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AUGMENTED S-NITROSYLATION CONTRIBUTES TO IMPAIRED RELAXATION IN ANGIOTENSIN II HYPERTENSIVE MOUSE AORTA: ROLE OF THIOREDOXIN REDUCTASE

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Abstract

Vascular dysfunction, including reduced endothelium-dependent dilation, is a major characteristic of hypertension. We previously investigated that TrxR inhibition impairs vasodilation via soluble guanylyl cyclase *S*-nitrosylation, but *S*-nitrosylation and TrxR function are not known in hypertension. We hypothesized that *S*-nitrosylation is associated with reduced vasodilation in hypertensive mice. Aortic rings from normotensive (sham) and angiotensin II (AngII)-induced hypertensive C57BL/6 mice were treated with a TrxR inhibitor, 1-chloro-2,4-dinitrobenzene (DNCB) for 30 min, and relaxation to acetylcholine (ACh) was measured in the rings following contraction with phenylephrine. DNCB reduced relaxation to ACh compared with vehicle in sham aorta but not in AngII (sham-vehicle $E_{\max}=77\pm2$, sham-DNCB $E_{\max}=59\pm4$, $*p<0.05$). DNCB shifted the concentration-response relaxation to sodium nitroprusside (SNP) to the right in both sham and AngII aortic rings (sham-vehicle $pD_2=8.8\pm0.1$, sham-DNCB $pD_2=8.4\pm0.1$, $*p<0.05$; AngII-vehicle $pD_2=8.5\pm0.1$, AngII-DNCB $pD_2=8.3\pm0.1$, $*p<0.05$). As downstream signaling of nitric oxide, cyclic GMP level was reduced by DNCB during activation with SNP. The effect of DNCB to increase *S*-nitrosylation was confirmed by the biotin-switch method and western blot analysis and total protein *S*-nitrosylation was increased in AngII aorta (1.5-fold) compared to sham. TrxR activity was inhibited in AngII aorta compared to sham. We conclude that increased *S*-nitrosylation contributes to impaired relaxation in aorta from AngII-induced hypertensive mice. AngII treatment resulted in inactivation of TrxR and increased *S*-nitrosylation, indicating that TrxR and *S*-nitrosylation may provide a critical mechanism in hypertension associated with abnormal vascular reactivity.

Keywords

S-Nitrosylation; Thioredoxin reductase; Vascular relaxation; Hypertension

INTRODUCTION

Vascular dysfunction, including reduced endothelium-dependent dilation and enhanced vasoconstriction, is a major feature of hypertension. This has been observed in hypertensive

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patients where forearm blood flow responses to vasodilators are impaired [1–3]. Compromised endothelium-dependent vascular relaxation, referred to as endothelial dysfunction, has been demonstrated in both conduit arteries and resistance arteries in several hypertensive animal models. Endothelium-dependent vasodilation is reduced in isolated arteries from spontaneously hypertensive rats [4, 5], deoxycorticosterone acetate salt-treated animals [6, 7], Dahl salt-sensitive rats [8], and in animal models of renovascular hypertension [9, 10].

Nitric oxide (NO) is an important signaling molecule in the cardiovascular system and a regulator of vascular tone. NO also reacts indirectly with biological molecules and these chemical reactions include *S*-nitrosylation, which is a post-translational modification forming *S*-nitrosothiol (SNO) on the cysteine residue of target proteins [11]. Since proteins with a critical role in vascular function could be targets for *S*-nitrosylation, studies on the effects of *S*-nitrosylation are essential to understand the regulation of the vascular signaling under both normal and pathological conditions. *S*-Nitrosylation is regulated by denitrosylation to decrease *S*-nitrosylation, and one of the enzymes for denitrosylation is the thioredoxin reductase (TrxR). TrxR reduces thioredoxin (Trx) using the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction. Reduction of Trx by TrxR degrades the *S*-nitrosylation from proteins [12]. Mammalian TrxR can reduce several substrates, such as glutaredoxin and glutathione [13, 14], but its main target is Trx. In addition to denitrosylation, TrxR serves in many cell functions, including: antioxidant formation, cell growth, inhibition of apoptosis, and gene transcription [15].

Abnormal *S*-nitrosylation is related to various human diseases, such as diabetes, heart failure, asthma and pulmonary hypertension [16–19]. However, an association between hypertension and *S*-nitrosylation has not been demonstrated. Therefore, the present study aims to investigate a novel regulatory mechanism, *S*-nitrosylation, modulating vascular function during hypertension. In this study, we hypothesized that increased *S*-nitrosylation is associated with impaired vasodilation in hypertension. To investigate this hypothesis, we tested vascular relaxation in aorta from normotensive and angiotensin II (AngII)-induced hypertensive mice. Specifically, we focused on TrxR function to demonstrate *S*-nitrosylation effects on vascular reactivity, using a TrxR inhibitor to stabilize the level of *S*-nitrosylation [12, 20].

METHODS

Drugs and reagents

AngII was obtained from Phoenix Pharmaceuticals (Burlingame, CA). 1-Chloro-2,4-dinitrobenzene (DNCB) and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). *S*-Nitrosocysteine (CysNO) was synthesized from L-cysteine using acidified nitrite. Auranofin was purchased from BioMol (Plymouth Meeting, PA).

Animals

Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME), 12–14 weeks of age, were used in this study. To induce AngII hypertension, osmotic minipumps (Durect Corporation,

Cupertino, CA) with AngII (3.6 µg/kg/min) were implanted subcutaneously into the dorsum for 14 days. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Georgia Health Sciences University Committee on the Use of Animals in Research and Education. The animals were housed on a 12-hour light/dark cycle and fed a standard chow diet with water *ad libitum*.

Isolation of aortic rings and functional studies

After mice were euthanized, thoracic aortae were excised, cleaned from fat tissue and cut into 2 mm length-rings in an ice-cold physiological salt solution consisting of the following: 130 mmol/L NaCl, 4.7 mmol/L KCl, 1.18 mmol/L KH₂PO₄, 1.18 mmol/L MgSO₄·7H₂O, 1.56 mmol/L CaCl₂·2H₂O, 14.9 mmol/L NaHCO₃, 5.6 mmol/L glucose, and 0.03 mmol/L EDTA. Aortic rings were mounted in a myograph (Danish Myo Technology A/S, Aarhus, Denmark) containing warmed (37°C), oxygenated (95% O₂/5% CO₂) physiological salt solution. The preparations were equilibrated for at least 1 hour under a passive force of 5 mN. After the equilibration period, arterial integrity was assessed by stimulation of vessels with 120 mmol/L KCl and, after contraction reached a plateau, the rings were washed. Subsequently, the rings were stimulated with phenylephrine (PE, 10⁻⁷ mol/L) followed by relaxation with acetylcholine (ACh, 10⁻⁶ mol/L). More than 70 % relaxation response to ACh was taken as evidence of an intact endothelium. Some aortic rings were incubated with DNCB (4 µmol/L) for 30 min or CysNO (10 µmol/L) for 15 min. Endothelium-dependent relaxation was performed on PE-contracted rings followed by cumulative concentration-response curves to ACh (10⁻⁹ to 3×10⁻⁵ mol/L) and endothelium-independent relaxation was tested with sodium nitroprusside (SNP, 10⁻¹¹ to 10⁻⁷ mol/L).

cGMP assay

Cleaned aortae were incubated with vehicle (ethanol, 0.1 %) or DNCB (4 µmol/L) for 1 h at 37 °C in Dulbecco's modified Eagle's medium (DMEM) and then treated with SNP (3×10⁻⁸ mol/L) for 30 min. Cyclic 3',5'-guanosine monophosphate (cGMP) was measured using a commercially available cGMP enzyme-linked immunoassay (cGMP EIA kit) according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

Detection of S-nitrosylation

Aortae were treated with vehicle (ethanol, 0.1 %) or DNCB (4 µmol/L) for 1 h at 37 °C in DMEM. The biotin-switch method to detect S-nitrosylation was performed with a S-nitrosylated protein detection assay kit (Cayman Chemical, Ann Arbor, MI) modified from the method of Jaffrey et al [21]. Biotin-labeled proteins were used for western blot analysis.

Western blot analysis

Proteins (40 µg) extracted from aortae or biotin-labeled proteins were eluted by boiling in Laemmli sample buffer (Bio-Rad, Hercules, CA) with or without mercaptoethanol, respectively. These proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membranes were blocked with 5% skim milk in Tris-buffered saline solution with Tween-20 for 1 h at room-temperature. Membranes for biotinlabeled proteins were then incubated with anti-biotin

antibody conjugated with horseradish peroxidase (HRP) (Cayman Chemical) overnight at 4°C. Other membranes were incubated with primary antibodies, such as TrxR1 and TrxR2 (Abcam, Cambridge, MA) overnight at 4°C. After incubation with secondary antibodies and washing of the membranes, signals were developed with chemiluminescence, visualized by autoradiography, and quantified densitometrically.

TrxR activity

Aortae were treated with vehicle (ethanol) or DNCB (4 µmol/L) for 1 h at 37 °C in DMEM. TrxR activity was measured using a commercially available TrxR assay kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions.

Recombinant protein delivery

To deliver recombinant protein, TrxR (American Diagnostic Inc., Stamford, CT), into the aortic rings, Chariot protein delivery reagent (Active Motif, Carlsbad, CA) was used according to the manufacturer's instruction as described previously [22, 23]. This transfection reagent is able to deliver protein and antibody into cells and has been used successfully to deliver proteins to lung and vascular tissue [24, 25]. In brief, 6 µl of Chariot reagent in 100 µl of 40% dimethyl sulfoxide (DMSO) were mixed with 9 µg of protein in 100 µl of phosphate-buffered saline (PBS) and incubated at room temperature for 30 minutes. The aortae were transferred to 400 µl of DMEM in a 24-well cell culture plate, and 200 µl of the protein/Chariot complexes were added. Aortae were incubated for 1 hour at 37 °C in a CO₂ incubator, followed by addition of DMEM (750 µl) and further incubated for 3 hours at 37 °C (CO₂ incubator). Subsequently, the aortic rings were mounted in the myograph, and functional studies were performed.

Statistical analysis

Values are mean ± standard error of the mean (SEM), and 'n' represents the number of animals used in the experiments. Experimental values were calculated relative to the maximal changes from the contraction produced by PE in each segment, which was taken as 0%. The baseline tension before addition of PE was considered as 100%. Concentration-response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 4.0; GraphPad Software, San Diego, CA), and two pharmacological parameters were obtained: the maximal effect generated by the agonist (or E_{max}) and EC₅₀ (molar concentration of agonist producing 50% of the maximum response) or pD₂ (−log EC₅₀). Statistical differences were calculated by Student's *t*-test or one-way ANOVA. Post hoc test was performed using Newman-Keuls analysis to compare the all the groups. A *P* value less than 0.05 was considered to be statistically significant.

RESULTS

Effects of TrxR inhibition on vascular relaxation

Considering that endothelium-dependent relaxation is impaired in mice models of AngII-induced hypertension, we first measured relaxation to ACh and EC₅₀ value was attenuated in the AngII compared to sham normotensive mouse aorta (Figure 1A and 1D). To test the hypothesis that TrxR inhibition contributes to vascular relaxation, we used a TrxR inhibitor,

DNCB [12, 20]. In endothelium-intact aortic rings incubated with DNCB, relaxation to ACh was significantly reduced in sham, but not in AngII aortic rings (Figure 1B and 1C). In endothelium-independent relaxation responses, EC₅₀ value of relaxation to SNP was reduced in AngII compared to sham (Figure 2A and 2D). In addition, DNCB shifted the concentration-response relaxation to SNP to the right in both sham and AngII aortic rings (Figure 2).

Effect of TrxR in cGMP concentration

Figure 3 shows that basal cGMP levels without SNP was not different between control and DNCB-treated aorta. However, in the SNP-stimulated condition, DNCB decreased cGMP levels in both sham and AngII aorta. Also, SNP-treated aorta from AngII-induced hypertensive mice exhibited lower cGMP levels than sham aorta.

Contribution of TrxR and S-nitrosylation in AngII

As an alternative approach to evaluate the role of TrxR in vascular relaxation, TrxR protein delivery using the Chariot system resulted in normalization of vascular relaxation to ACh and SNP in AngII aortic rings (Figure 4).

To compare TrxR levels in sham and AngII aorta, TrxR protein expression and activity were measured. Protein expression of the cytosolic form TrxR1 and mitochondrial TrxR2 was not different between sham and AngII aorta (Figure 5A). However, TrxR activity was significantly less in AngII aorta when compared to sham (Figure 5B). To consider whether the attenuated TrxR activity in AngII is associated with the level of protein S-nitrosylation, total S-nitrosylated proteins were measured as described in the methods. Total S-nitrosylated proteins were increased in AngII aorta compared to sham (Figure 6).

Effects of S-nitrosylation on vascular relaxation

CysNO is used to increase directly the S-nitrosylation of proteins [26]. We investigated whether directly augmented S-nitrosylation contributes to relaxation and whether further increased S-nitrosylation affects relaxation in aortic rings from AngII mice. We observed that concentration-dependent responses to ACh and SNP were attenuated in the presence of CysNO in aortic rings from both sham and AngII (Figure 7).

DISCUSSION

The major finding of this study is that TrxR, through S-nitrosylation, plays a role in vascular relaxation during hypertension. Our results demonstrate that augmented S-nitrosylation is associated with impaired vasodilation in hypertensive mice. TrxR has various cellular functions including S-nitrosylation, which is a redox-based post-translational modification, leading to the modification of several proteins and alteration in their activity. However, the functional importance of TrxR and S-nitrosylation has not been elucidated in hypertension. Therefore, these findings provide a new concept, linking TrxR-mediated S-nitrosylation and impaired vascular reactivity in hypertension.

ACh stimulates relaxation of vascular smooth muscle by binding to the endothelial muscarinic receptor, which stimulates increased intracellular calcium to activate endothelial NO synthase (eNOS), ultimately leading to production of NO in endothelial cells. NO then diffuses into the vascular smooth muscle and stimulates soluble guanylyl cyclase (sGC) to convert guanosine triphosphate (GTP) to cGMP, which acts as a second messenger, affecting ion channels and proteins, and resulting in vascular relaxation [27]. Our data show that TrxR inhibition impaired endothelium-dependent relaxation in only sham, but not in AngII aortic rings. We speculate these data that in AngII, TrxR function is more important in vascular smooth muscle. Due to the hypertensive condition, in which endothelial dysfunction already exists [4–10], TrxR might already be inactivated in endothelial cells, and thus, DNCB did not change ACh-induced relaxation in hypertensive mice. In endothelium-independent relaxation, DNCB attenuated the concentration-response relaxation to SNP in both sham and AngII, suggesting that relaxation related to TrxR inhibition may be associated with impaired signaling of vascular smooth muscle proteins. As shown in Figure 3, cGMP levels were reduced by DNCB in SNP-treated aortae from both sham and AngII, indicating that sGC activity was desensitized following inhibition of TrxR [28]. Additionally, we determined that the cGMP concentration was decreased in AngII aorta compared to sham, and these results demonstrate that endothelium-independent relaxation is impaired in AngII. Taken together, these data suggest that AngII and inhibition of TrxR contribute to decreased cGMP, resulting in impaired relaxation.

There are a large number of Trx and TrxR functions related to human disease, such as cancer, inflammation, viral infection, and cardiovascular diseases [29]. Further, the expression of the Trx system proteins has been found to be changed in many disease conditions [30]. Also, TrxR activity is inhibited in the oxidized state [31, 32] and selenium is essential for the activity of TrxR [33]. We showed that in AngII-induced hypertension, the protein expression of TrxR in the aorta was not changed compared to normotensive aorta. However, investigation of TrxR activity showed that the activity was decreased in AngII compared to sham. Even though TrxR protein level was not different between normotensive and AngII hypertensive condition, it is possible that AngII hypertension could manipulate its oxidized state and reduce selenium availability, inducing inactivated TrxR. These data were consistent with our protein S-nitrosylation results showing that in AngII, augmented S-nitrosylation is associated with inactivation of TrxR, due to reduced denitrosylation by TrxR.

Deficiencies and/or an excess of NO can lead to dysregulation of S-nitrosylation in specific cellular targets and signaling pathways. In vascular reactivity, augmented S-nitrosylation impaired both endothelium-dependent and -independent relaxation in sham and AngII (Figure 7). In addition to relaxation, augmented S-nitrosylation inactivates PKC and inhibits PKC-mediated vasoconstriction [34]. Accordingly, S-nitrosylation within the vasculature may be a critical regulatory step to investigate as well as a potential pharmacological target in disease conditions related to vascular dysfunction. The possibility for therapeutic alterations of S-nitrosylation levels is receiving increasing attention, and potential agents that affect S-nitrosylation are being explored [35]. Thus, research on the molecular

mechanisms of dysregulated *S*-nitrosylation may be important for determining potential therapeutic targets.

Research into *S*-nitrosylation and diseases has shown that the level of *S*-nitrosylation is increased or decreased in various disease states. For example, in diabetes, the levels of *S*-nitrosylation are elevated and evidence has linked insulin resistance in type 2 diabetes with protein *S*-nitrosylation [17, 36]. Inducible NOS (iNOS)-mediated *S*-nitrosylation inactivates insulin signaling proteins, such as protein kinase B (PKB, Akt) and insulin receptor substrate 1 (IRS-1). On the other hand, protein *S*-nitrosylation is decreased in pulmonary hypertension and heart failure [16, 18]. In the heart, dysregulated *S*-nitrosylation may explain some of the characteristic dysfunction of the failing heart, because the cardiac ryanodine receptor/calcium channel (RyR2) and L-type calcium channel are known to be *S*-nitrosylated [37, 38]. Furthermore, as a reservoir of NO, SNO promotes circulating low-molecular-weight *S*-nitrosylation mediated by serum albumin and lowers vasodilatory NO levels in the vasculature [39]. Increased albumin *S*-nitrosylation can raise blood pressure in preeclamptic patients via deficiency of ascorbate, releasing NO, in plasma [40]. Additionally, increased SNO concentrations in plasma predict cardiovascular risk in patients with end-stage renal disease [41].

In conclusion, to date the level of protein *S*-nitrosylation has been incompletely examined in hypertension; however, the present study is the first to demonstrate that protein *S*-nitrosylation is increased, and related to impaired relaxation in aorta from AngII hypertensive mouse. Specifically, these effects are accompanied by TrxR inactivation, which is important in mediating redox-based protein signaling. Thus, TrxR and *S*-nitrosylation may represent a key mechanism and a new therapeutic approach in hypertension associated with abnormal vascular reactivity.

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Reference List

1. Benndorf RA, Appel D, Maas R, Schwedhelm E, Wenzel UO, Boger RH. Telmisartan improves endothelial function in patients with essential hypertension. *J Cardiovasc Pharmacol*. 2007; 50(4): 367–371. [PubMed: 18049303]
2. Panza JA, Quyyumi AA, Brush JE Jr, Epstein SE. Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N Engl J Med*. 1990; 323(1):22–27. [PubMed: 2355955]
3. Widlansky ME, Price DT, Gokce N, Eberhardt RT, Duffy SJ, Holbrook M, et al. Short-and long-term COX-2 inhibition reverses endothelial dysfunction in patients with hypertension. *Hypertension*. 2003; 42(3):310–315. [PubMed: 12874094]
4. Yang D, Gluais P, Zhang JN, Vanhoutte PM, Feletou M. Endothelium-dependent contractions to acetylcholine, ATP and the calcium ionophore A 23187 in aortas from spontaneously hypertensive and normotensive rats. *Fundam Clin Pharmacol*. 2004; 18(3):321–326. [PubMed: 15147283]
5. Luscher TF, Aarhus LL, Vanhoutte PM. Indomethacin improves the impaired endothelium-dependent relaxations in small mesenteric arteries of the spontaneously hypertensive rat. *Am J Hypertens*. 1990; 3(1):55–58. [PubMed: 2302329]

6. Cordellini S. Endothelial dysfunction in DOCA-salt hypertension: possible involvement of prostaglandin endoperoxides. *Gen Pharmacol.* 1999; 32(3):315–320. [PubMed: 10211585]
7. Jimenez R, Lopez-Sepulveda R, Kadmiri M, Romero M, Vera R, Sanchez M, et al. Polyphenols restore endothelial function in DOCA-salt hypertension: role of endothelin-1 and NADPH oxidase. *Free Radic Biol Med.* 2007; 43(3):462–473. [PubMed: 17602962]
8. Zhou MS, Kosaka H, Tian RX, Abe Y, Chen QH, Yoneyama H, et al. L-Arginine improves endothelial function in renal artery of hypertensive Dahl rats. *J Hypertens.* 2001; 19(3):421–429. [PubMed: 11288812]
9. Heitzer T, Wenzel U, Hink U, Krollner D, Skatchkov M, Stahl RA, et al. Increased NAD(P)H oxidase-mediated superoxide production in renovascular hypertension: evidence for an involvement of protein kinase C. *Kidney Int.* 1999; 55(1):252–260. [PubMed: 9893134]
10. Quaschnig T, Hochoer B, Ruhl S, Kraemer-Guth A, Tilgner J, Wanner C, et al. Vasoepitidase inhibition normalizes blood pressure and restores endothelial function in renovascular hypertension. *Kidney Blood Press Res.* 2006; 29(6):351–359. [PubMed: 17139187]
11. Gow AJ, Farkouh CR, Munson DA, Posencheg MA, Ischiropoulos H. Biological significance of nitric oxide-mediated protein modifications. *Am J Physiol Lung Cell Mol Physiol.* 2004; 287(2):L262–L268. [PubMed: 15246980]
12. Benhar M, Forrester MT, Hess DT, Stamler JS. Regulated protein denitrosylation by cytosolic and mitochondrial thioredoxins. *Science.* 2008; 320(5879):1050–1054. [PubMed: 18497292]
13. Johansson C, Lillig CH, Holmgren A. Human mitochondrial glutaredoxin reduces S-glutathionylated proteins with high affinity accepting electrons from either glutathione or thioredoxin reductase. *J Biol Chem.* 2004; 279(9):7537–7543. [PubMed: 14676218]
14. Lillig CH, Lonn ME, Enoksson M, Fernandes AP, Holmgren A. Short interfering RNA-mediated silencing of glutaredoxin 2 increases the sensitivity of HeLa cells toward doxorubicin and phenylarsine oxide. *Proc Natl Acad Sci U S A.* 2004; 101(36):13227–13232. [PubMed: 15328416]
15. Mustacich D, Powis G. Thioredoxin reductase. *Biochem J.* 2000; 346(Pt 1):1–8. [PubMed: 10657232]
16. McMahon TJ, Ahearn GS, Moya MP, Gow AJ, Huang YC, Luchsinger BP, et al. A nitric oxide processing defect of red blood cells created by hypoxia: deficiency of S-nitrosohemoglobin in pulmonary hypertension. *Proc Natl Acad Sci U S A.* 2005; 102(41):14801–14806. [PubMed: 16203976]
17. Carvalho-Filho MA, Ueno M, Hirabara SM, Seabra AB, Carnevali JB, de Oliveira MG, et al. S-nitrosation of the insulin receptor, insulin receptor substrate 1, and protein kinase B/Akt: a novel mechanism of insulin resistance. *Diabetes.* 2005; 54(4):959–967. [PubMed: 15793233]
18. Whalen EJ, Foster MW, Matsumoto A, Ozawa K, Violin JD, Que LG, et al. Regulation of beta-adrenergic receptor signaling by S-nitrosylation of G-protein-coupled receptor kinase 2. *Cell.* 2007; 129(3):511–522. [PubMed: 17482545]
19. Nozik-Grayck E, McMahon TJ, Huang YC, Dieterle CS, Stamler JS, Piantadosi CA. Pulmonary vasoconstriction by serotonin is inhibited by S-nitrosoglutathione. *Am J Physiol Lung Cell Mol Physiol.* 2002; 282(5):L1057–L1065. [PubMed: 11943671]
20. Holmgren A. Biochemistry. SNO removal. *Science.* 2008; 320(5879):1019–1020. [PubMed: 18497281]
21. Jaffrey SR, Snyder SH. The biotin switch method for the detection of S-nitrosylated proteins. *Sci STKE.* 2001; 2001(86):11.
22. Giachini FR, Chiao CW, Carneiro FS, Lima VV, Carneiro ZN, Dorrance AM, et al. Increased activation of stromal interaction molecule-1/Orai-1 in aorta from hypertensive rats: a novel insight into vascular dysfunction. *Hypertension.* 2009; 53(2):409–416. [PubMed: 19075091]
23. Clarke CJ, Forman S, Pritchett J, Ohanian V, Ohanian J. Phospholipase C-delta1 modulates sustained contraction of rat mesenteric small arteries in response to noradrenaline, but not endothelin-1. *Am J Physiol Heart Circ Physiol.* 2008; 295(2):H826–H834. [PubMed: 18567701]
24. Aoshiba K, Yokohori N, Nagai A. Alveolar wall apoptosis causes lung destruction and emphysematous changes. *Am J Respir Cell Mol Biol.* 2003; 28(5):555–562. [PubMed: 12707011]

25. Keller M, Lidington D, Vogel L, Peter BF, Sohn HY, Pagano PJ, et al. Sphingosine kinase functionally links elevated transmural pressure and increased reactive oxygen species formation in resistance arteries. *FASEB J.* 2006; 20(6):702–704. [PubMed: 16476702]
26. Greco TM, Hodara R, Parastatidis I, Heijnen HF, Dennehy MK, Liebler DC, et al. Identification of S-nitrosylation motifs by site-specific mapping of the S-nitrosocysteine proteome in human vascular smooth muscle cells. *Proc Natl Acad Sci U S A.* 2006; 103(19):7420–7425. [PubMed: 16648260]
27. Carvajal JA, Germain AM, Huidobro-Toro JP, Weiner CP. Molecular mechanism of cGMP-mediated smooth muscle relaxation. *J Cell Physiol.* 2000; 184(3):409–420. [PubMed: 10911373]
28. Choi H, Tostes RC, Webb RC. THIOREDOXIN REDUCTASE INHIBITION REDUCES RELAXATION BY INCREASING OXIDATIVE STRESS AND S-NITROSYLATION IN MOUSE AORTA. *J Cardiovasc Pharmacol.* 2011
29. Holmgren A, Lu J. Thioredoxin and thioredoxin reductase: current research with special reference to human disease. *Biochem Biophys Res Commun.* 2010; 396(1):120–124. [PubMed: 20494123]
30. Lillig CH, Holmgren A. Thioredoxin and related molecules--from biology to health and disease. *Antioxid Redox Signal.* 2007; 9(1):25–47. [PubMed: 17115886]
31. Gorlatov SN, Stadtman TC. Human thioredoxin reductase from HeLa cells: selective alkylation of selenocysteine in the protein inhibits enzyme activity and reduction with NADPH influences affinity to heparin. *Proc Natl Acad Sci U S A.* 1998; 95(15):8520–8525. [PubMed: 9671710]
32. Sun QA, Wu Y, Zappacosta F, Jeang KT, Lee BJ, Hatfield DL, et al. Redox regulation of cell signaling by selenocysteine in mammalian thioredoxin reductases. *J Biol Chem.* 1999; 274(35):24522–24530. [PubMed: 10455115]
33. Berggren M, Gallegos A, Gasdaska J, Powis G. Cellular thioredoxin reductase activity is regulated by selenium. *Anticancer Res.* 1997; 17(5A):3377–3380. [PubMed: 9413175]
34. Choi H, Tostes RC, Webb RC. S-Nitrosylation inhibits protein kinase C-mediated contraction in mouse aorta. *J Cardiovasc Pharmacol.* 2010
35. Foster MW, Hess DT, Stamler JS. Protein S-nitrosylation in health and disease: a current perspective. *Trends Mol Med.* 2009; 15(9):391–404. [PubMed: 19726230]
36. Kaneki M, Shimizu N, Yamada D, Chang K. Nitrosative stress and pathogenesis of insulin resistance. *Antioxid Redox Signal.* 2007; 9(3):319–329. [PubMed: 17184170]
37. Xu L, Eu JP, Meissner G, Stamler JS. Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. *Science.* 1998; 279(5348):234–237. [PubMed: 9422697]
38. Sun J, Picht E, Ginsburg KS, Bers DM, Steenbergen C, Murphy E. Hypercontractile female hearts exhibit increased S-nitrosylation of the L-type Ca²⁺ channel α_1 subunit and reduced ischemia/reperfusion injury. *Circ Res.* 2006; 98(3):403–411. [PubMed: 16397145]
39. Rafikova O, Rafikov R, Nudler E. Catalysis of S-nitrosothiols formation by serum albumin: the mechanism and implication in vascular control. *Proc Natl Acad Sci U S A.* 2002; 99(9):5913–5918. [PubMed: 11983891]
40. Gandle RE, Tyurin VA, Huang W, Arroyo A, Daftary A, Harger G, et al. S-nitrosoalbumin-mediated relaxation is enhanced by ascorbate and copper: effects in pregnancy and preeclampsia plasma. *Hypertension.* 2005; 45(1):21–27. [PubMed: 15569857]
41. Massy ZA, Fumeron C, Borderie D, Tuppin P, Nguyen-Khoa T, Benoit MO, et al. Increased plasma S-nitrosothiol concentrations predict cardiovascular outcomes among patients with end-stage renal disease: a prospective study. *J Am Soc Nephrol.* 2004; 15(2):470–476. [PubMed: 14747395]

Figure 1.

Concentration-response relaxation to ACh was attenuated by DNCB only in sham aortic rings but not in AngII. A: Relaxation to ACh was measured in vehicle-treated rings from sham (n=6) and AngII (n=9). B and C: Relaxation to ACh was measured after incubation with DNCB (4 μ mol/L) for 30 min in sham (B; n=6) or AngII (C; n=9) aortic rings. D: Representative pD₂ values from graphs A, B, and C. Relaxation responses were calculated relative to the maximal PE contraction, which was taken as 100%. Results are presented as mean $\pm$ SEM in each experimental group. Student's *t* test (A–C) and one-way ANOVA plus Newman-Keuls (D) were used to analyze. **p*<0.05 compared with sham-vehicle in pD₂ (A) or E_{max} (B).

Figure 2.

Vascular relaxation to SNP was shifted to the right by DNCB in sham and AngII. A: Relaxation to SNP was measured in vehicle-treated rings from sham (n=5) and AngII (n=8). B and C: Concentration-dependent relaxations to SNP were measured after incubation with vehicle or DNCB (4 μ mol/L, 30 min) in sham (B; n=5 to 6) or AngII (C; n=8) aortic rings. D: Representative pD₂ values from graphs A, B, and C. Relaxation responses were calculated relative to the maximal PE contraction, which was taken as 100%. Results are presented as mean $\pm$ SEM in each experimental group. Student's *t* test (A–C) and one-way ANOVA plus Newman-Keuls (D) were used to analyze. **p*<0.05 compared with sham-vehicle in pD₂. †*p*<0.05 compared between vehicle and DNCB in the AngII group in pD₂.

Figure 3.

DNCB decreased cGMP concentrations in the SNP-treated condition. cGMP levels were measured in mouse aorta to determine sGC activity. Aortae were incubated with or without DNCB (4 $\mu\text{mol/L}$, 1 h) in the presence or absence of SNP (3×10^{-8} mol/L, 30 min). Results are presented as mean $\pm$ SEM in each experimental group (n=3 to 6). One-way ANOVA plus Newman-Keuls were used to analyze. * $p < 0.05$ compared to control with SNP in each sham or AngII group. † $p < 0.05$ compared to control without SNP in each sham or AngII group. ** $p < 0.05$.

A **B** **C** **D**

Figure 4.

Increased TrxR normalized relaxation to ACh and SNP in AngII aortic rings. Concentration-dependent relaxations to ACh (A–D) or SNP (E–H) were measured after incubation of the aortic rings with empty chariot (control) or chariot-TrxR complex (TrxR). D and H: Bar graphs are representative pD₂ values from the ACh or SNP-mediated relaxation curves, respectively. Relaxation responses were calculated relative to the maximal PE contraction, which was taken as 100%. Results are presented as mean ± SEM in each experimental group (n= 6 to 8). Student's *t* test (A–C, E–G) and one-way ANOVA plus Newman-Keuls (D and H) were used to analyze. **p*<0.05 compared with sham-control (A; E_{max}, E; pD₂). †*p*<0.05 compared between control and TrxR in AngII (C and G; pD₂).

A **B** **Figure 5.**

TrxR protein expression was not different between sham and AngII, but TrxR activity was diminished in AngII aorta compared to sham. A: Representative western blot images of TrxR1 and TrxR2 proteins in aorta (Top). Bar graphs show the relative content of TrxR1 or TrxR2 proteins after normalization to β-actin expression (Bottom; n=4). B: TrxR activity was measured in mouse aorta as described in methods section. Results are presented as mean ± SEM in each experimental group and Student's *t* test was used to analyze. **p*<0.05 compared to control in sham (n=5 to 8).

A **B** **Figure 6.**

S-Nitrosylated proteins (SNO) were increased in AngII aorta compared to sham. A: Representative western blot images of *S*-nitrosylated proteins. B: Bar graphs showing the relative content of *S*-nitrosylated proteins after normalization to β-actin expression. Results are presented as mean ± SEM in each experimental group and Student's *t* test was used to analyze. **p*<0.05 compared with sham (n=4 to 5).

Figure 7.

CysNO decreased relaxation in both sham and AngII aortic rings. Concentration-dependent relaxations to ACh (A and B) or SNP (C and D) were measured after incubation of the aortic rings with vehicle or CysNO (10 μ mol/L, 15 min). Relaxation responses were calculated relative to the maximal PE contraction, which was taken as 100%. Results are presented as mean $\pm$ SEM in each experimental group and Student's *t* test was used to analyze (*n*=6). **p*<0.05 compared with sham-vehicle (A; $E_{\max}$, C; pD_2). †*p*<0.05 compared with AngII-vehicle (B; $E_{\max}$, D; $E_{\max}$ and pD_2).