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# Acute inhibition of glutathione biosynthesis alters endothelial function and blood pressure in rats

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**Abstract**

The cardiovascular and biochemical responses during acute oxidative stress induced by D,L-buthionine-(*S*,*R*)-sulfoximine (BSO) were investigated in Sprague–Dawley rats. Mean arterial pressure, heart rate and vascular reactivity were measured after subcutaneous injection of BSO (4 mmol/kg). Control rats received saline. Levels of GSH and GSSG in blood and tissues as well as renal superoxide were determined. Nitric oxide, prostacyclin and thromboxane $A_2$ in plasma and aorta, and isoprostane in plasma were also measured. Blood pressure was elevated at 24 h (121 ± 2 vs. 104 ± 2 mm Hg), with increased reactivity to phenylephrine (by a 59 ± 4 vs. 45 ± 2 mm Hg change), and impaired response to sodium nitroprusside (by a −35 ± 2 vs. −63 ± 2 mm Hg change), $P<0.05$. The GSH:GSSG ratio was reduced at 8 and 24 h in blood (4.1 ± 0.6 and 5.1 ± 0.3, respectively, vs. 8.5 ± 0.2), and at 8 h in the aorta (1.0 ± 0.2 vs. 2.9 ± 0.5), heart (1.6 ± 0.3 vs. 2.3 ± 0.1) and kidney (2.1 ± 0.2 vs. 3.7 ± 0.4), $P<0.05$. Superoxide fluorescence was increased at 24 h via NADH (4131 ± 194 vs. 2853 ± 199), NADPH (2874 ± 272 vs.1479 ± 257) and succinate (2475 ± 133 vs. 1594 ± 2150), $P<0.05$. Plasma prostacyclin was reduced at 8 and 24 h (36 ± 4 and 52 ± 13, respectively, vs. 310 ± 44 pg/ml), $P<0.001$, whereas nitric oxide was reduced at 24 h (6.4 ± 1 vs. 22 ± 2 μM), $P<0.01$. Also at 24 h, thromboxane $A_2$ was increased both in plasma (374 ± 154 vs. 61 ± 10 pg/ml) and the aorta (174.4 ± 38 vs. 27 ± 3.4 pg/mg), $P<0.05$. Thus, acute BSO-induced oxidative stress alters blood pressure and endothelial function by mechanisms involving increased plasma levels and aortic release of thromboxane $A_2$ and reduced nitric oxide and prostacyclin.


*Keywords:* Oxidative stress; Glutathione; Blood pressure; Endothelial function

## 1. Introduction

Oxidative stress due to thiol depletion has been shown to play a role in the pathogenesis of various cardiovascular disorders such as hypertension (Vaziri et al., 2000; Sharma et al., 1992) and ischemia–reperfusion injury (Kim and Phyllis, 1998). Glutathione is the most abundant nonprotein intracellular thiol with multiple roles as an antioxidant agent (Halliwell and Gutteridge, 1989). It acts to scavenge reactive oxygen species as well as to regenerate other antioxidants from their oxidized forms (Dhalla et al., 2000). Consistent with this notion, administration of antioxidant compounds has been shown to provide protection against oxidative cardiovascular injury (Peng and Li, 2002). Administration of buthionine sulfoximine (BSO), a selective inhibitor of γ-glutamyl-cysteine synthetase, an enzyme in the glutathione biosynthetic pathway, induces glutathione depletion and oxidative stress (Meister, 1984). In this process, glutathione is converted to its oxidized form (GSSG) which must be reduced by the combination of glutathione reductase and NADPH. Therefore, an index of cellular oxidative events is the ratio of the levels of the reduced and oxidized forms of glutathione. Also, reactive oxygen species, such as superoxide radicals, form vasoconstrictor isoprostanes from non-enzymatic peroxidation of arachidonic acid (Tesfamariam and Cohen, 1992), and may inactivate nitric oxide (Vaziri et al., 2000). Endothelial cells are known to generate nitric oxide and prostacyclin, which are vasodilators, and thromboxanes, which mediate vasoconstriction. (Davidge, 2001).

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It is also now clear that nitric oxide plays a critical role in the maintenance of blood pressure and peripheral vascular resistance and contributes to the resting tone of conductance and resistance arteries (Quyyumi et al., 1995). Thus, vascular endothelial dysfunction has been associated with decreased formation, release or action of nitric oxide; decreased formation and release of prostacyclin; and increased formation of vasoconstrictor prostanoids (Taylor, 2001). Also, oxidant stress influences blood pressure via alterations in the production, release or effect of endothelium-derived paracrine substances (Schiffrin, 2001).

In this study, we investigated the effect of inhibition of glutathione biosynthesis, by BSO, on blood pressure and endothelial function. Levels of GSH and GSSG in blood, the aorta, heart and kidney as well as superoxide anion production in the kidney were determined. We also measured levels of nitric oxide, prostacyclin, thromboxane $A_2$ and isoprostane in plasma, in addition to the aortic capacity to produce nitric oxide and prostanoids.

## 2. Materials and methods

### 2.1. Experimental design

Male Sprague–Dawley rats (4–5 weeks, Harlan Sprague–Dawley, Indianapolis, IN) were used in these experiments. Upon arrival, they were grouped five per cage in our animal facility that has 12-h light/dark cycles with the temperature controlled at 21–23 °C. Rodent Lab. Chow® (Purina Mills, IN) and water were made available ad libitum.

Following 1 week of acclimatization, each animal was anesthetized (using 70 mg/kg ketamine and 10 mg/kg xylazine, i.m.) and the carotid artery and jugular vein were cannulated using polyethylene tubing containing heparin (20 IU/ml) in 0.9% sodium chloride. The artery was used for the measurement of direct mean arterial pressure, heart rate, and free-flow blood sample collection. The cannulae were externalized in the posterior cervical region and occluded with a metal plug, and flushed with heparinized saline every 12 h. The animals were allowed to recover for 24 h, after which the basal mean arterial pressure and heart rate were measured. Following the basal measurements, the rats were divided into four groups ($n=6$ each) for the control, 4, 8 and 24 h studies. Each animal was administered with BSO at a dose of 4 mmol/kg (or ~ 890 mg/kg) of body weight s.c. (dissolved in 2 ml 0.9% sodium chloride, with the aid of 0.1 N sodium hydroxide, final pH 8.5). This dose was previously demonstrated to be optimal for depletion of hepatic glutathione in rats (Drew and Miners, 1984). Control animals received saline (2 ml s.c. for 4 h). At the end of each time point, mean arterial pressure and heart rate were measured. Also at 24 h, vascular reactivity was assessed in a separate set of unanesthetized free-moving animals ($n=5$ per group).

The Institutional Animal Care and Use Committee at Morehouse School of Medicine has approved all experiments and procedures.

### 2.2. Direct mean arterial blood pressure, heart rate and vascular reactivity measurement in conscious rats

In order to take readings in the fully conscious state, the plugs were replaced with metal tubing on the cannulae connected to 23-gauge needles on tuberculin syringes containing heparinized saline. Mean arterial pressure and heart rate were measured through the arterial cannulae connected to a blood pressure transducer (Gould Model P23, Gould, Oxnard, CA) that was coupled to a Cardiomax-II (CMX2-780-K, Columbus Instruments Intern, Columbus, OH). For every individual animal, after stabilization, readings were collected each minute for 5 min and an average value obtained.

Vascular reactivity was assessed in unanesthetized free-moving animals as described previously (Bayorh et al., 2001). Briefly, intravenous bolus injections (0.2 ml) of phenylephrine (1, 3 and 9 μg/kg) were followed by sodium nitroprusside (1, 3, and 9 μg/kg) injection, each followed by a 0.2-ml saline flush. Sufficient time (10–15 min) between injections allowed both mean arterial pressure and heart rate to return to basal values. The data were expressed as the change from the resting mean arterial pressure and heart rate for each dose.

### 2.3. Collection and storage of blood samples

Blood samples (1 ml) for plasma nitric oxide, thromboxane and prostacyclin measurements were collected by free flow (for basal levels and at each time point) via the cannulae in the carotid artery into heparinized and indomethacin (100 mM)-rinsed (for prostaglandin samples) tubes. The volume was replaced with normal saline. To assess glutathione and isoprostane concentrations, blood samples (5–10 ml) were withdrawn via cardiac puncture from all animals (under ketamine/xylazine i.v.) prior to sacrifice. Both samples were centrifuged at $3000 \times g$ for 25 min at 4 °C.

For the isoprostane assay, butylated hydroxytoluene was added to 1 ml of plasma to a final concentration of 0.005% (v/v), and then sample was frozen and stored at − 80 °C until analysis.

For the glutathione assay, 0.5 ml of the blood sample was added to an Eppendorf tube containing an equal volume of ice-cold 10% perchloric acid containing 0.2 M boric acid and 10 μM γ-glutamylglutamate (PCA/BA/γGG) solution. It was then centrifuged at $3000 \times g$ for 25 min at 4 °C, to remove protein. The supernatant was collected, frozen in aliquots and stored at − 80 °C until analysis.

### 2.4. Tissue harvesting for in vitro studies

Immediately following cardiac puncture, the heart and both kidneys were harvested from all animals and frozen in

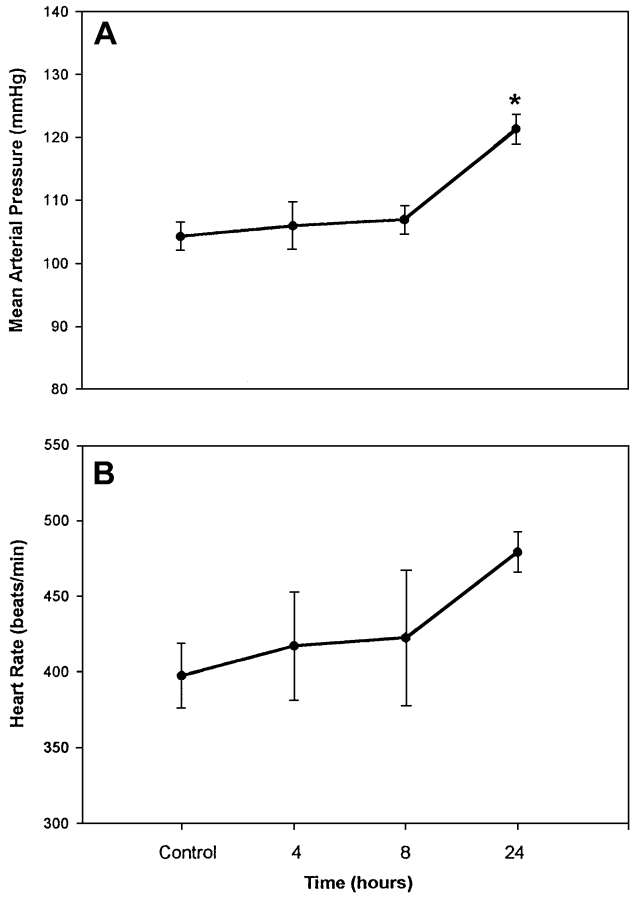


Fig. 1. Effect of administration of BSO for 24 h on mean arterial pressure and heart rate in Sprague–Dawley rats. Data are represented as mean ± S.E.M. for six animals per group. Significant difference ($P<0.05$) from control is denoted by asterisk (*).

liquid nitrogen and stored at −80 °C. The descending thoracic aortas were put in Hank's balanced salt solution (HBSS) containing HEPES and kept at 4 °C prior to release studies.

### 2.5. Measurement of plasma prostanoids and nitric oxide

Enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI), were used to measure plasma levels of thromboxane $A_2$ (as thromboxane $B_2$), prostacyclin (as 6-keto-prostaglandin $F_{1\alpha}$) and total 8-isoprostanes (free and esterified), after sample purification on a C-18 column, as described by the manufacturer. Nitric oxide (as nitrates + nitrites) levels in plasma were quantitated by a colorimetric assay kit (Cayman Chemical), which utilizes the Greiss reagent.

### 2.6. Determination of whole blood and tissue levels of glutathione

Total reduced and oxidized glutathione in whole blood was simultaneously measured by high performance liquid chromatography fluorescence detection as described previously (Jones et al., 1995). The tissues (heart, kidney and aorta) were mixed 1:1 (w/v) with ice-cold PCA/BA/γGG solution and homogenized. The mixture was then centrifuged at 3000 × *g* for 25 min at 4 °C. The supernatant was collected and frozen at −80 °C until assayed. An aliquot was taken for protein determination by the BioRad method (Bradford, 1976). Total reduced and oxidized tissue glutathione was measured as described for plasma.

### 2.7. Measurement of renal superoxide production

Fluorescence spectrometry of renal superoxide production was performed as described previously (Zou et al., 2001). Briefly, whole kidneys were minced and homogenized with a glass homogenizor in ice-cold HEPES buffer containing 25 mM HEPES, 1 mM ethylenediaminetetracetic acid and 0.1 mM phenylmethylsulfonyl fluoride, in a 1:1 (w/v) ratio. After centrifugation at 6000 × *g* for 5 min at 4 °C, the supernatant was collected, frozen and stored at −80 °C until use. Protein was determined in an aliquot of each homogenate by the BioRad method (Bradford, 1976). Superoxide production was measured in the following reaction mixture: 10 μM

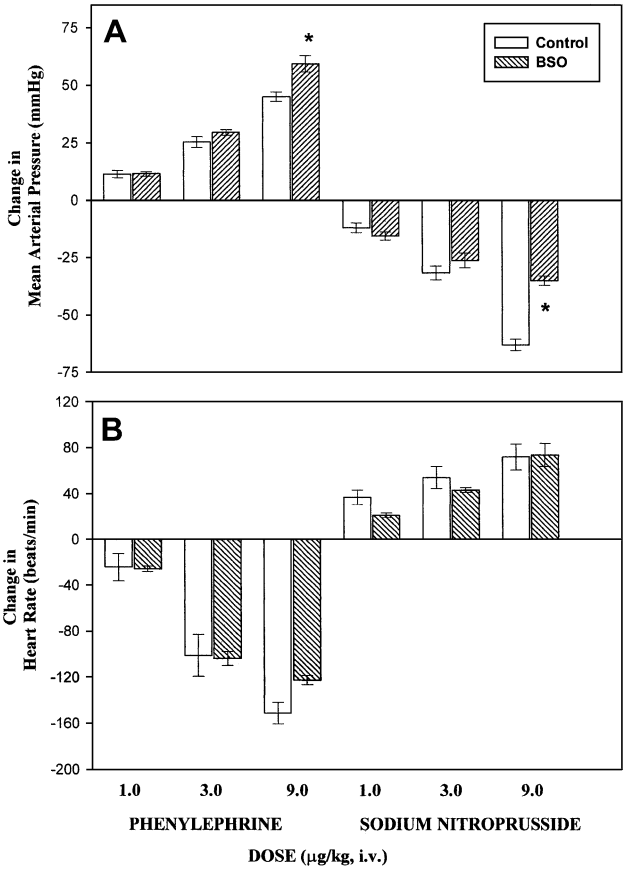


Fig. 2. Effect of administration of BSO for 24 h on vascular reactivity to intravenous doses of phenylephrine and sodium nitroprusside in Sprague–Dawley rats. Data are represented as the change in mean arterial pressure and heart rate from resting values at each dose. Each bar represents mean ± S.E.M. for five animals per group. Significant difference ($P<0.05$) from control is denoted by asterisk (*).

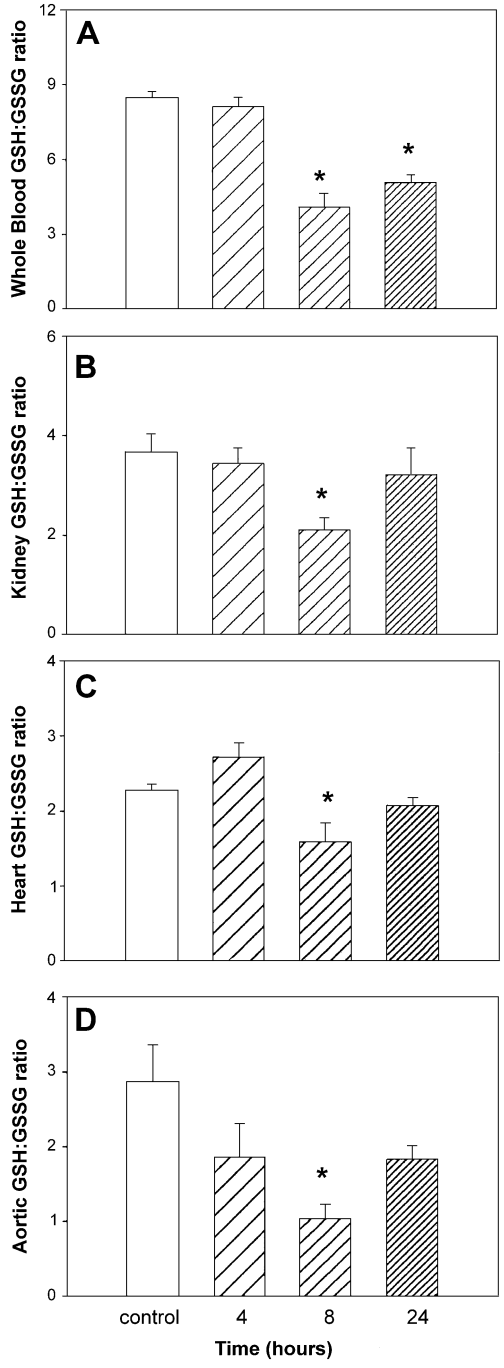


Fig. 3. Effect of administration of BSO for 24 h on whole blood, kidney, heart and aortic GSH:GSSG ratio in Sprague–Dawley rats. Data are represented as mean ± S.E.M. for six animals per group. Significant difference ($P<0.05$) from control is denoted by asterisk (*).

dihydroethidium, 0.5 mg/ml salmon testes DNA, 10 μg of homogenate protein, and the appropriate substrate for either NADH/NADPH oxidase (0.1 mM NADH or NADPH), mitochondrial respiratory enzymes (5 mM succinate) or xanthine oxidase (0.1 mM xanthine), in a total volume of 0.2 ml. This mixture was incubated on a 96-well microplate for 30 min at 37 °C. Ethidium/DNA fluorescence was measured at an excitation of 475 nm and an emission of 610 nm on a CytoFluor™ II Microplate Reader (Biosearch Products, Bedford, MA). The data was expressed as fluorescence units/min/mg protein.

### 2.8. Vascular capacity for generation of prostanoids and nitric oxide

The vascular capacity for generation of prostanoids and nitric oxide was determined as described by Uehera et al. (1991). Briefly, the aortas were placed in HBSS containing HEPES and rinsed repeatedly with fresh media. The surrounding connective tissues were carefully removed. A segment of the aorta was dissected and incubated in 2 ml of HBSS at 37 °C for 30 min. Prostanoids and nitric oxide released into the media were directly assayed as described above for plasma. The aortic strips were then dried at 80 °C for 18 h and weighed. The amount of prostanoids and nitric oxide produced was normalized to the dry weight of the aorta.

### 2.9. Statistical analysis

Values were expressed as mean ± S.E.M, and $n$ refers to the number of animals used. Statistical significance ($P<0.05$) was evaluated using either Student's $t$-test or, for multiple groups, analysis of variance (ANOVA), followed by the Tukey–Kramer multiple comparison test using the INSTAT program (GraphPad Software, San Diego, CA).

## 3. Results

### 3.1. Effect of BSO on mean arterial pressure and heart rate

Blood pressure was significantly elevated at 24 h (121 ± 2 mm Hg), compared to the other time points (104 ± 2, 106 ± 3 and 107 ± 2 mm Hg, for the control, 4 and 8 h, respectively, $P<0.05$), as shown in Fig. 1A. There were no significant changes in the heart rate from the control (398 ± 21 vs.

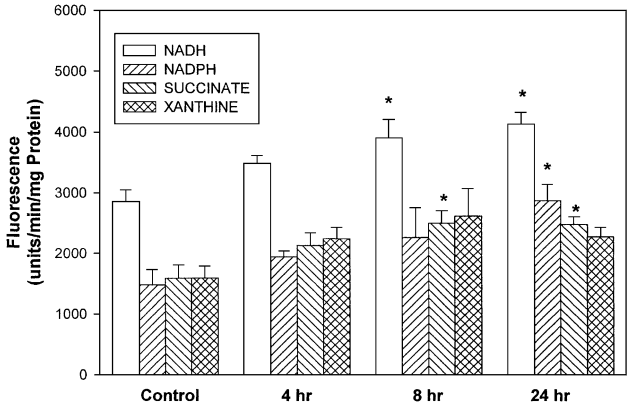


Fig. 4. Effect of administration of BSO for 24 h on renal production of superoxide anion, via different pathways, in Sprague–Dawley rats. Data are represented as mean ± S.E.M. for six animals per group. Significant difference ($P<0.05$) from control is denoted by asterisk (*).

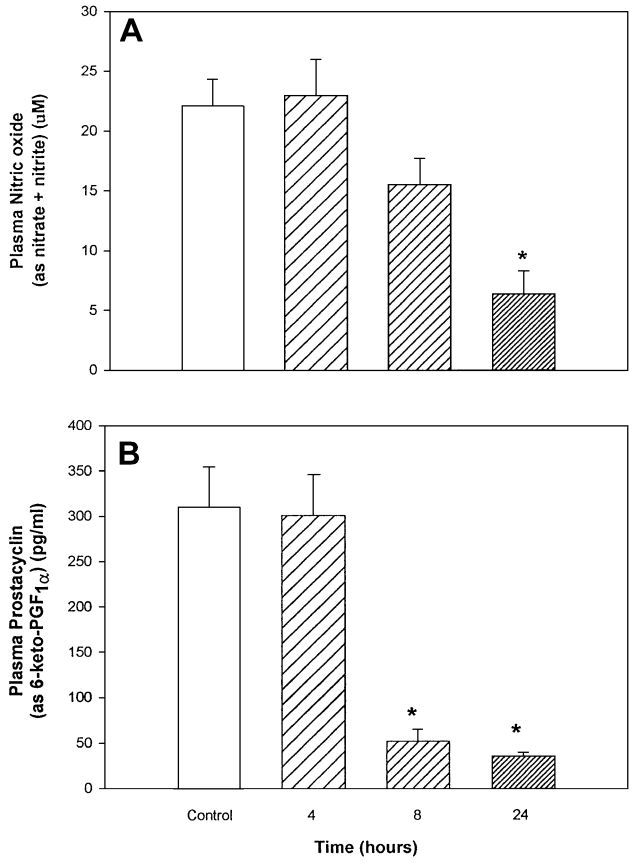


Fig. 5. Effect of administration of BSO for 24 h on plasma nitric oxide and prostacyclin levels in Sprague–Dawley rats. Data are represented as mean ± S.E.M. for six animals per group. Significant difference ($P<0.05$) from control is denoted by asterisk (*).

417 ± 36, 423 ± 45 and 479 ± 14 beats/min for the 4, 8 and 24 h, respectively), as shown in Fig. 1B.

### 3.2. Effect of BSO on vascular reactivity

Phenylephrine produced dose-dependent increases in mean arterial pressure and corresponding decreases in the heart rate, with a significantly bigger increase in blood pressure at a dose of 9 μg/kg, in the BSO group (59 ± 4 vs. 45 ± 2 mm Hg), $P<0.05$. Sodium nitroprusside induced dose-dependent decreases in mean arterial pressure and increases in heart rate, with a significantly less reduction in blood pressure at the 9 μg/kg dose ( − 35 ± 2 vs. − 63 ± 2 mm Hg), $P<0.05$, as shown in Fig. 2A. The heart rate responses were not statistically different between the two groups, as shown in Fig. 2B.

### 3.3. Effect of BSO on whole blood and tissue GSH:GSSG ratio

The GSH:GSSG ratio was significantly reduced in whole blood at 8 and 24 h (4.1 ± 0.6 and 5.1 ± 0.3, respectively), compared to the control (8.5 ± 0.2), $P<0.05$, in Fig. 3A. In the kidney, heart and aorta, the GSH:GSSG ratio was also significantly reduced at 8 h ($P<0.05$) in Fig. 3b–d. The kidney GSH:GSSG ratio was 2.1 ± 0.2 at 8 h compared to 3.7 ± 0.4 for control, 3.4 ± 0.3 at 4 h and 3.2 ± 0.5 at 24 h. In the heart, the GSH:GSSG ratio was 1.6 ± 0.3 at 8 h, compared to 2.3 ± 0.1 for control, 2.7 ± 0.2 at 4 h and 2.1 ± 0.1 at 24 h. The aortic GSH:GSSG ratio was 1.0 ± 0.2 at 8 h, compared to 2.9 ± 0.5 for control, 1.9 ± 0.4 at 4 h and 1.8 ± 0.2 at 24 h.

### 3.4. Effect of BSO on renal superoxide production

The formation of superoxide was increased in the reaction mixtures compared to the control: at 8 h via NADH oxidase and succinate, 3905 ± 298 vs. 2853 ± 199 and 2500 ± 207 vs. 1594 ± 215, respectively; at 24 h via NADH, NADPH oxidases and succinate, 4131 ± 194 vs. 2853 ± 199, 2874 ± 273 vs. 1479 ± 257 and 2475 ± 133 vs. 1594 ± 215, respectively, $P<0.05$, as shown in Fig. 4.

### 3.5. Effect of BSO on plasma nitric oxide and prostacyclin levels

There was a gradual decrease in the levels of plasma nitric oxide and prostacyclin at 8 and 24 h compared to the

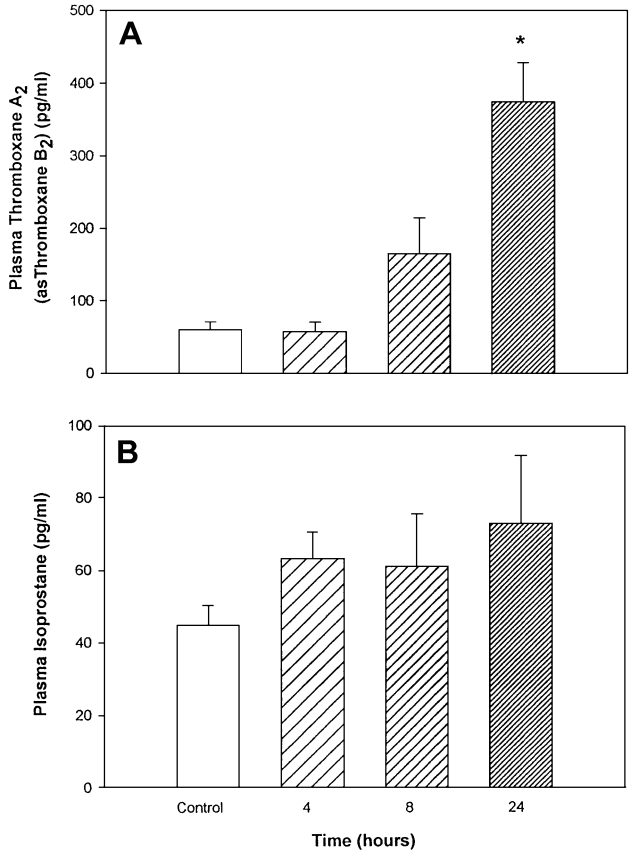


Fig. 6. Effect of administration of BSO for 24 h on plasma thromboxane and total isoprostane levels in Sprague–Dawley rats. Data are represented as mean ± S.E.M. for six animals per group. Significant difference ($P<0.05$) from control is denoted by asterisk (*).

Table 1
Effect of BSO for 24 h on aortic production of nitric oxide, prostacyclin and thromboxane $A_2$ in Sprague–Dawley rats

| | Aortic production (per mg dry weight) of nitric oxide, prostacyclin and thromboxane $A_2$ | | | |
|---|---|---|---|---|
| | Control | 4 h | 8 h | 24 h |
| Nitric oxide (μM/mg) | 1.6 ± 0.1 | 2.4 ± 0.3 | 1.8 ± 0.2 | 1.6 ± 0.6 |
| Prostacyclin (pg/mg) | 59.7 ± 16.5 | 26.7 ± 4.8 | 43.7 ± 8.3 | 59.9 ± 19.3 |
| Thromboxane $A_2$ (pg/mg) | 27.0 ± 3.4 | 32.9 ± 3.6 | 51.2 ± 7.6 | 174.4 ± 38.0[a] |

[a] $P<0.05$ compared to control, 4 and 8 h.

control. Nitric oxide was significantly reduced to 6.4 ± 1.9 μM at 24 h ($P<0.01$), compared to 22 ± 2, 23 ± 3 and 16 ± 2 μM, for the control, 4 and 8 h, respectively, as shown in Fig. 5A. Prostacyclin levels were reduced to 36 ± 4 pg/ml at 24 h and 52 ± 13 pg/ml at 8 h ($P<0.001$), compared to 310 ± 44 pg/ml for the control and 302 ± 45 pg/ml at 4 h, as shown in Fig. 5B.

### 3.6. Effect of BSO on plasma thromboxane $A_2$ and isoprostane levels

Thromboxane $A_2$ levels were significantly elevated at 24 h (374 ± 154 pg/ml) compared to earlier time points, 61 ± 10 pg/ml for control, 58 ± 13 pg/ml at 4 h and 166 ± 49 pg/ml at 8 h ($P<0.05$), as shown in Fig. 6A. Plasma isoprostane levels were higher at 24 h (73 ± 19 pg/ml) compared to the control (45 ± 5 pg/ml), 4 h (63 ± 7 pg/ml) and 8 h (61 ± 15 pg/ml) values, but this elevation was not statistically significant, as shown in Fig. 6B.

### 3.7. Effect of BSO on vascular capacity to produce prostanoids and nitric oxide

Levels of thromboxane $A_2$ released from the aorta were significantly elevated at 24 h (174.4 ± 38 vs. 27 ± 3.4 pg/mg), $P<0.05$; however, there was no difference in the aortic production of nitric oxide and prostacyclin, as shown in Table 1.

## 4. Discussion

Data from the present study show that treatment with BSO causes significant changes in glutathione homeostasis, reflected by a reduction of the GSH:GSSG ratio for blood, aorta, kidney and heart during 24 h. This pattern of change in GSH and GSSG levels is consistent with previous observations in studies on the interorgan translocation, turnover and metabolism of glutathione in rats following BSO administration (Griffith and Meister, 1979, 1997). Furthermore, renal superoxide formation was increased via NADH/NADPH and succinate, which are substrates for NADH/NADPH oxidases and mitochondrial respiratory chain enzymes, respectively. These enzymes have been demonstrated to represent the major source of superoxide formation in the kidney (Zou et al., 2001). Cowley et al. (1995) demonstrated that increased oxidant stress or impaired antioxidant mechanisms in the kidney may lead to reduction of renal medullary blood flow and sodium excretion and hypertension. The reduction in the GSH:GSSG ratio and increase in renal superoxide formation in our study were associated with increased mean arterial blood pressure. Thus, the current data underscores the central role of the kidney in the regulation of blood pressure. The effect of BSO on blood pressure was associated with an augmented pressor response to phenylephrine and a reduced hypotensive responsive to sodium nitroprusside, suggesting impaired endothelial function and, thus, a reduction in vascular relaxation.

Administration of BSO was also associated with gradual reductions in the plasma levels of vasodilatory compounds, as reflected by the changes in nitric oxide and prostacyclin. During oxidative stress, nitric oxide and superoxide react to form peroxynitrite, a potent cytotoxic oxidant, hence reducing the available nitric oxide (Halliwell, 1997). Likewise, a recent study suggested that oxidative stress-induced hypertension is not caused by either structural abnormality of or depressed nitric oxide synthase expression. Instead, it may be associated with and perhaps partially related to enhanced nitric oxide inactivation by reactive oxygen species and diminished nitric oxide bioavailability (Zhou et al., 2002). We found that BSO-induced depletion of glutathione after 4 h had no effect on plasma prostacyclin, consistent with what was previously reported (Maynard et al., 1992). However, we demonstrated that at longer periods of treatment, plasma prostacyclin levels are significantly reduced. This effect may be related to the inhibitory effect of superoxide on the production of prostacyclin (Katusic and Vanhoutte, 1989).

It was also very interesting to observe a gradual time-dependent increase of the aortic production and plasma levels of thromboxane $A_2$. Previous studies in spontaneously hypertensive rats suggested that oxygen-derived free radical-induced vasoconstriction in the rat aorta is caused by thromboxane $A_2$ and prostaglandin $H_2$ release in smooth muscles (Michitaka et al., 1999). The increase in aortic production of thromboxane $A_2$ and reduction in prostacyclin may be related to the differential expression of these prostanoids in the vascular tissue (Szekacs et al., 1996). The GSH–GSSG system is capable of activating and/or inactivating many enzymes and may upregulate thromboxane $A_2$ and downregulate prostacyclin synthesis. Oxidative stress has also been associated with elevated plasma isoprostane levels due to increased free radical catalyzed peroxidation of arachidonic acid (Schnackenberg and Wilcox, 1999). We observed a tendency towards increased levels of isoprostane over the treatment period.

Thus, the acute BSO-induced oxidative stress and pressor response is associated with vascular endothelial dysfunction as evidenced by altered reactivity, increased plasma levels and aortic production of thromboxane $A_2$ and reductions in nitric oxide and prostacyclin. These findings have implications for the use of antioxidative therapy in hypertension.

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