Induction of Oxidative Stress by Glutathione Depletion Causes Severe Hypertension in Normal Rats

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Abstract—Several recent studies have shown that certain forms of genetic or acquired hypertension are associated with oxidative stress and that animals with those types of hypertension respond favorably to antioxidant therapy. We hypothesize that oxidative stress may cause hypertension via (among other mechanisms) enhanced oxidation and inactivation of nitric oxide (NO). To test this hypothesis, Sprague-Dawley rats were subjected to oxidative stress by glutathione (GSH) depletion by means of the GSH synthase inhibitor buthionine sulfoximine (BSO, 30 mmol/L in drinking water) for 2 weeks. The control group was given drug-free drinking water. In parallel experiments, subgroups of animals were provided vitamin E–fortified chow and vitamin C–supplemented drinking water. The BSO-treated group showed a 3-fold decrease in tissue GSH content, a marked elevation in blood pressure, and a significant reduction in the urinary excretion of the NO metabolite nitrate plus nitrite, which suggests depressed NO availability. These characteristics were associated with a significant accumulation in various tissues of nitrotyrosine, which is the footprint of NO inactivation by reactive oxygen species. Administration of vitamin E plus vitamin C ameliorated hypertension, improved urinary nitrate-plus-nitrite excretion, and mitigated nitrotyrosine accumulation (despite GSH depletion) in the BSO-treated animals but had no effect in the control group. In conclusion, GSH depletion resulted in perturbation of the NO system and severe hypertension in normal animals. The effects of BSO were mitigated by concomitant antioxidant therapy despite GSH depletion, which supports the notion that oxidative stress was involved in the pathogenesis of hypertension in this model. (Hypertension. 2000;36:142-146.)

Key Words: blood pressure n nitric oxide n antioxidants n hypertension, genetic n hypertension, essential

Oxidative reactions yield high-energy compounds that fuel various biochemical, biophysical, and mechanical functions of aerobic organisms. These reactions are a continuous source of potentially cytotoxic reactive oxygen species (ROS). Under physiological conditions, ROS produced in the course of normal metabolism are fully inactivated by an elaborate cellular and extracellular antioxidant defense system.1,2 However, in certain pathological conditions, increased generation of ROS and/or depletion of antioxidant capacity leads to enhanced ROS activity and oxidative stress. By promoting lipid peroxidation, DNA damage, and protein modification, oxidative stress can cause cellular injury and tissue damage.1,3 These processes have been implicated in the pathogenesis of various lesions observed in patients with ischemia, inflammation, aging, degenerative diseases, and numerous other disorders.4–6

Several recent studies7–10 have provided convincing evidence of enhanced ROS activity in patients with various hypertensive disorders. We have found increased ROS activity in rats with lead-induced hypertension and in rats with chronic renal failure.11 In addition, oxidative stress has been demonstrated in rats with cyclosporine-induced hypertension,12,13 spontaneously hypertensive rats,14–16 Dahl salt-sensitive rats,17,18 and women with pre-eclampsia.19 Oxidative stress may contribute to the generation and maintenance of hypertension via the inactivation of NO (which is also termed “endothelium-derived relaxing factor”),7,10,11,14 the nonenzymatic generation of vasoconstrictive isosprotanes from arachidonic acid peroxidation16,20–22 and direct vasoconstrictor action.23,24 Antioxidant administration improves NO metabolism and ameliorates hypertension in rats with lead-induced hypertension,7,10 chronic renal failure,11 or spontaneous hypertension.14,16 In addition to oxidative stress, each of the conditions cited above is characterized by a complex set of biochemical, hemodynamic, and/or genetic disorders that can contribute to the development and maintenance of hypertension. This study is designed to test the hypothesis that oxidative stress per se can lead to arterial hypertension.

Methods

Animal Model

Male Sprague-Dawley rats with an average weight of 275 g (Harlan Sprague-Dawley Inc, Indianapolis, Ind) were housed in a climate-controlled, light-regulated space with 12-hour day (~500 lux) and night (~5 lux) cycles. They were fed a low-nitrate rat chow and water ad libitum. The rats were randomly assigned to either the

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oxidative stress group or the placebo-treated control group. The drinking water in the former group was supplemented with the glutathione (GSH) synthase inhibitor buthionine sulfoximine (BSO, Sigma Chemical Inc) 30 mmol/L (BSO-treated group) for 2 weeks. The BSO dosage used here was based on previous studies conducted in the rat. This treatment was intended to raise ROS activity by depleting GSH, which is a major component of the natural antioxidant defense system. The control group was provided with regular water.

After BSO or placebo had been administered for 2 weeks, the animals were killed by exsanguination via cardiac puncture while under general anesthesia (sodium pentobarbital [Nembutal], 50 mg/kg IP). The kidney, liver, heart and thoracic aorta were immediately removed, frozen in liquid nitrogen, and stored at −70°C until they were processed. In addition, plasma was separated and stored at −70°C. The animals in another subgroup were treated with either BSO or placebo for 2 weeks, after which therapy was stopped and the animals were observed for several weeks.

To discern the effect of oxidative stress, subgroups of BSO-treated and control animals were fed a vitamin-E fortified chow that contained 5000 U/kg of tocopherol rather than regular chow, which contained 40 U/kg of tocopherol. In addition, the drinking water of the vitamin E–treated groups was supplemented with ascorbic acid (3 mmol/L). During the observation period, tail arterial blood pressure was measured and overnight fasting urine collections were obtained by means of individual metabolic cages. Hematocrit and serum and urine creatinine concentrations were measured by standard techniques.

**Measurement of Blood Pressure**

Arterial blood pressure was measured by tail plethysmography (Harvard Apparatus Inc) as previously described. Conscious rats were placed in a restrainer on a heated pad and were allowed to rest inside the cage for 15 minutes before blood pressure measurements were obtained. The procedure was performed in a climate-controlled room with an ambient temperature of 70°F. Rat tails were placed inside a tail cuff, and the cuff was inflated and released several times to allow conditioning of the animals to the procedure. A minimum of 4 consecutive measurements was taken, and the measurements were recorded by a student oscillograph (Harvard Apparatus). The data were then averaged for presentation.

**Tissue Glutathione Assay**

The total GSH content of the hepatic tissue was determined by means of Cayman’s GSH assay kit (Cayman Chemical Co). The carefully optimized enzymatic recycling method of that assay uses GSH reductase, which enables the sulfhydryl group of GSH to react with DTNB (5,5'-dithiobis-2-nitrobenzoic acid) and Ellman’s reagent to produce a yellow-colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB, which is concomitantly produced, is reduced by GSH reductase to recycle GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the concentration of GSH in the sample. Thus measurement of TNB at 405 or 412 nm provides an accurate estimate of GSH in the sample. It should be noted that oxidized GSH is converted to GSH by GSH reductase in this system, which is used to measure total GSH.

**Total Nitrate and Nitrite Assay**

Urinary excretion of the total nitrite and nitrate (NOx) was determined as described in our previous studies by means of the purge system of a Sievers Instruments Model 270B Nitric Oxide Analyzer.

**Measurement of Nitrotyrosine**

The plasma, heart, liver, aorta, and kidneys of the animals studied were processed to determine nitrotyrosine abundance. The tissues (25% w/vol) were homogenized in a solution containing 50 mmol/L Tris-HCl (pH 7.4); 1% NP-40; 0.25% sodium deoxycholate; 150 mmol/L NaCl; 1 mmol/L EGTA; aprotinin, leupeptin, pepstatin [1 μg/mL each]; 1 mmol/L NaVO₃; and 1 mmol/L NaF at 0° to 4°C by means of a polytron homogenizer. Homogenates were centrifuged at 12,000g for 5 minutes at 4°C, and the supernatant was used to determine nitrotyrosine abundance. Protein concentration was determined by means of a bicinchoninic protein assay kit (Pierce, Inc). Plasma and tissue nitrotyrosine abundance was determined by Western blot analysis that used an anti-nitrotyrosine monoclonal antibody (Upstate Biotechnology, Inc) as described in our earlier studies.

**Data Analysis**

ANOVA, Student’s t test, and regression analysis were used in statistical analysis of the data, which are presented as mean±SEM. A probability value <0.05 was considered significant.

**Results**

**Effects of BSO Administration**

The BSO-treated animals exhibited a nearly 3-fold reduction in the total GSH content of the liver tissue and a marked increase in arterial blood pressure (Figure 1). After cessation of BSO administration, blood pressure gradually decreased to baseline value by week 9 (130±9 versus 124±16 mm Hg, \(P=NS\)). No significant difference was found in either creatinine clearance (7.1±0.9 mL·min⁻¹·kg⁻¹ body weight in the BSO-treated group and 6.1±0.7 mL·min⁻¹·kg⁻¹ body weight in the control group), hematocrit (44.8±0.6% versus 45.1±0.9%, respectively) or body weight (323±10 versus 324±5 g, respectively) between the 2 groups studied at 2 weeks. The BSO-treated animals showed a sharp decrease in urinary nitrate plus nitrite (NOx) excretion (Figure 1), which returned to baseline level 7 weeks after the discontinuation of
A negative correlation was found between arterial blood pressure and urinary NOx excretion in the study groups \( (r=0.77, P<0.001) \). The BSO-treated animals showed a significant increase in nitrotyrosine abundance in the kidney, aorta, heart, liver, and plasma (Figures 2 to 6). These findings point to enhanced NO interaction with ROS and NO sequestration as nitrotyrosine in all tested tissues.

**Effects of Vitamin E Plus Vitamin C Administration**

As expected, vitamin E plus vitamin C supplementation did not prevent BSO-induced GSH depletion (Figure 1). However, antioxidant therapy with vitamin E plus vitamin C significantly ameliorated the BSO-induced hypertension. Vitamin E plus vitamin C administration also mitigated the BSO-induced decrease in urinary NOx excretion (Figure 1). In addition, antioxidant therapy with vitamin E plus vitamin C mitigated the accumulation of nitrotyrosine in the tested plasma and tissues of the kidney, aorta, liver, and heart (Figures 2 to 6). These findings point to enhanced NO availability and decreased NO inactivation and sequestration as a result of antioxidant therapy, despite GSH depletion. In contrast to the effects seen in the BSO-treated group, vitamin E plus vitamin C supplementation had no significant effect on either blood pressure or urinary NOx excretion and did not alter either GSH (Figure 7) or nitrotyrosine abundance in the control animals (data not shown).

**Figure 2.** Representative Western blot and group data depicting nitrotyrosine abundance in the kidney of rats treated with BSO, BSO plus vitamin E plus vitamin C (B+Vit. E, C), and those treated with inactive vehicle (CTL) for 2 weeks. \( n=6 \) in each group. \( *P<0.01 \) by ANOVA.

**Figure 3.** Representative Western blot and group data depicting nitrotyrosine abundance in the aorta of rats treated with BSO, BSO plus vitamin E plus vitamin C (B+Vit. E, C), and those treated with inactive vehicle (CTL) for 2 weeks. \( n=6 \) in each group. \( *P<0.01 \) by ANOVA.

**Figure 4.** Representative Western blot and group data depicting nitrotyrosine abundance in the heart of rats treated with BSO, BSO plus vitamin E plus vitamin C (B+Vit. E, C), and those treated with inactive vehicle (CTL) for 2 weeks. \( n=6 \) in each group. \( *P<0.01 \) by ANOVA.

**Figure 5.** Representative Western blot and group data depicting nitrotyrosine abundance in the liver of rats treated with BSO, BSO plus vitamin E plus vitamin C (B+Vit. E, C), and those treated with inactive vehicle (CTL) for 2 weeks. \( n=6 \) in each group. \( *P<0.01 \) by ANOVA.
Discussion

As noted in the introduction, several forms of experimental and clinical hypertension are associated with oxidative stress. The causal association of oxidative stress and hypertension has been supported by the observation that antioxidant therapy ameliorates hypertension in those models.7,10,11,14,16 In addition, chronic consumption of a high-fat diet, which causes hyperlipidemia and oxidative stress, has been recently shown to cause hypertension and endothelial dysfunction, which are reversed by resumption of a regular diet in genetically normotensive rats.28,29 However, because of numerous concurrent biochemical and hemodynamic disturbances that can potentially contribute to hypertension in those models, it is difficult to attribute the associated hypertension to a direct effect of oxidative stress per se. We therefore undertook the present study, in which oxidative stress was induced in otherwise intact genetically normotensive animals. Administration of the GSH synthase inhibitor BSO led to a 3-fold reduction in tissue GSH content and to a significant increase in tissue nitrotyrosine, which is a strong indicator of oxidative stress. Induction of oxidative stress by GSH depletion with BSO resulted in a marked elevation of arterial blood pressure in otherwise genetically intact normotensive animals. After the cessation of BSO administration, blood pressure gradually declined to baseline levels by week 9. Serum creatinine concentration, creatinine clearance, and urinary protein excretion were identical in the BSO-treated and placebo-treated groups, which excludes discernible renal disease in this model.

The increase in arterial blood pressure in animals with BSO-induced hypertension was accompanied by a marked reduction in tissue GSH content and to a significant increase in tissue nitrotyrosine, which is a strong indicator of oxidative stress. Induction of oxidative stress by GSH depletion with BSO resulted in a marked elevation of arterial blood pressure in otherwise genetically intact normotensive animals. After the cessation of BSO administration, blood pressure gradually declined to baseline levels by week 9. Serum creatinine concentration, creatinine clearance, and urinary protein excretion were identical in the BSO-treated and placebo-treated groups, which excludes discernible renal disease in this model.

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The increase in arterial blood pressure in animals with BSO-induced hypertension was accompanied by a marked reduction in uronic excretion of NO metabolites (NOx), which suggests diminished NO availability. This was accompanied by a widespread tissue accumulation of nitrotyrosine, which is the footprint of NO interaction with ROS. Interaction of ROS, particularly that of superoxide with NO, leads to the production of peroxynitrite (ONOO\(^-\)), which is a highly cytotoxic reactive compound.30,31 Peroxynitrite can in turn react with DNA and with lipid and protein molecules.30 For instance, peroxynitrite reacts with the tyrosine residues in various proteins to produce nitrotyrosine. Alternatively, ROS can initially activate tyrosine residues to produce tyrosyl radicals that can in turn oxidize NO to produce nitrotyrosine.32,33 In addition, nitrotyrosine can be formed from the interaction of tyrosine with other reactive nitrogen species.30,32 However, the contribution of the latter reactions to total tissue nitrotyrosine abundance is limited. As a result, nitrotyrosine abundance is largely a function of ROS interaction with NO.31,34 The observed accumulation of nitrotyrosine in the BSO-treated animals points to the effectiveness of BSO in generating the intended oxidative stress in the study animals. In addition, the increased tissue nitrotyrosine burden was indicative of the inactivation and sequestration of NO. This could contribute to the reduction of urinary NOx excretion and NO availability. If this hypothesis is true, then reduced NO availability resulting from enhanced NO inactivation by ROS could have contributed to the pathogenesis of hypertension in the BSO-treated animals.

Concomitant antioxidant therapy with vitamin E plus vitamin C prevented BSO-induced reductions in the urinary excretion of NOx as well as tissue nitrotyrosine accumulation and also ameliorated hypertension without affecting the associated GSH deficiency. These observations point to the role of oxidative stress in the pathogenesis of hypertension and altered NO metabolism as opposed to an unrelated effect of BSO. The given antioxidant regimen had no effect on either urinary NOx excretion, tissue nitrotyrosine abundance, or blood pressure in the normal control animals, which confirms our earlier observations.10,35 These findings also...
suggest that in the absence of oxidative stress, the given antioxidant therapy has no effect on either NO metabolism or arterial blood pressure. Thus the observed effect of vitamin E plus vitamin C administration in BSO-treated rats was probably mediated by alleviation of oxidative stress rather than by an unrelated action of that vitamin combination.

Antioxidant therapy with vitamin E plus vitamin C in the given amounts significantly ameliorated but did not completely reverse hypertension in the GSH-depleted animals. This observation suggests that GSH is a necessary component of the natural antioxidant system and is not entirely replaceable.

In conclusion, we have demonstrated that chronic oxidative stress can lead to the induction and maintenance of severe hypertension in genetically normotensive rats. This was accompanied by and was perhaps in part due to the inactivation and sequestration of NO (mediated by ROS), which led to diminished NO availability. The role of oxidative stress in the pathogenesis of these abnormalities is supported by the efficacy of concomitant antioxidant therapy in this model. These observations strongly support the notion that oxidative stress can cause hypertension. We believe that the new model of acquired hypertension introduced in the this study will be useful in future investigations of the mechanism, pathophysiology factors, and treatment of hypertension.

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References