 for human sequence) was determined from tryptic fragment $_{681}$LLQLGQGDELC*$_{691}$GQEEAFR$_{698}$. The percentage of S-glutathionylation on Cys 691 with 80 μM BCNU treatment was > 50% (52-60%). S-glutathionylation of Cys908 also appeared when IP eNOS was treated with 2 mM GSSG. The ratio between GS- modified Cys689 and C908 was 3 to 1. From these studies, we see that Cys689 is a major site of modification in these cells, while Cys908 modification was also detected.



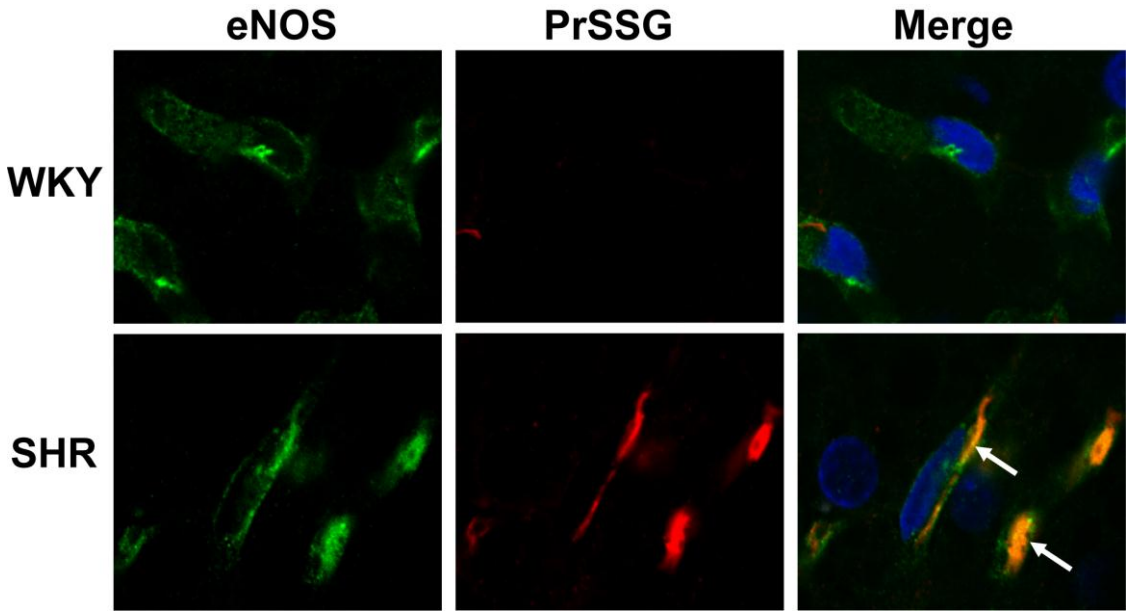
Supplementary Figure 6. Graph of BCNU induced $\cdot O_2^-$ generation from BAECs (Figure 3a). Bars from left to right are corresponding to control, 25 μM BCNU, 80 μM BCNU, and 25 μM BCNU + 1 mM DTT. DHE fluorescence was quantified and plotted as mean ± SE from three experiments. n=5.



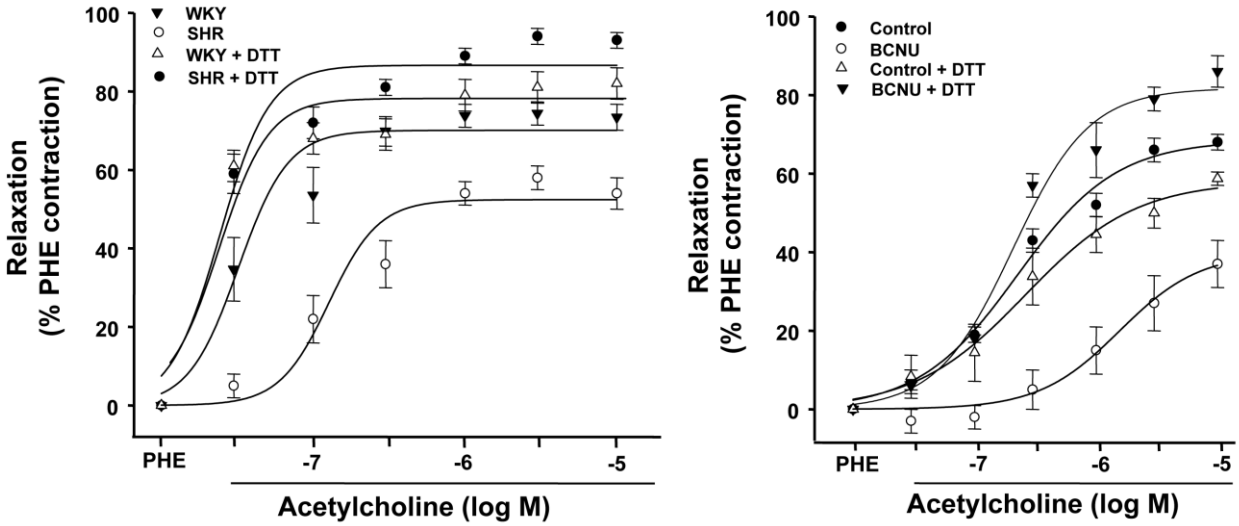
Supplementary Figure 7. NO generation from BAECs measured by EPR spin trapping using Fe^{2+}-MGD. Spectra are shown in the right panel and results from a series of experiments as a bar graph in the left panel. While a NO triplet signal is seen in untreated cells, BCNU dose-dependently decreased this NO generation (* $P < 0.05$, ** $P < 0.01$ vs. respective controls). Data were expressed as mean ± SEM, n=3.



Supplementary Figure 8. Importance of eNOS C689/C908 in S-glutathionylation-induced $\cdot O_2^-$ generation in cells. COS7 cells were transiently transfected with mammalian expression plasmids containing either wild type (WT) or mutant (C689A/C908A) eNOS using Effectine (Qiagen Inc.). 72 hours after transfection the cells were treated/untreated with BCNU 80 μM for 3 hrs and incubated with dihydroethidium (DHE). Briefly, the cells were incubated with or without CaI 10 μM for 10 min prior to 5 μM DHE (543 nm) for 15 min at 37°C, in a light-protected humidified chamber. Cells were washed, fixed, permeabilized and probed for eNOS simultaneously (488 nm). The images were obtained with a laser scanning confocal microscope (Olympus Filter Flow View 1000). DHE fluorescence was quantified by automated image analysis using Image J software (National Institutes of Health) and normalized for the eNOS expression in the WT-eNOS and C689A/C9089A group. BCNU treatment that induces eNOS S-glutathionylation increased $\cdot O_2^-$ generation in cells with WT-eNOS but not in cells with C689A/C908A eNOS confirming that S-glutathionylation at these residues triggers $\cdot O_2^-$ generation. Data were expressed as mean ± SEM, n=5.



Supplementary Figure 9. En face immunohistology of eNOS and PSSG in aorta of control WKY and SHR rats. Tissues were cryosectioned en face and probed for eNOS (green) and PrSSG (red) as described in the methods section. Merged images reveal areas of overlap (yellow-orange color, white arrows) where co-mapping of eNOS and protein glutathionylation is clearly seen.



Supplementary Figure 10. Endothelium-dependent vasorelaxation studies in SHR and BCNU-treatment models showing the effects of DTT in reversing vascular dysfunction. **Left panel:** Endothelium-dependent vasorelaxation was determined in SHR and control (WKY) aortic rings. SHR aortic rings displayed a marked decrease in endothelium-dependent relaxation to ACh. However, when treating the same SHR rings with DTT (1 mM) the endothelium–dependent response to ACh is reestablished. DTT treatment of control WKY rings only modestly increased the observed relaxation. Aortic relaxation expressed as % decrease of phenylephrine (PHE)-induced contraction (y-axis); vs. molar concentration of agonist on a logarithmic scale (x-axis). Results are expressed as mean ± SEM; $P < 0.05$ for SHR vs WKY or DTT treatment groups. n=4. **Right panel:** Endothelium-dependent vasorelaxation was determined in control and BCNU (80 μM) treated rat aortic rings. BCNU markedly decreased endothelium-dependent relaxation of acetylcholine (ACh). DTT (1 mM) reversed the BCNU induced inhibition of relaxation. DTT treatment of control rings had no significant effect on relaxation. Aortic relaxation expressed as % decrease of phenylephrine (PHE)-induced contraction (y-axis); vs. molar concentration of agonist on a logarithmic scale (x-axis). Results are expressed as mean ± SEM; $P < 0.05$ for BCNU vs. respective control or DTT treatment groups. The data were fit to the Hill equation using non-linear regression global curving fitting. n=4.