Nitric Oxide Synthase Expression in Hypertension Induced by Inhibition of Glutathione Synthase

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ABSTRACT

Induction of chronic oxidative stress by glutathione (GSH) depletion has been shown to cause hypertension in normal rats. This was accompanied by and perhaps in part due to inactivation and sequestration of NO by reactive oxygen species (ROS), leading to diminished NO bioavailability. This study was designed to examine renal histology, nitric oxide synthase (NOS) isotype expression, and nitrotyrosine distribution in this model. Sprague-Dawley rats were subjected to oxidative stress by administration of the GSH synthase inhibitor buthionine sulfoximine (BSO; 30 mM/l in drinking water) for 2 weeks. The controls were given tap water. Blood pressure, renal histology, tissue expression of endothelial and inducible NOS (eNOS and iNOS) and nitrotyrosine, tissue GSH content, and urinary excretion of NO metabolites (NOx) were examined. 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 NOx. Histological examination of kidneys revealed no significant abnormalities in either group. In addition, no significant differences were observed in either intensities or localizations of eNOS and iNOS in the kidney. However, the BSO-treated group exhibited intense accumulation in the renal tissue of nitrotyrosine, which is the footprint of NO oxidation by ROS. These observations suggest that oxidative stress-induced hypertension is not caused by either structural abnormality of or depressed NOS expression by the kidney in this model. Instead, it is associated with and perhaps partially related to enhanced renal NO inactivation by ROS and diminished NO bioavailability.

Several forms of experimental and clinical hypertension are associated with oxidative stress. For instance, we have found increased reactive oxygen species (ROS) activity in rats with lead-induced hypertension (Gonick et al., 1997; Vaziri et al., 1997, 1999; Ding et al., 1998) and rats with chronic renal failure (Vaziri et al., 1998b). In addition, oxidative stress has been demonstrated in rats with cyclosporine-induced hypertension (Lopez-Ongil et al., 1998; Navarro-Antolin et al., 1998), spontaneously hypertensive rats (Tschesuhi et al., 1996; Schnackenberg et al., 1998; Schnackenberg and Wilcox, 1999; Vaziri et al., 2000a), Dahl salt-sensitive rats (Atarashi et al., 1997; Swei et al., 1997), diet-induced hypertension (Roberts et al., 2000, 2001), patients with essential hypertension (Kumar and Das, 1993; Lacy et al., 1998), and women with preeclampsia (Roggensack et al., 1999). Oxidative stress can potentially contribute to generation and maintenance of hypertension via inactivation of NO (Vaziri et al., 1997, 1999b, 1999; Schnackenberg et al., 1998), a nonenzymatic generation of vasoconstrictive isoprostanes from arachidonic acid peroxidation (Takahashi et al., 1992; Schnackenberg and Wilcox, 1999) and a direct vasopressor action (Atzori et al., 1991; Tesfamariam and Cohen, 1992). In fact, antioxidant administration has been shown to improve NO metabolism and ameliorate hypertension in rats with lead-induced hypertension (Vaziri et al., 1997, 1999), chronic renal failure (Vaziri et al., 1998b), and spontaneous hypertension (Schnackenberg et al., 1998; Schnackenberg and Wilcox, 1999; Vaziri et al., 2000a). However, due to the presence of numerous concurrent biochemical, hemodynamic, and/or genetic disturbances that can contribute to the development of hypertension in these models, it is difficult to attribute the associated hypertension to a direct effect of oxidative stress per se. To test the hypothesis that oxidative stress per se can cause hypertension, recently Vaziri et al. (2000b) carried out a study in which normal Sprague-Dawley rats were subjected to oxidative stress by glutathione (GSH) depletion using the GSH synthase inhibitor buthionine sulfoximine (BSO) for 2 weeks. The BSO-treated group showed a dramatic fall in tissue GSH content, marked elevation in blood pressure, and a significant reduction in urinary excretion of NO metabolites, nitrate plus nitrite (NOx), suggesting depressed NO

ABBREVIATIONS: ROS, reactive oxygen species; GSH, glutathione; BSO, buthionine sulfoximine; NOx, NO metabolites; NOS, nitric oxide synthase; TNB, 5-thio-2-nitrobenzoic acid; eNOS, endothelial NOS; iNOS, inducible NOS.
bioavailability. This was associated with a significant accumulation in various tissues of nitrotyrosine, which is the footprint of NO inactivation by ROS. They further demonstrated a marked amelioration of hypertension together with improved urinary NOx excretion and reduced nitrotyrosine accumulation (despite GSH depletion) in the BSO-treated animals by concomitant antioxidants therapy, supporting the notion that oxidative stress was involved in the pathogenesis of hypertension in this model.

The reduction in urinary NOx excretion may be due to either diminished NO production and/or enhanced NO sequestration. With respect to the latter possibility, the increased nitrotyrosine accumulation, a secondary by-product of interactions of NO, ROS, and tyrosine residues of proteins (Eiserich et al., 1995a, 1998; Halliwell, 1997), reflects ROS-mediated NO inactivation and sequestration in this model. A second possible mechanism for depressed urinary NOx excretion in this model is diminished NO generation. This can in turn be due to diminished l-arginine availability, quantitative NO synthase (NOS), deficiency, NOS inhibition, or loss of renal parenchyma (Vaziri et al., 1998a). This study was intended to examine renal histology, NOS protein expression, and nitrotyrosine distribution in rats rendered hypertensive by oxidative stress using glutathione depletion.

Materials and Methods

Animal Model

Male Sprague-Dawley rats with an average weight of 275 g (Harlan Sprague-Dawley, Inc., Indianapolis, IN) were housed in a climate-controlled, light-regulated space with 12-h day (750 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 oxidative stress group or the placebo-treated control group. The drinking water in the former group was supplemented with the GSH synthase inhibitor BSO (Sigma Chemical Company, St. Louis, MO), 30 mM/l (BSO-treated group) for 2 weeks (Vaziri et al., 2000b). 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 2 weeks of BSO or placebo treatments and under general anesthesia (Nembutal; 50 mg/kg i.p.), the animals were killed by exsanguination using cardiac puncture. Kidneys were immediately exsanguinated for morphological studies. Immediately after exsanguination, the kidneys were removed, promptly sectioned, and separately postfixed in 10% buffered formalin. The fixed tissue was embedded in paraffin and sectioned. The sections were stained with Masson’s trichrome stain, periodic acid-Schiff stain, and hematoxylin and eosin and were examined under light microscopy.

Immunoperoxidase Studies. Immunohistochemical staining for eNOS, iNOS, and nitrotyrosine were performed using standardized streptavidin-biotin-peroxidase method on formalin-fixed, paraffin-embedded renal tissues, as described previously (Zhou et al., 2000a,b). Except the incubation with the primary antibodies, all incubations were at room temperature and were separated by washes with phosphate-buffered saline. After deparaffinization, sections were treated with 1.25% hydrogen peroxide to block endogenous peroxidase activity. After precuoination with 10% normal horse or swine serum for 20 min, sections were incubated with primary antibodies overnight at 4°C, followed sequentially with biotinylated horse anti-mouse (Vector Laboratories, Burlingame, CA) or biotinylated swine anti-rabbit (Dako Corporation, Carpinteria, CA) antibodies for 20 min and streptavidin-peroxidase complex (Dako, Carpinteria, CA) for 30 min. The working concentrations for the mouse anti-eNOS monoclonal antibody (Transduction Laboratories, Lexington, KY), rabbit anti-iNOS antibody (Transduction), and rabbit antinitrotyrosine antibody (Upstate Biotechnology, Inc., Lake Placid, NY) were 0.1, 0.05, and 5.9 μg/ml, respectively. For negative controls, a monoclonal mouse IgG1 (Bethyl Laboratories, Inc., Montgomery, TX) or normal rabbit serum was used at equivalent concentrations. Diaminobenzidine (Sigma Chemical Company) was used as chromogen, and hematoxylin was used for nuclear counterstain. To further verify the specificity of immunostaining for eNOS and iNOS, antibodies from different vendors (eNOS from Upstate Biotechnology; iNOS from Cayman Chemicals) were used, and immunostaining was repeated.

Data Analysis. Data were presented as mean ± S.E.M. Student’s t test and regression analysis were used in evaluation of the data as appropriate. P values equal to or less than 0.05 were considered significant.

Results

General Data and Biochemical Measurements (Table 1). The BSO-treated animals exhibited a 3-fold reduction in total GSH content of the liver tissue and a marked rise in arterial blood pressure. No significant difference was found
either in creatinine clearance or hematocrit between the two groups. The BSO-treated animals showed a sharp fall in urinary NOx excretion. A negative correlation was found between arterial blood pressure and urinary NOx excretion. A negative correlation was found between arterial blood pressure and urinary NOx excretion. A negative correlation was found between arterial blood pressure and urinary NOx excretion. A negative correlation was found between arterial blood pressure and urinary NOx excretion. A negative correlation was found between arterial blood pressure and urinary NOx excretion.

Renal Histology (Fig. 1). The morphological appearance of glomeruli, arteries, arterioles, and tubulointerstitium in BSO-treated animals was normal and indistinguishable from that of the control animals.

**Immunoperoxidase Studies for eNOS and iNOS.** The intensities and distributions of eNOS were similar between the two groups (Fig. 2, a and b). In both groups, there was mild to moderate staining of endothelial and smooth muscle cells in the small arteries and arterioles. Focally, the cortical proximal and distal tubules also showed positivity for eNOS in both groups. However, some collecting ducts and S3 segments revealed mild eNOS positivity in the BSO-treated group, which showed slightly less intensity in the control group.

A similar staining pattern and intensity for iNOS was observed in the two groups of animals (Fig. 2, c and d). The signals were localized in the endothelial and smooth muscle cells of small arteries and arterioles, scattered glomerular cells, peritubular capillaries, and scattered collecting ducts. To further verify the specificity of immunostaining for NOS isoforms, antibodies for eNOS and iNOS from different vendors were used and similar results were observed.

**Immunoperoxidase Studies for Nitrotyrosine.** In normal control rats (Fig. 2e), mild nitrotyrosine expression was seen in some of the proximal and distal tubules of renal cortex (1+). The S3 segments of the proximal tubules were diffusely positive (1+). Collecting ducts in the inner stripe of the outer medulla and in the inner medulla were also positive (1+). Mild (1+) smooth muscle staining was observed in some of the arterial and arteriolar walls. The endothelial cells of vasa recta were mildly positive (1+). In addition, there was scattered staining (1+) in some of the glomerular visceral epithelial and endothelial cells. The glomerular mesangial cells were essentially negative.

In contrast, the BSO-treated animals (Fig. 2f) showed diffuse strong nitrotyrosine expression in the renal cortex in virtually all the proximal and distal tubules (3+). The S3 segments of the proximal tubules and the collecting ducts in the inner stripe of the outer medulla and in the inner medulla were strongly positive (3+). The smooth muscle cells of the arteries and the arterioles and the endothelial cells demonstrated moderate staining (2+). The endothelial cells of vasa recta were positive (1 to 2+). The glomeruli revealed positive staining in scattered visceral epithelial cells (1+), mesangial cells (1+), and occasional endothelial cells (1+). It is of interest to note that the nitrotyrosine staining was stronger in the area underneath the luminal brush borders (where the endocytic lysosomal apparatus is distributed) than in the basal part of the tubular cells (where numerous mitochondria are located).

**Discussion**

GSH is an endogenous and ubiquitous sulfhydryl-containing tripeptide that is synthesized by two enzymes (acting in concert), γ-glutamylcysteine synthase and GSH synthase (Poot et al., 1995). GSH has been implicated in a variety of cellular processes, including prevention of free radical formation and accompanying tissue injury (Yu, 1994). BSO inhibits γ-glutamylcysteine synthase irreversibly and consequently depletes tissue GSH content (Griffith and Meister, 1979). In the present study, administration of BSO led to a marked reduction in tissue GSH content and a significant increase in tissue nitrotyrosine, which is a strong indicator of NO oxidation by ROS. Induction of oxidative stress by GSH depletion with BSO resulted in a marked elevation of arterial blood...
pressure in otherwise intact genetically normotensive animals. These observations confirmed the results of our earlier studies (Vaziri et al., 2000b). In addition, the serum creatinine concentration and creatinine clearance were identical in the BSO-treated and placebo-treated groups. Furthermore, no morphologic abnormalities were observed in the kidneys by light microscopy in the BSO-treated rats. These observations suggest that oxidative stress-induced hypertension in this model is not associated with discernible renal disease and/or structural abnormalities of the kidney.

The rise in blood pressure in BSO-treated animals was associated with a marked reduction in urinary NOx excretion. This phenomenon could be due to depressed NO production capacity. To this end, we studied renal eNOS and iNOS expressions by immunohistochemical methods. We found no significant differences in either intensities or localizations of eNOS or iNOS signals by immunohistochemical methods. In addition, the eNOS and iNOS distributions in normal rats were similar to those described earlier (Kone, 1999; Zhou et al., 2000a,b). Therefore, the reduction in urinary NOx excretion cannot be attributed to a quantitative NOS deficiency. Likewise, it was not due to reduced dietary L-arginine content because food intake and body weight were similar in the two groups.

A plausible explanation for the decline in urinary NOx excretion in the face of normal NOS isotype expressions is the potential ROS-mediated oxidation and sequestration of NO. In fact, we found a marked increase in renal tissue nitrotyrosine (the footprint of NO interaction with ROS) by immunohistochemical methods, which confirmed the results of our previous studies using Western blot analysis (Vaziri et al., 2000b). It is of note that generation of NO from L-arginine by NOS depends on the presence of the NOS cofactor tetrahydrobiopterin, which itself is readily oxidized by ROS. Thus, in addition to direct oxidation/inactivation of NO, oxidative stress can limit NO production by NOS via depletion of its cofactor tetrahydrobiopterin.

Interaction of ROS, particularly that of superoxide with NO, leads to the production of peroxynitrite, which is a highly cytotoxic reactive compound (Beckman and Koppenol, 1996; Halliwell, 1997). Peroxynitrite can in turn react with DNA, lipid, and protein molecules (Halliwell, 1997). 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 (Eiserich et al., 1995b). In addition, nitrotyrosine can be formed from the interaction of tyrosine with other reactive nitrogen species (Eiserich et al., 1995b; Halliwell, 1997). However, the contribution of the latter reactions to total...
tissue nitrotyrosine abundance is limited. As a result, nitrotyrosine abundance was minimal in the glomeruli of BSO-treated animals, pointing 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 NO excretion and NO bioavailability. Reduced NO bioavailability resulting from enhanced NO inactivation by ROS could in turn contribute to the pathogenesis of hypertension in the BSO-treated animals. The role of oxidative stress in the pathogenesis of hypertension in this model is further supported by the efficacy of concomitant antioxidant therapy in our earlier study (Vaziri et al., 2000b). We demonstrated that concomitant antioxidant therapy with vitamin E plus vitamin C prevented BSO-induced renal nitrotyrosine accumulation (by Western blot analysis) and ameliorated hypertension without effecting the associated GSH deficiency. These observations point to the role of oxidative stress in the pathogenesis of hypertension as opposed to an unrelated effect of BSO.

Nitrotyrosine was primarily distributed in the proximal tubules, distal tubules, and collecting duct. These observations are consistent with a previous report by Bian et al. (1999). The above structures heavily depend on oxidative metabolism for energy-dependent sodium transport. In contrast, nitrotyrosine abundance was minimal in the glomeruli in which H_2O and solute transport is passive. Generation of superoxide by mitochondria is a function of oxygen consumption in the given tissue. Thus, high oxygen consumption by renal tubules is predictably coupled with a high superoxide generation, which in the presence of NO (since the tubule epithelium is the location for both eNOS and iNOS) and depressed natural antioxidant capacity (GSH depletion) can lead to local oxidative stress and nitrotyrosine formation. This is clearly evidenced by the intense accumulation of nitrotyrosine in those locations in the BSO-treated animals. Another source of nitrotyrosine may be the reabsorption of nitrosated proteins by the endocytic lysosomal apparatus of the proximal tubules. In fact, we found that the strongest staining of nitrotyrosine is not in the basal mitochondria region but rather in the area underneath the luminal brush borders where the endocytic lysosomal apparatus is distributed. Thus, the cellular location of nitrotyrosine staining suggests that a large portion of nitrotyrosine may come from the reabsorption of nitrosated proteins (Bian et al., 1999).

In conclusion, we have confirmed the results of our earlier study (Vaziri et al., 2000b) in which we stated that chronic oxidative stress per se can lead to enhanced NO inactivation and induction of severe hypertension in genetically normotensive rats. We have further shown that eNOS and iNOS expressions and renal morphology are normal in this model, suggesting that oxidative stress-induced hypertension is not caused by discernible renal injury or quantitative NO deficit in this model. Instead, depressed urinary NO excretion and increased nitrotyrosine accumulation in the face of normal NO isoform expression point to enhanced ROS-mediated NO inactivation and functional NO deficiency in BSO-treated animals.

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