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Endothelium-dependent Vasorelaxation is Inhibited by *in vivo* Depletion of Vascular Thiol Levels: Role of Endothelial Nitric Oxide Synthase

JØRN BECH LAURSEN*, SØREN BOESGAARD, SIMON TRAUTNER, INGER RUBIN, HENRIK E. POULSEN and JAN ALDERSHVILE

Medical Department B, Division of Cardiology, Institute of Biochemistry and Department of Clinical Pharmacology, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark

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Thiols like glutathione may serve as reducing cofactors in the production of nitric oxide (NO) and protect NO from inactivation by radical oxygen species. Depletion of thiol compounds reduces NO-mediated vascular effects *in vitro* and *in vivo*. The mechanisms underlying these actions are not clear, but may involve decreased synthesis of NO and/or increased degradation of NO. This study investigates the effect of glutathione depletion on the response to NO-mediated vasodilation induced by acetylcholine (Ach, 10 μg/kg), endothelial NO synthase (eNOS) activity and potential markers of vascular superoxide anion ($O_2^{\bullet-}$) production in conscious chronically catheterized rats. Thiol depletion induced by buthionine sulfoximine (BSO, 1 g ip within 24 h) decreased the hypotensive effect of Ach by 30% (MAP reduction before BSO 27 ± 3 mmHg, 19 ± 3 mmHg after BSO, (mean ± SEM), $p < .05$, $n = 8$). The impaired effect of Ach was associated with a significant reduction in eNOS activity (control: 7.7 ± 0.8, BSO: 3.9 ± 0.4 pmol/min/mg protein ($p < .05$), $n = 6$). In contrast, neither NADH/NADPH driven membrane-associated oxidases nor lucigenin reductase activity were significantly ($p < .05$) affected by BSO (BSO: 4415 ± 123, control: 4105 ± 455 counts/mg, $n = 6$) in rat aorta. It is concluded that *in vivo* thiol depletion results in endothelial dysfunction and a reduced receptor-mediated vascular relaxation. This effect is caused by reduced endothelial NO formation.

Keywords: Nitric oxide, thiols, free radicals, eNOS, vasorelaxation

INTRODUCTION

Nitric oxide (NO) generated from L-arginine acts as a potent vasodilator and plays a critical role in regulation of vascular tone.[1] Some *in vitro* studies show that thiol compounds (e.g. cysteine and/or glutathione (GSH), may inhibit NO-mediated vasorelaxation[2,3] while other *in vitro* data suggest that thiol compounds potentate NO-mediated vasodilation.[4–11] Thiols may facil-

* Corresponding author. Fax: + 45 35 45 25 13. E-mail: jbl37@hotmail.com.

itate endothelial L-arginine transport and act as reducing cofactors in the production of NO.[4–6] The antioxidant effects of thiols may prevent inactivation of NO synthase isoenzymes or NO itself by ($O_2^{\bullet-}$) formation.[5,7] In addition thiols may increase formation of stable and potent vasoactive nitrosothiols like nitrosoglutathione or other storage forms of NO and thus increase the bioavailability of NO.[8–10] Finally, thiols are necessary for activation of guanylate cyclase which mediates the vasodilator effect of NO.[8,11] Thus, thiol compounds may play a role both in the formation, bioactivity and stability of NO *in vitro*.

In vivo, recent clinical studies suggest that thiol supplementation may improve endothelium-dependent NO-mediated vasorelaxation in patients with coronary artery atherosclerosis.[12,13] This condition may be associated with increased oxidative stress, decreased cellular GSH content and reduced bioavailability of NO. However, the extent to which intracellular thiol levels regulate NO-mediated vasorelaxation *in vivo* is not clear at present. In fact, little is known about the effects of thiol depletion, and thus the thiol-dependency of NO-mediated vasodilation *in vivo*.

The primary aims of the present study were (1) to determine how *in vivo* thiol depletion affects NO-mediated vasodilation in healthy conscious rats and; (2) to study whether thiol depletion affects endothelial NO formation (i.e. eNOS activity) or affects factors responsible for the vascular bioactivity of NO (i.e. vascular $O_2^{\bullet-}$ formation) *in vivo*.

MATERIAL AND METHODS

Animals

Experiments were performed in female Wistar rats (200–230 g). Rats were anesthetized with 1–3% halothane and N_2O/O_2 (2:1) and underwent a chronic catheterization procedure. One catheter was implanted with its tip in the ascending aorta through the left carotid artery and two catheters were placed in the superior vena cava via the right jugular vein. Each catheter was externalized through the neck skin and postoperatively the rats were housed individually and exposed to a 12/12 h light–dark cycle with free access to standard rat chow and tap water. Postoperatively, rats were allowed to recover from surgery until they had regained their preoperative weight and appeared healthy (6–8 days). Details about this rat model with chronically catheterized conscious rats have been described previously.[14] All experiments conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of health (NIH publication No. 85–23, revised 1996).

Hemodynamic Measurements

NO-mediated reduction in mean arterial blood pressure (MAP) was induced by the endothelium-dependent vasodilator acetylcholine (Ach) and the endothelium-independent vasodilator sodium nitroprusside (SNP). Ach (10 μg/kg) and SNP (20 μg/kg) were administered as a bolus iv infusion (0.4 ml) lasting for 60 s and each infusion was separated by a 20 min interval allowing MAP to recover to the baseline value. The doses were chosen based on previous dose-response curves (data not shown) to elicit a fall in MAP of approximately 30 mmHg in normal untreated rats. Isotonic saline was used as control. Blood pressure during baseline conditions and after Ach, SNP and saline was recorded continuosly by pressure transducers (Baxter Corp, Uden, Holland) connected to the arterial catheter. Tracings were displayed on a Graphtec linear recorder (Watanabe Instruments Corp, Japan).

Estimation of Vascular $O_2^{\bullet-}$ Production

Lucigenin-enhanced chemiluminescence was measured as a marker of vascular $O_2^{\bullet-}$ formation. The details of this assay have been published previously.[15,16] Briefly, after removal, the

aortas were placed in a chilled modified Krebs/HEPES buffer (mM content; NaCl, 99.01; KCl, 4.69; $CaCl_2$, 1.87; $MgSO_4$, 1.20; K_2HPO_4, 1.03; $NaHCO_3$, 25.0; Na-HEPES, 20.0 and glucose, 11.1; pH 7.4), cleaned of adventitial tissue, and cut into 5 mm ring segments and allowed to equilibrate for 30 min at 37 °C. Scintillation vials containing two ml of Krebs/HEPES buffer with 250 µM lucigenin were placed into a scintillation counter switched to the out-of-coincidence mode. After dark adaptation, background counts were recorded and a vascular segment was added to the vial. Scintillation counts were recorded every 2 min for 15 min, and the respective background counts were subtracted. The vessels were then dried by placing them in a 90 °C oven for 24 h for determination of dry weight. In this assay, 10 pmoles of $O_2^{\bullet-}$ generated from known amounts of xanthine and xanthine oxidase yielded approximately 4,700 counts.

Vascular Oxidase Activity

Aortas were homogenized and prepared as described above. Five mm segments of rat aorta were placed in scintilation vials containing 250 µM lucigenin dissolved in Krebs-HEPES buffer. Light emission was detected using a scintilation counter switched to an out of coincidence mode. Counts taken 15 min after addition of the vessel segments were used to estimate steady-state $O_2^{\bullet-}$ production. In some studies, membrane fractions were separated from cytosolic fractions by centrifuging at 100,000 g for 15 min. Twenty µg of protein was added to the lucigenin-containing buffer and $O_2^{\bullet-}$ was stimulated by addition of either xanthine, arachidonic acid, NADPH or NADH. Details of this assay have been published previously.[15,16]

Endothelial NOS Activity

The NOS assay was based on the conversion of L-arginine to L-citrulline.[17] All animals were decapitated to diminish the blood content in the sample. Endothelial cells were prepared by removing the entire aorta, which was opened longitudinally and carefully washed free of blood with cold saline. To avoid contamination from non-endothelial tissue, the inner surface was scraped with the tip of a scalpel by a single movement to avoid the underlying layer of connective tissue. The sample, containing the endothelial cell layer, was placed in an Eppendorf tube containing 50 µl of ice cold Tris buffer and immediatly frozen in liquid nitrogen and stored at −80 °C. Samples from two identically treated animals were pooled. For determination of NOS activity the samples were frozen and thawed five times to break intracellular membranes. The mixture was not centrifuged because most if not all NOS in endothelial cells is membrane associated. Ten µl of the mixture was added to the reaction buffer for determination of NOS activity. The reaction buffer was containing: 50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer (pH 7,4) containing 1 mM EDTA, 1.25 mM $CaCl_2$, 1 mM DTT, 1 µM FAD, 1 µM calmodulin, 15 µM 6R-tetrahydrobiopterin and 1 µM L-(3H)arginine (1 µCi final concentration). The supernatant was added to obtain a final concentration of 15–30 µg in a reaction volume of 150 µl. The reaction was initiated by adding cofactor NADPH to a final concentration of 1 mM. Samples were incubated for 30 min at 37 °C. The reaction was terminated by adding 1 ml of ice-cold 100 mm HEPES buffer (pH 5.5) containing 10 mM ethylene glycol-bis(b-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) (stop buffer). The total volume (1 ml) was applied to a 0.5 ml Dowex AG50 WX-8 COLUMN (Na^+ form) that had been equilibrated with the stop buffer (minus EGTA). L-(2,3-3H)citrulline was eluted twice with 0.5 ml stop buffer (minus EGTA), and radioactivity was determined by liquid scintillation counting. *In vitro*, addition of BSO (0.5–5.0 mM) did not affect the enzyme activity (BSO (5 mM): $V_{max} = 243$ pmol/min/mg, $K_m = 1.8$ µM; control: $V_{max} = 251$ pmol/min/mg, $K_m = 1.8$ µM, $p > .05$). In contrast, V_{max}

and K_m were markedly reduced by approximately 50% after *in vitro* addition of NAC (0.5 mM). Based on this NAC/assay interaction, it was decided not to determine the effect of NAC administration on eNOS activity.

Study Design

Thiol Depletion

Thiol depletion was induced by administration of L-buthionine SR-sulfoximine (BSO) (0.33 g i.p. 3 times within 24 h) in 3 groups of rats. In one group the hypotensive response to Ach, SNP and saline were investigated immediately before BSO and two hours after the last BSO dose ($n = 8$). Likewise, the effects of BSO on endothelial eNOS activity ($n = 12$), lucigenin chemiluminescence and oxidase activity ($n = 6$) were examined 2 h after the last dose of BSO in two other groups of rats. In each of the three experiments an untreated control group ($n = 6$–12 in each group) was included.

Thiol Supplementation

N-acetylcysteine (NAC) and N-acetylserine (NAS, control) were infused in equimolar amounts of 5 mmol/kg/hour in a volume of 1.5 ml/hr for 3 h. The hypotensive effects of Ach, SNP and saline were recorded immediately before the beginning of the experiment and repeated after 2 h of thiol/control administration (NAC = 7, NAS = 8). Lucinogenin chemiluminescence and vascular oxidase activity were measured after 2 h of NAC/control infusion in two seperate groups ($n = 6$ in both groups).

Drugs

NAS, BSO, Ach and SNP were purchased from Sigma Chemical Co, St Louis, USA. NAC (MucomystR) was kindly delivered by ASTRA/ZENECA A/S, Copenhagen, Denmark. All solutions were prepared in 0.9% saline and adjusted to pH 7.4 with NaOH.

Statistical Analysis

Mean arterial blood pressure (MAP) was estimated as diastolic pressure plus (systolic minus diastolic pressure)/3 in mmHg. The reported alterations in MAP to Ach and SNP represent the difference between the baseline MAP value (immediately before Ach, SNP and saline) and the nadir on the blood pressure curve after Ach, SNP and saline respectively. All data are presented as mean ± SEM. Differences between pre- and posttreatment means within each treatment group were determined by Student's paired t-test. Comparisons between experimental groups were done by unpaired Student's t-tests. Statistical significance was assumed when $p < .05$.

RESULTS

Hemodynamic Effets of Thiol Depletion and Thiol Supplementation

BSO-induced thiol depletion significantly reduced the hypotensive effect of Ach (from 27 ± 3 to 19 ± 3 mmHg, $p < .05$), (Figure 1). In contrast, intracellular thiol depletion does not affect vaso-

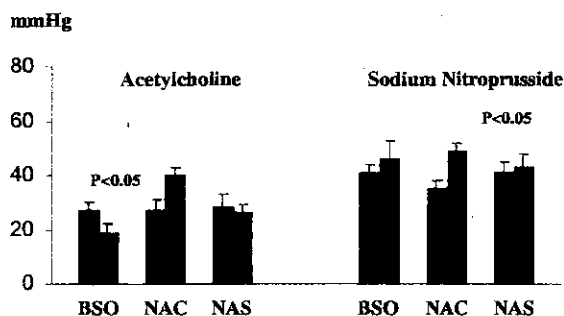

FIGURE 1 Blood pressure responses to ACh and SNP. Bar graph showing the hypotensive (mmHg) effect (mean ± SEM) of acetylcholine (10 µg/kg) and sodium nitrprusside (20 µg/kg) before (black bars) and after (white bars) a 2 h infusion of N-acetylserine (NAS, 5 mmol/kg/hr, $n = 8$), N-acetylcysteine (NAC, 5 mmol/kg/h, $n = 7$) or buthionine sulfoximine (BSO, 1 g i.p. within 24 h, $n = 8$) in conscious rats. $p < .05$: Significantly different from values before infusion of thiol-modulating agents.

dilation induced by SNP (41 ± 3 vs 46 ± 7 mmHg, $p > .05$) (Figure 1). Thiol supplementation significantly increased the hypotensive effect of ACh by 48% (from 27 ± 4 mmHg to 40 ± 3 mmHg, $p < .05$) (Figure 1). NAC also augmented the fall in MAP to SNP (from 35 ± 3 to 49 ± 3 mmHg, $p < .05$) (Figure 1). Baseline MAP values (before Ach, SNP, NaCl) were similar in all treatment groups and not affected by administration of thiol modulating agents (data not shown). Bolus infusions of saline caused no significant changes in MAP in any of the groups. Infusion of NAS did not significantly alter the hypotensive response to Ach (28 ± 5 vs 25 ± 3 mmHg, $p > .05$) or SNP (41 ± 4 vs 43 ± 5 mmHg, $p > .05$).

Endothelial eNOS Activity

BSO treatment significantly impaired eNOS activity in rat aorta by 50% (Figure 2) (BSO; 3.87 ± 0.5 pmol/min/mg protein, control; 7.7 ± 0.8 pmol/min/mg protein, $p < .05$).

Estimates of Vascular $O_2^{\bullet -}$ Formation and Oxidase Activity

Lucigenin counts from aortic segments of control rats averaged 4105 ± 154 counts/mg protein.

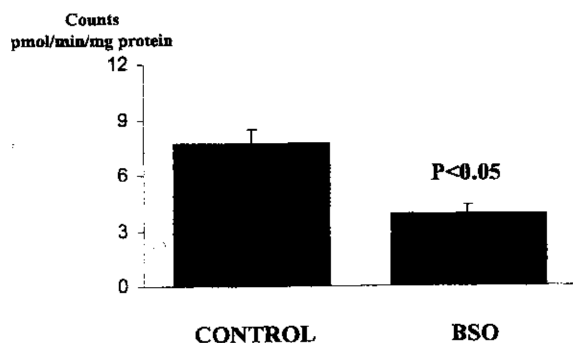

FIGURE 2 Nitric oxide synthase activity. Endothelial eNOS activity (pmol/min/mg prot) in normal healthy rats (control, $n = 12$) and after treatment with the thiol depleting agent buthionine sulfoximine (BSO) (1 g i.p. within 24 h, $n = 12$). $p < .05$: significantly different from control.

Neither treatment with NAC (4283 ± 415 counts/mg/protein) nor BSO (4415 ± 122 counts/mg/protein) altered the lucigenin counts significantly ($p > .05$). NADH or NADPH dependent oxidases were not affected by thiol supplementation (NAC) or thiol depletion (BSO) as compared to controls (Table I).

DISCUSSION

The major finding of this study is (1) that *in vivo* thiol depletion inhibits receptor-mediated endothelium dependent NO-mediated vasodilation (Ach) without affecting the response to exogenous NO (SNP) and; (2) that this effect is most likely mediated through inhibition of eNOS.

Intracellularly, GSH is present in high concentrations (0.5–10 mM) and is synthesized from glutamate, glycine and the thiol providing amino acid L-cysteine by the consecutive actions of γ-glutamylcysteine synthetase (the rate limiting step) and GSH synthetase.[18] A significant amount of cellular antioxidant protection is delivered by GSH and the reducing capacity of GSH represents a critical aspect of the interaction between GSH and hydrogen peroxide and other oxidants and products of peroxidation. In general, cellular thiol levels can be decreased by several oxidizing agents and compounds reacting with both the protein and non-protein thiol pools but their use is limited by their lack of specificity.[19] This problem can be solved by the use of specific inhibitors of GSH synthesis such as BSO which specificly inhibits the γ-glutamylcysteine synthetase and prevents the formation of GSH.[19,20] In contrast, thiol levels are readily increased by several agents e.g. NAC which leads to increased cysteine and GSH levels after de-acetylation.[21] NAS is identical to NAC except that it contains a hydroxyl group in place of the thiol group. Consequently, NAS, in this study is used as a thiol placebo substance in the described experiments.

TABLE I NADH- and NADPH-oxidase activity in control rats and rats treated with NAC or BSO ($N = 6$ in each group)

Thiol modulating agent	NADH-oxidase activity Counts/mg/min	NADPH-oxidase activity Counts/mg/min
control	8.97 ± 0.69	17.69 ± 0.96
N-Acetylcysteine	8.85 ± 0.53	16.14 ± 1.37
L-buthionine SR-sulfoximine	10.05 ± 0.47	16.34 ± 0.58

Based on *in vitro* experiments several mechanisms by which thiols may play a role in NO-mediated vasorelaxation have been proposed.[4–11] Among these are the existence of thiols as (1) necessary cofactors for NO synthase (NOS) or components of NOS; (2) compounds interacting with NO to produce S-nitrosothiols; (3) anti-oxidants protecting NO from inactivation by other radical intermediates; (4) determinants of guanylate cyclase activity; and (5) modulators of a broad range of enzyme activities.

The present results suggest that GSH is a necessary co-factor for endothelial NO synthesis and that intracellular thiol levels, in conditions with low thiol availability (e.g. oxidative stress) may be a rate-limiting factor in the synthesis of NO. We have previously shown that BSO treatment reduces the vascular GSH content to less than 25% of normal values in this experimental setup.[21] In the present study such GSH depletion significantly inhibits the effect of Ach on MAP suggesting that *in vivo* NO-mediated endothelium-dependent vasodilation specifically requires GSH. This is further substantiated by three findings. Firstly, the response to the endothelium-independent exogenous NO-donor substance SNP, which bypasses the endogenous steps of NO synthesis, is unaffected by thiol depletion. Secondly, *in vivo* thiol depletion was accompanied by a marked reduction in eNOS activity in the present experiments. Thirdly, *in vivo* thiol depletion was not associated with increased vascular oxidase activity or changes in lucigenin chemiluminescence, suggesting that the reduced effect of Ach during thiol depletion is not caused by reduced bioavaliability of NO due to interactions between NO and $O_2^{\bullet -}$. The interpretation, however, assumes that the degree of alteration in eNOS activity in the aorta reflects qualitatively similar changes in other vascular districts responsible for the hypotensive effect of Ach (e.g. resistance vessels). Although this has not been studied in detail, our finding that thiol depletion may significantly affect endothelium-dependent vasorelaxation is compatible with previous *in vitro* data from different vascular areas.[6,20,22–24] Endothelial L-arginine transport may depend on sufficient intracellular GSH levels.[20] *In vitro* studies has demonstrated that both the inducible NOS and the neuronal NOS have maximum enzymatic activity in the presence of GSH[22,23] and *in vitro* thiol depletion has been shown to reduce cellular NO production.[6] In addition, GSH may directly stimulate NOS.[23]

A major effect of GSH is to regulate the intracellular redox state. It acts both as a scavenger and detoxifier of free oxygen species and may protect against inactivation of NOS or oxidation of necessary cofactors (e.g. tetrahydrobiopterin).[25] Thiol depletion is generally associated with an increased oxidative stress.[19] Intriguingly, GSH depletion in the present study was not followed by enhanced lucigenin chemiluminescence. Although, we cannot be completely confident that this assay really measures $O_2^{\bullet -}$, it is widely used to estimate $O_2^{\bullet -}$ formation[15,16] and has been been shown to compare favorably to spin trap measurement of $O_2^{\bullet -}$.[26] Thus, our finding may suggest that the *in vivo* thiol defence mechanism against oxidative stress was either not exhausted by BSO or replaced by other mechanisms like ascorbic acid which can spare GSH

from oxidation.[27] Furthermore, activation of vascular oxidases may require more prolonged thiol depletion. The unaltered hemodynamic response to exogenous NO (SNP) and thus presumably unaltered vascular NO bioavailability after BSO treatment is in aggreement with the assumption that BSO treatment did not significantly affect the vascular level of $O_2^{\bullet-}$.

NAC administration in the present amount leads to a large increase in plasma thiol levels.[21,28] e finding that such changes are accompanied by an augmented hypotensive response to both Ach and SNP confirms and extends previous findings in anesthetized rats.[29] NO may combine with tissue thiols to produce nitrosothiols (e.g. S-nitrosocysteine, S-nitroso-GSH) which are more potent and more stable than NO.[9] Thiols may reduce and regenerate NO from its metabolite NO_2 and scavenge free oxygen species, thus preventing inactivation of NO.[7,30,31] In addition, thiols may stimulate guanylate cyclase directly. The similar response pattern of SNP and Ach to thiol supplementation suggests a common target for interaction between NO and excess thiol levels beyond the endogenous formation of NO itself. In addition, NAC did not affect lucigenin chemiluminescence which is compatible with a NO/thiol mechanism of interaction unrelated to a thiol-mediated $O_2^{\bullet-}$ scavenging mechanism. Thus, possible explanations of the effect of supraphysiological thiol levels on NO-mediated vasodilation may likely involve increased regeneration of NO and/or production of nitrosothiols and/or direct activation of guanylate cyclase.

In summary, this is the first study to show the *in vivo* effects of thiol depletion on endothelium dependent vasodilation. The results suggest that the hypotensive effect of Ach *in vivo* is inhibited by depletion of intracellular GSH content (BSO). This effect is mediated through inhibition of eNOS. It is concluded that *in vivo* thiol depletion results in endothelial dysfunction and a reduced receptor-mediated vascular relaxation. This effect is caused by reduced endothelial NO formation.

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