Effects of aortic coarctation on aortic antioxidant enzymes and NADPH oxidase protein expression

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Abstract

Abdominal aortic coarctation above the renal arteries leads to severe hypertension above the stenotic site and provides a model for simultaneous testing of the effects of increased and decreased pressure and consequently shear stress in the same animal. The effects of increased pressure, per se, on oxidative stress and antioxidant enzyme expression is unknown. We studied the protein expressions of antioxidant enzymes and NADPH oxidase (gp91\textsuperscript{phox} subunit) in the aortic segments above and below the stenosis site in sham-operated control and aortic-banded rats at four weeks postoperatively. Compared with the control group, the banded group showed significant up-regulation of NADPH oxidase, catalase (CAT), Cu/Zn superoxide dismutase (SOD) and Mn SOD protein content in the thoracic aorta. In contrast, Mn SOD, Cu/Zn SOD and NADPH oxidase protein abundance were unchanged in the abdominal aortic segment below the stricture where blood pressure is not elevated, whereas CAT protein abundance was also elevated in the abdominal aorta. No changes were noted for glutathione peroxidase (GPX) protein content either in the thoracic or abdominal aortic segments. Coarctation-induced hypertension is associated with increased aortic CAT, Cu/Zn SOD, Mn SOD and NADPH oxidase protein expression. The up-regulation of NADPH oxidase increases reactive oxygen species (ROS) generation noted in the present study and contributes to inactivation of nitric oxide (NO) as shown previously in this model. Upregulation of antioxidant enzymes may be a compensatory response in the
face of elevated pressure and oxidative stress. The normality of protein abundance in the abdominal aorta wherein blood pressure is not elevated points to the role of baromechanical factors, as opposed to circulating humoral factors that were similar in both segments, as a mechanism responsible for increased antioxidant enzyme expression.

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Introduction

Excessive generation of reactive oxygen species (ROS) leads to oxidative stress, which in turn causes macromolecular damage, lipid peroxidation and tissue damage (Yu, 1994). In the past two decades, it has become increasingly clear that oxidative stress plays a major role in the pathogenesis of a number of human diseases including hypertension, atherosclerosis, chronic renal failure, ischemia/reperfusion injury, neurodegenerative diseases, cancer and diabetes mellitus (Yu, 1994). The primary antioxidant enzyme system includes, but is not limited to, mitochondrial and cytoplasmic superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidase (GPX).

Elevation of systemic blood pressure is associated with increased ROS activity and enhanced nitric oxide (NO) inactivation in various models of genetic and acquired hypertension (Rajagopalan et al., 1996; Bouloumie et al., 1997; Swei et al., 1997; Schnackenberg et al., 1998; Vaziri et al., 1999; Vaziri et al., 2000; Roberts et al., 2000, 2001). Coarctation of the abdominal aorta above the renal arteries is known to cause severe hypertension proximal to the level of constriction primarily as a consequence of reduced renal perfusion and subsequent activation of the renin-angiotensin system (Fernandes et al., 1976; Parker et al., 1982). A previous study from this laboratory examined the role of hypertension, per se, on reactive oxygen species mediated nitric oxide (NO) inactivation in rats with abdominal aortic banding (Barton et al., 2001a), which causes severe regional hypertension in the arterial system proximal to the coarctation while sparing the arterial tree below the coarctation. Noted were marked up-regulations of endothelial nitric oxide synthase (eNOS) in heart and thoracic aorta and of neuronal NOS (nNOS) in the brain of rats with severe aortic coarctation (Barton et al., 2001b).

Although baromechanical forces have been shown to stimulate NO production through upregulation of eNOS, the role, if any, of baromechanical forces in regulation of antioxidant enzymes is unknown. We hypothesized that the occurrence of severe hypertension, caused by enhanced inactivation of NO by ROS, may be associated with altered NADPH oxidase and antioxidant enzyme protein expression. To test this hypothesis, we determined the abundance of Cu/Zn superoxide dismutase (SOD), Mn SOD, catalase (CAT), glutathione peroxidase (GPX) and NADPH oxidase (gp91phox subunit) in aortic tissue proximal and distal to the coarctation in aortic banded rats and the matching tissues in the sham-operated controls four weeks postoperatively. Since in this model circulating humoral factors are identical on both sides of the coarctation, possible differences in protein abundance in aortic tissue proximal and distal to the coarctation reasonably could be attributed to the effect of baromechanical forces and local as opposed to systemic biochemical events.
Methods

Animal model

Male Sprague-Dawley rats with an average weight of 150 g were randomly assigned to the abdominal aorta constriction and sham-operated control groups. Under general anesthesia with nembutal (50 mg/kg, intraperitoneally), the abdomen was opened, and the abdominal aorta was surgically dissected from the inferior vena cava at a site slightly above the renal arteries. A 21-gauge blunt needle was then placed along the side of the isolated aorta segment. Thereafter, a 2-0 suture was tightly tied around the aorta and the overlying needle. The needle was then removed, thus producing severe aortic constriction above the renal arteries. Animals assigned to the control group underwent the same procedures without actual ligation of the aorta. The animals were then observed for four weeks, during which they were allowed access to regular rat chow and water ad libitum. They were housed in a climate-controlled, light-regulated space with 12-hour light and dark cycles. At the conclusion of the four-week observation period, the animals were placed in metabolic cages for a timed urine collection. They were then anesthetized with an intraperitoneal injection of nembutal (50 mg/kg) and were killed by exanguination using cardiac puncture between the hours of 8 a.m. and 11 a.m. Aorta segments above and below the banding site were resected. Surrounding adventitial tissues were removed, and the segments were washed with ice-cold phosphate-buffered saline and immediately frozen in liquid nitrogen. Harvested tissues were then stored at −70°C until processed. Six animals were included in each group. The experimental protocol employed in the study was approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Immunoblotting for antioxidant enzymes and NADPH oxidase protein abundance

Homogenates (20% w/v) of thoracic and abdominal aorta were prepared in 10 mM HEPES buffer, pH 7.4, containing 320 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 10 μg/ml leupeptin, 2 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF) at 0–4°C with a polytron homogenizer. Homogenate was centrifuged at 9000 × g for 10 min at 4°C to remove nuclear fragments and tissue debris without precipitating membrane fragments. A portion of the supernatant was used for the determination of total protein concentration by using a Bio-Rad kit (Hercules, CA).

Immunoblotting was performed as described in our earlier publication (Sindhu et al., 2003). Briefly, total cellular protein (20 μg) was electrophoresed in 4–20% Tris-glycine sodium dodecyl sulfate (SDS) polyacrylamide gels (Novex, San Diego, CA). Proteins were transferred onto polyvinyl diethyl fluoride (PVDF) membranes (Millipore Corp., Bedford, MA), blocked in 5% dry milk in T-TBS (0.02 M Tris/0.15 M NaCl, pH 7.5 containing 0.1% Tween 20) at room temperature (RT) for 3 hours, washed 3× with T-TBS and incubated with the primary antibodies to the Cu/Zn SOD (1:20,000, Calbiochem), Mn SOD (1:1000, Upstate), CAT (1:2000, Calbiochem), GPX (1:250, Cortex) and gp91phox (1:1000, Transduction Labs) for 3 hours at RT. After washing 5× with T-TBS, the blots were incubated with secondary antibodies (anti-sheep for Cu/Zn SOD, Mn SOD and GPX, 1:2000; anti-rabbit for CAT, 1:2000 and anti-mouse for gp91phox, 1:1000) conjugated with horseradish peroxidase at RT for 2 hours. After washing 5× with T-TBS, the membranes were developed using enhanced chemiluminescent reagent (ECL) (Amersham Life Science Inc., Arlington Heights, IL, USA) and subjected to
autoluminography for 1 to 5 min. The autoluminographs were scanned with a laser densitometer (Model PD 1211, Molecular Dynamics, Sunnyvale, CA) to determine the relative optical densities of the bands.

All the proteins of interest were positively identified by running parallel to the molecular weight markers and by eliminating the primary and secondary antibodies. Normalization to β-actin (anti-actin, 1:5000, Chemicon International, Temecula, CA) or glyceraldehyde-3 phosphate dehydrogenase (GAPDH) (anti-GAPDH, 1:2500, Chemicon International, Temecula, CA) was used to verify the uniformity of protein load and transfer efficiency across the tested tissues (data not shown).

**Data analysis**

An ANOVA was used in statistical analysis of the data, which are presented as mean ± SEM. Differences were considered statistically significant at P < 0.05. When significant F values were noted, post hoc analyses were performed using a Newman-Keuls Multiple Comparison Test. Values reported are means ± SEM with 6 rats per group unless otherwise indicated.

**Results**

**General data**

No significant differences were found for body weight between the banded and control groups (293 ± 11 vs. 301 ± 4 p < 0.05).

**NADPH oxidase protein abundance**

The aortic segment proximal to the banding site showed a significant up-regulation of gp91phox subunit of NADPH oxidase, with no change in the abdominal aorta (Fig. 1). The protein expression of

![Fig. 1. Representative Western blot of NADPH oxidase (gp91phox subunit) protein content in aortic tissue from banded and control groups. Group data is below, illustrating relative optical densities of gp91phox protein bands in the study animals. Data is presented as mean±SEM. CT: control thoracic; BT: banded thoracic CA: control abdominal BA: banded abdominal. N=6 in each group. †p<0.05 vs. control abdominal aorta. **p<0.01 compared to control thoracic aorta.](image)
gp91phox subunit was significantly higher in control abdominal aorta compared to the control thoracic aorta (Fig. 1). Thus, aortic stenosis above renal arteries resulted in a marked up-regulation of antioxidant enzymes and superoxide generating NADPH oxidase in vascular tissue exposed to high pressure.

Antioxidant enzymes protein abundance

Compared with the sham-operated controls, the banded group showed a significant rise in CAT in both the thoracic (p < 0.01) and abdominal (p < 0.05) aorta (Fig. 2). Furthermore, the protein expression of CAT was significantly higher in control abdominal aorta compared to the control thoracic aorta (p < 0.01). Both Cu/Zn SOD (Fig. 3) and Mn SOD (Fig. 4) protein expression were markedly up-regulated in the thoracic aorta of the banded animals (p < 0.05), but was not changed in the abdominal aorta. Like CAT, the protein expression of both the SODs was significantly higher in control abdominal aorta as compared to the control thoracic aorta (p < 0.01). GPX protein expression in both segments of the aorta was similar between the two groups (Fig. 5). However, GPX protein abundance was significantly higher in control abdominal aorta compared to the control thoracic aorta (p < 0.05).

Discussion

Antioxidant/oxidant balance is well established as an important physiological regulator of arterial pressure, and recently, its role in the pathogenesis of various forms of hypertension has been substantiated (Rajagopalan et al., 1996; Bouloumie et al., 1997; Swei et al., 1997; Schnackenberg et al., 1998; Vaziri et al., 1999; Vaziri et al., 2000; Roberts et al., 2000, 2001). For example, a number of studies have described reduced antioxidant and/or increased NADPH oxidase expression in selected forms of hypertension, such as lead-induced hypertension (Vaziri et al., 2003b), in the spontaneously hypertensive rat (Chen et al., 2001) and in renal insufficiency (Vaziri et al., 2003a). Additionally, humans with essential hypertension have reduced SOD activity (Russo et al., 1998). While a reduction in vascular antioxidant enzyme expression and increase in oxidant enzyme expression could be interpreted

![Fig. 2. Representative Western blot of catalase protein content in aortic tissue from banded and control groups. Group data is below, illustrating relative optical densities of catalase protein bands in the study animals. Data is presented as mean±SEM. CT: control thoracic; BT: banded thoracic CA: control abdominal BA: banded abdominal. N=6 in each group. *p<0.01 compared to control thoracic aorta. †p<0.05 vs. control abdominal aorta. **p<0.01 compared to control thoracic aorta.](image-url)
as evidence that an antioxidant/oxidant imbalance is involved in the pathogenesis of hypertension, it is also plausible that elevated enzyme expression may be present as the result of endothelial dysfunction secondary to elevated pressure per se. The present study utilized abdominal aortic banding to examine antioxidant enzyme expressions in tissues proximal to the level of coarctation (thoracic aorta), as well as in tissues distal to the coarctation (abdominal aorta). For comparison, the proteins were measured in the corresponding segments obtained from the sham-operated rats.

The aortic banded animals exhibited a markedly increased CAT, Mn SOD, Cu/Zn SOD and gp91phox protein content in the aorta segment above the stenotic site compared with the corresponding aortic segments in the sham-operated controls. However, GPX protein expression in the aorta segment above the stenotic site did not change. Additionally, below the banding site protein content of Mn SOD, Cu/Zn SOD, gp91phox and GPX were similar. These data clearly demonstrate that up-regulation of aortic antioxidant enzymes and NADPH oxidase is generally confined to aortic segments exposed to elevated arterial blood pressure in this model. This selective up-regulation of protein expression in
aortic segments exposed to the sustained baromechanical effects of hypertension is consistent with previous findings. For example, Bouloumie et al. (1997), utilizing a coarctation-induced hypertensive model of complete ligation of the abdominal aorta between the right and left renal arteries, found a near twofold increase in superoxide anion production after two weeks and a marked increase in eNOS protein and mRNA expressions coupled with impaired endothelium-dependent relaxation after six weeks of aortic banding in the aortic segment proximal to the coarctation. Studies from this laboratory showed marked up-regulation of eNOS expression in the heart and the aortic segment proximal to the coarctation and of nNOS expression in brain tissue in this model (Barton et al., 2001a). In contrast, there was no increase in eNOS expression in the aortic segment distal to the level of coarctation (Barton et al., 2001a). The present data combined with these findings suggests that elevated pressure may upregulate proteins exposed to shear, which is well-known to increase NOS expression (Sessa et al., 1994; Laughlin, 1995; Uematsu et al., 1995). Exercise training has been documented to increase SOD protein content, and this is hypothesized to be due to an increase in shear (Rush et al., 2000, 2003). Elevation of CAT, Mn SOD, Cu/Zn SOD and gp91phox protein abundance in tissues exposed to high pressure and its normality in the abdominal aorta, which was not exposed to high blood pressure, points to the role of baromechanical and local biochemical factors as opposed to circulating humoral factors, which were uniform in both aortic segments. It is of interest to note that the protein expression of the oxidant as well as all the antioxidant enzymes was higher in control abdominal aorta compared to the control thoracic aorta. The reason(s) for this could not be discerned in the present study and require further investigation.

Oxidative stress and enhanced NO inactivation by ROS have been implicated in the pathogenesis of various forms of hypertension in experimental animals and humans (Rajagopalan et al., 1996; Bouloumie et al., 1997; Swei et al., 1997; Schnackenberg et al., 1998; Vaziri et al., 1999; Vaziri et al., 2000; Roberts et al., 2000, 2001). The present data documenting an increase in the protein content of gp91phox subunit of NADPH oxidase in banded aorta of rats provides evidence for the increase in oxidative stress consequent to elevated pressure. Cuccurullo et al. (1991) noted an increase in thiobarbituric acid-reactive substances proximal to aortic stenosis and a significant increase in glutathione-related antioxidant defenses in the hypertensive aortic wall of rabbits. Accordingly, the
data suggest that increased ROS production in the vascular tissues exposed to elevated pressure in this model may account for the constellation of increased nitrotyrosine accumulation, regional hypertension, and endothelial dysfunction seen in this model. It is of note that pulsatile stretch has been shown to increase superoxide production and activate nuclear factor-κB (NF-κB) in vascular smooth muscle cells (Hishikawa et al., 1997). NF-κB, in turn, promotes transcription of numerous factors, including the proinflammatory cytokines. Thus, increased pulsatile stretch associated with hypertension can raise ROS production directly as well as indirectly via stimulation of proinflammatory cytokines.

In conclusion, these data clearly demonstrate that up-regulation of aortic CAT, Mn SOD, Cu/Zn SOD and gp91phox protein expression was confined to aortic segments exposed to elevated arterial blood pressure in animals with abdominal aortic banding. These findings demonstrate the important role of hemodynamic factors and related baromechanical forces on the regulation of antioxidant and oxidative stress related enzymes. In contrast, our data showed no significant change in GPX expression of the aorta on either side of the coarctation, suggesting that GPX regulation in the aorta may be independent of intravascular pressure.

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References


