Superoxide-Dependent Hypertension in Male and Female Endothelin B Receptor–Deficient Rats

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Evidence for endothelin (ET) involvement in the control of fluid volume balance and arterial pressure has been derived in part from the observations that rats lacking the ET_B receptor develop hypertension when placed on a high-salt (HS) diet. The present study was designed to determine the effect of superoxide on salt-induced hypertension in male and female ET_B-deficient (sl/sl) and wild-type control (wt) rats. After 14 days on a HS (8% NaCl) diet, female sl/sl rats had significantly elevated arterial pressure (183 ± 2 mm Hg, tail cuff) compared with female wt rats (134 ± 2 mm Hg). The response to a HS diet was lower in male sl/sl rats (166 ± 6 mm Hg) yet was significantly greater than that in male wt controls (135 ± 3 mm Hg). Separate groups of male and female sl/sl and wt rats were given tempol (1 m*M* in drinking water) during HS treatment. Arterial pressures were 149 ± 5 mm Hg in male and 143 ± 3 mm Hg in female sl/sl rats treated with tempol, values that were similar to those of controls on a normal salt diet. After 14 days, however, male and female sl/sl rats recovered from the blood pressure–lowering effects of tempol. On Day 15, arterial pressures in female sl/sl rats on a HS diet were 160 ± 6 mm Hg and 177 ± 6 mm Hg in tempol-treated and untreated groups, respectively. In male sl/sl rats, arterial pressures were 155 ± 3 mm Hg and 165 ± 5 mm Hg in tempol-treated and untreated groups, respectively. On Day 15, no differences among groups with or without tempol were observed in plasma thiobarbituric acid–reactive substance (TBARS) concentrations or in urinary excretion of TBARS. Plasma ET-1 concentrations were significantly higher in female vs. male sl/sl rats. These results indicate that the early stages of salt-dependent hypertension produced by ET_B receptor deficiency are dependent on superoxide and that the elevated pressure in the female rats may be due to elevated circulating levels of ET-1. Exp Biol Med 231:818–823, 2006

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Introduction

The endothelin (ET) system is an important regulator of blood pressure. Binding of vascular smooth muscle cell ET_A receptors by ET-1 results in vasoconstriction, while ET-1 activation of ET_B receptors on endothelial and renal tubular cells results in vasodilation and natriuresis (1–3). It has been previously demonstrated that treatment with a high-salt (HS) diet in rats following chronic blockade of the ET_B receptor or in ET_B receptor–deficient rats results in the development of hypertension and elevated plasma and urinary ET-1 (4–8). From these studies, it has been concluded that the lack of ET_B receptor function results in a salt-sensitive form of hypertension.

Other results have suggested that salt-dependent hypertension in ET_B receptor–deficient rats may be linked with an ET_A receptor–dependent increase in reactive oxygen species (5). Elmarakby *et al.* (5) observed that ET_A receptor blockade prevented the hypertension and increase in oxidative stress in ET_B receptor–deficient rats placed on a HS diet. ET-1 has been shown to stimulate superoxide production in the vasculature; therefore, increased ET-1 in salt-sensitive hypertension may contribute to an increase in oxidative stress and an elevation in blood pressure (9–11). This is supported by the finding that the elevated blood pressure with ET_B receptor blockade or long-term infusion of exogenous ET-1 may be attenuated by antioxidant treatment (8, 12). It has recently been reported that treatment with the superoxide dismutase (SOD) mimetic tempol attenuates the development of hypertension in the initial days of treatment in rats on a HS diet given an ET_B receptor antagonist, supporting the hypothesis that superoxide contributes to the maintenance of arterial pressure in rats on a HS diet (8).

A gender difference exists in the development of salt-sensitive hypertension in ET_B receptor–deficient rats, with females having a greater rise in blood pressure and a larger

increase in plasma ET levels in response to a HS diet compared with male ET_B receptor–deficient rats (6). Therefore, we hypothesize that the greater rise in blood pressure in female ET_B receptor–deficient rats on a HS diet is due to the stimulation of superoxide. This hypothesis was tested by treating male and female ET_B receptor–deficient rats with tempol in conjunction with a HS diet.

Materials and Methods

Tail-Cuff Measurements and Metabolic Cage Studies. Male and female wild-type (wt) and homozygous (sl/sl) rats deficient of ET_B receptors on a Wistar-Kyoto genetic background (13) were obtained from our local breeding colony. Experiments were conducted (at a mean age of 13 weeks) in accord with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and was approved and monitored by the Medical College of Georgia Institutional Animal Care and Use Committee. Rats were housed in temperature and humidity–controlled light-cycled quarters. All rats were placed on a HS (8% NaCl) diet, and a subset of male and female rats of both strains received tempol (1 m*M*) in the drinking water. This concentration of tempol has been shown to reduce superoxide levels in the kidney and vascular tissue (14, 15). Blood pressures were confirmed using the tail-cuff method (16). Before beginning treatment and at 7 and 14 days after starting treatment, rats were placed in metabolic cages (Nalgene Co., Rochester, NY) to allow quantitative measurement of food and water intake and to facilitate 24-hr urine collection. Under sodium pentobarbital anesthesia (65 mg/kg ip), a terminal blood sample was obtained and centrifuged, and plasma was frozen at −80°C.

Assays and Chemicals. Urinary ET levels were determined using radioimmunoassay (GE Healthcare Bio-Sciences, Piscataway, NJ). Plasma ET concentrations were measured using enzyme-linked immunosorbent assay (ELISA) (QuantiGlo; R&D Systems, Minneapolis, MN). Plasma and urinary thiobarbituric acid–reactive substance (TBARS) concentrations were determined by measuring the reaction of malondialdehyde with thiobarbituric acid (OXItek; ZeptoMetrix Corporation, Buffalo, NY). Urinary H_2O_2 excretion was determined using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Eugene, OR). Plasma 8-isoprostane levels were determined using enzyme immunoassay (Cayman Chemical, Ann Arbor, MI). The urinary microalbumin level was measured using a competitive ELISA (Nephrat; Exocell, Philadelphia, PA).

Statistical Analysis. Two-factor analysis of variance with repeated measures was used to evaluate tail-cuff pressure and excretion data. *Post hoc* contrasts were used to compare individual means using Bonferroni correction. One-way analysis of variance was used to compare data obtained from analysis of plasma taken at the end of the experiment. Values are reported as means ± SEM. $P < 0.05$ was considered statistically significant.

Results

Before the start of the HS diet, male and female rats had similar water intake (data not shown). Water intake was increased in male and female rats in response to a HS diet, although the increase in water intake was greater in male rats. Tempol treatment did not affect water or food intake in male or female rats.

As previously shown (6), a HS diet resulted in an increase in systolic arterial pressure in male and female sl/sl rats but not in wt rats (Fig. 1). The increase in pressure was greater in female sl/sl rats compared with male sl/sl rats. In both genders, treatment with tempol delayed the rise in arterial pressure in response to a HS diet and abolished the gender difference. Tempol did not, however, block a rise in pressure. Following 14 days of a HS diet, male and female sl/sl rats were recovering from the blood pressure–lowering effects of tempol.

Microalbumin excretion was measured as an index of renal injury. In male sl/sl rats, excretion of microalbumin was elevated following 14 days of a HS diet, and this increase was blocked by treatment with tempol. Neither HS nor tempol treatment altered microalbumin excretion in wt males. Similarly, microalbumin excretion was elevated following 14 days of a HS diet in female sl/sl rats. Treatment with tempol, however, had no effect on microalbumin excretion. In all groups studied, male rats had more microalbumin excretion compared with female rats at baseline (Fig. 2).

Plasma ET levels were greater in sl/sl rats, male and female, compared with wt rats (Fig. 3). Treatment with tempol had no effect on plasma ET levels in male rats but lowered ET levels in female sl/sl rats. A HS diet increased urinary ET excretion irrespective of genotype and gender (Table). The increase was more pronounced in wt males compared with sl/sl males. Tempol did not alter ET excretion in any group.

The SOD mimetic tempol was used in this study to determine if increased production of superoxide could account for the observed gender difference in response to a HS diet in sl/sl rats. Tempol was given in the drinking water and, given its ability to penetrate cell membranes, would be expected to affect total body oxidative stress. Oxidative stress was assessed using plasma levels and urinary excretion rates for TBARS, a measure of lipid peroxidation and a nonspecific indicator of oxidative stress, plasma 8-isoprostane, and urinary excretion of H_2O_2. Plasma TBARS and 8-isoprostane concentrations (data not shown) and urinary TBARS measurements (Table 1) were comparable with respect to genotype and gender; tempol had no effect on these measurements. H_2O_2 excretion was increased in response to a HS diet in wt and sl/sl male and female rats. The increase in H_2O_2 excretion was greater in sl/sl males compared with wt males. Treatment with tempol decreased H_2O_2 excretion in sl/sl rats only. Among females, there was no difference with regard to genotype in the

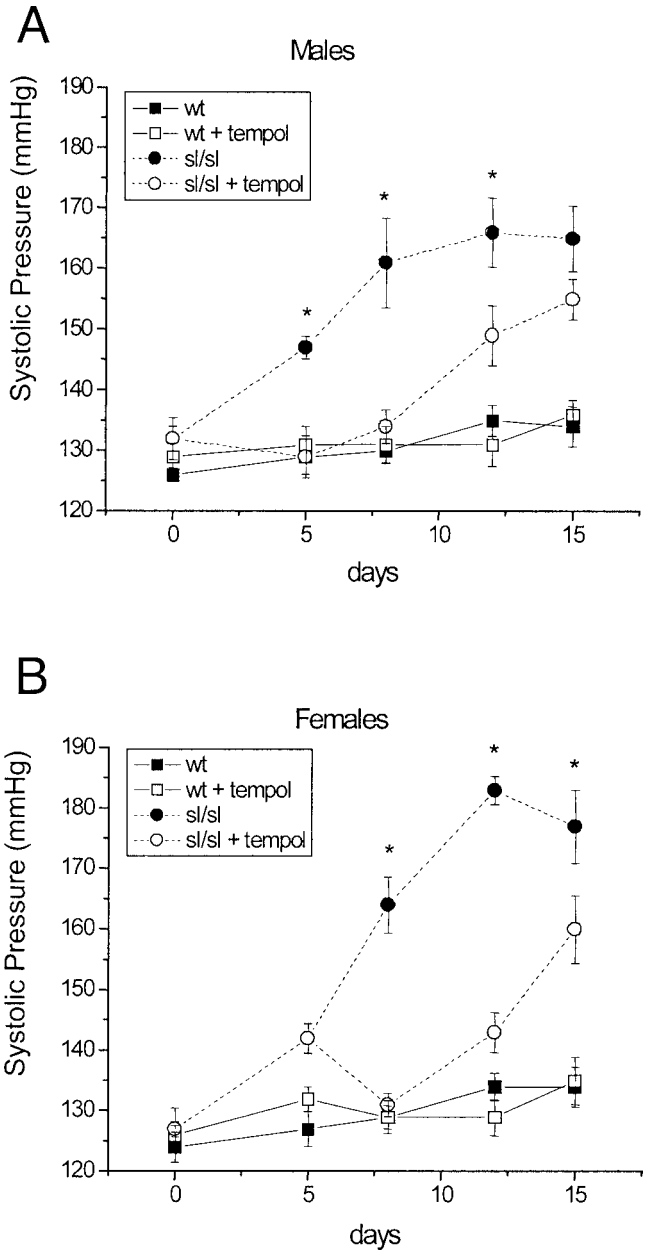

Figure 1. Systolic arterial pressure in male (A) and female (B) rats (n = 6–7 for males and 8–13 for females). A HS diet induced a greater increase in systolic arterial pressure in female compared with male sl/sl rats. Tempol attenuated the development of hypertension in male and female rats. *Significant difference from sl/sl + tempol on the same day.

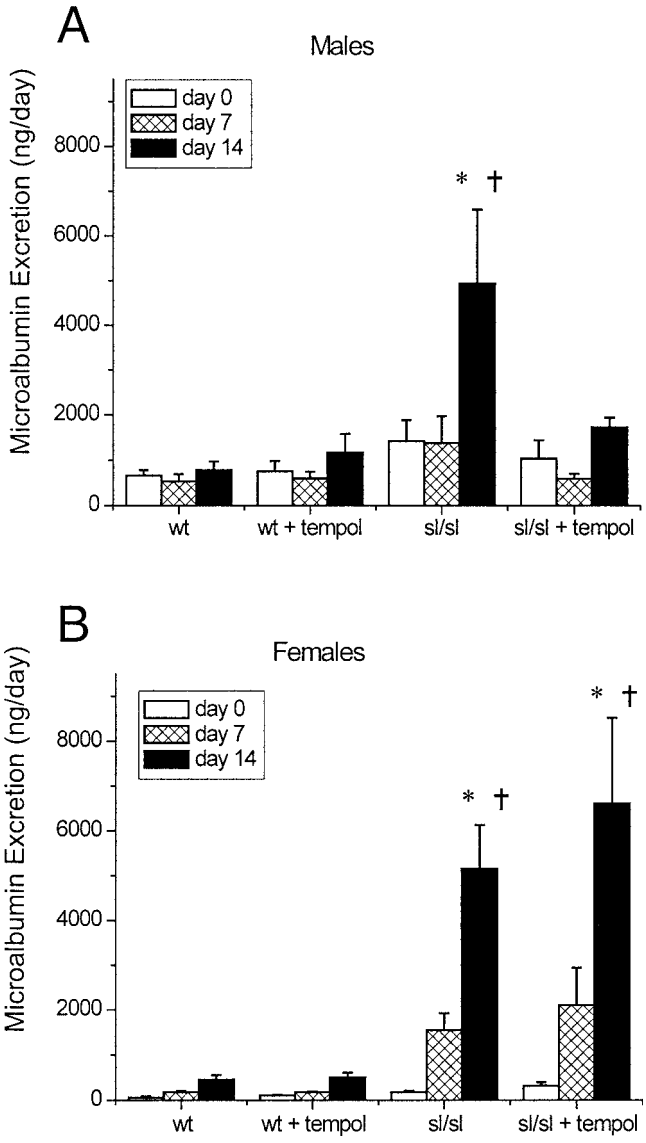

Figure 2. Microalbumin excretion in male (A) and female (B) rats (n= 6–8 for males and 5–9 for females). A HS diet increased urinary microalbumin excretion in male and female sl/sl rats but not wt rats. Tempol blocked the increase in microalbumin excretion in male but not female sl/sl rats. *Significant difference from Day 0. †Significant difference from wt on the same day.

increase in H_2O_2 excretion; however, similar to males, tempol reduced H_2O_2 in female sl/sl rats only.

Discussion

The ET_B receptor–deficient rat has elevated plasma levels of ET-1 and is a model of salt-sensitive hypertension that can be prevented by ET_A receptor blockade (4, 5). In the past few years, accumulating evidence has indicated that ET-1 can stimulate increases in oxidative stress, particularly superoxide (11, 12, 17, 18). In contrast to most models of hypertension, female ET_B receptor–deficient rats develop more severe hypertension in response to a HS diet compared with male rats (6). Therefore, the present study addressed two primary questions: first, whether salt-dependent hypertension in the ET_B receptor–deficient rat was dependent on superoxide and, second, whether the gender difference in the hypertension in this model could be due to differences in superoxide activity. Treatment with tempol blunted the development of salt-dependent hypertension in male and female ET_B receptor–deficient rats. This finding supports a role for superoxide to contribute to the development of salt-dependent hypertension in ET_B receptor–deficient rats.

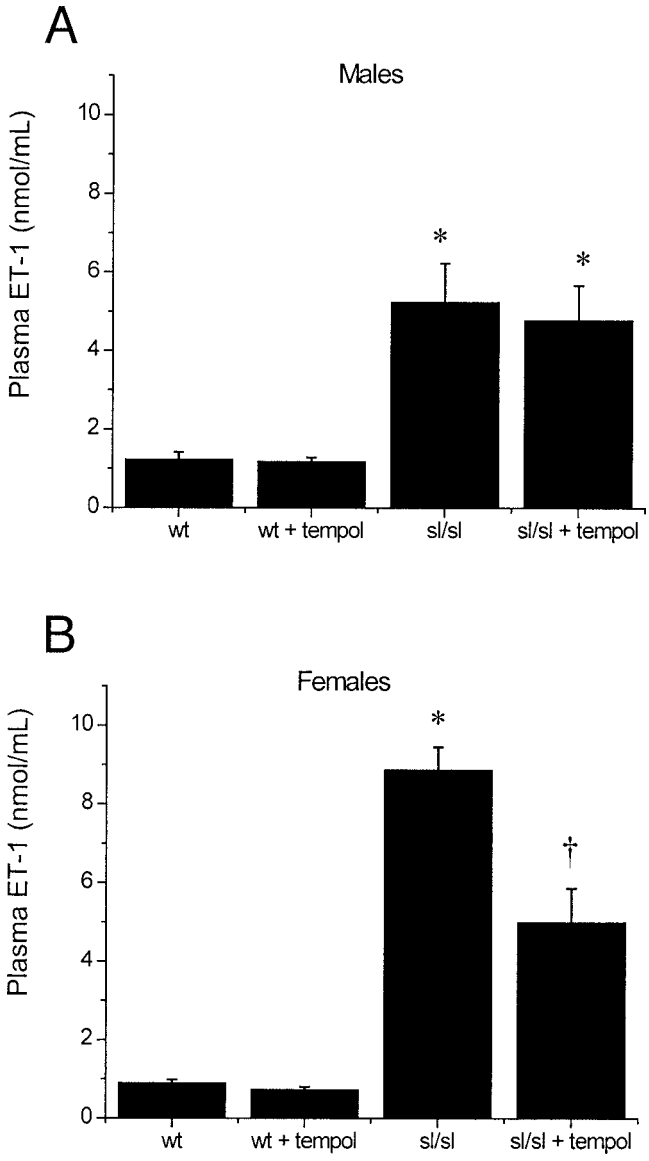

Figure 3. Plasma ET-1 levels in male (A) and female (B) rats ($n = 6$–8 for males and 7–13 for females). Plasma ET-1 levels were significantly elevated in male and female sl/sl rats compared with wt controls and were higher in female sl/sl compared with male. Tempol reduced ET-1 levels in female sl/sl rats. *Significant difference from wt. †Significant difference from sl/sl.

Furthermore, these data support the hypothesis that female sl/sl rats produce more superoxide or are more sensitive to the blood pressure–raising effects of superoxide compared with males.

In male and female sl/sl rats, tempol severely blunted the development of salt-induced hypertension, abolishing the gender difference in blood pressure. In both sexes, there appeared to be an "escape" from the blood pressure–lowering effects of tempol toward the end of the second week of treatment. This phenomenon was similar to previous observations in Sprague-Dawley rats in which hypertension was produced by chronic ET_B receptor blockade and a HS diet (8). In contrast to the present study, it was previously observed that tempol increased H_2O_2 levels following chronic ET_B receptor blockade and a HS diet (8). Tempol is an SOD mimetic and as such can increase the generation of H_2O_2 derived from superoxide. Antioxidants such as catalase and glutathione peroxidase can then convert H_2O_2 to water. With short-term ET_B receptor blockade, this antioxidant capacity may be overwhelmed, resulting in increased H_2O_2 levels. In contrast, a decrease in H_2O_2 in sl/sl rats may be the result of an increase in antioxidant potential to catalyze the breakdown of H_2O_2 that may be established as the ET_B receptor–deficient rats grow and develop. Future studies will need to investigate the level of antioxidant capacity during genetic and pharmacologic disruption of ET_B receptor function.

Measures of oxidative stress in the present study, however, did not reveal a clear gender difference in the degree of oxidative stress. It was somewhat surprising that sl/sl rats of either sex did not have any greater degree of oxidative stress compared with wt control rats, but this could be due to the indirect nature of these measurements or, as already mentioned, an upregulation of antioxidant mechanisms in this strain. Moreover, there was no direct evidence of tempol lowering measures of oxidative stress; yet, tempol treatment delayed the rise in blood pressure in male and female sl/sl rats. It is possible that the blood pressure–lowering effects of tempol are occurring independent of lowering superoxide levels. However, we find a more likely explanation is that tempol lowers local superoxide levels in the kidney or in peripheral nerves that would not result in changes in plasma reactive oxygen species, allowing for better blood pressure control. These findings unfortunately reflect the reality that all of the current means of assessing superoxide *in vivo* have severe limitations.

The lack of a gender difference in blood pressure during tempol treatment in ET_B receptor–deficient rats on a HS diet is consistent with the hypothesis that superoxide contributes to the salt-induced hypertension in this model and that the higher pressures seen in the female rats can be accounted for by superoxide. As previously reported, female ET_B receptor–deficient rats not only have an exaggerated salt-dependent hypertension compared with male rats, but this is associated with higher levels of circulating ET-1 compared with males (6). Given that ET_A receptor blockade can normalize blood pressure in this model (5), it is reasonable to postulate that the elevated ET-1 in female rats accounts for the elevated arterial pressure. ET_A receptor blockade can prevent or reverse the hypertension produced by chronic ET_B receptor blockade (7, 19). The mechanism for why female rats on a HS diet have higher ET-1 levels is not clear but may be related to increased oxidative stress. For the most part, ET-1 levels appear to be highly sensitive to the activity of ET_B receptors, which have even been referred to as clearance receptors. Because neither female or male sl/sl rats have functional ET_B receptors within the circulation, differences in ET_B receptor function cannot

Table 1. Urinary Excretion of Endothelin, TBARS, and H_2O_2 in wt and sl/sl Rats With and Without Tempol Treatment[a]

Excretion	Male Rats(n = 6–8)			Female Rats(n = 5–9)		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
ET excretion (fmol/day)						
wt	538 ± 112	1702 ± 164[b]	1851 ± 98[b]	549 ± 35	938 ± 64[b]	1164 ± 43[b]
wt + Tempol	479 ± 81	1886 ± 152[b]	1809 ± 134[b]	512 ± 44	949 ± 60[b]	1174 ± 67[b]
sl/sl	544 ± 57	1045 ± 92[b,c]	1267 ± 104[b,c]	622 ± 45	823 ± 92[b]	1314 ± 116[b]
sl/sl + Tempol	532 ± 85	1139 ± 126[b]	1572 ± 99[b]	560 ± 51	856 ± 94[b]	1169 ± 101[b]
TBARS excretion (nmol/day)						
wt	193 ± 9	236 ± 38	187 ± 15	224 ± 11	147 ± 19	144 ± 25
wt + Tempol	163 ± 13	187 ± 27	167 ± 24	212 ± 13	139 ± 24	214 ± 25
sl/sl	246 ± 11	171 ± 50	207 ± 55	240 ± 26	78 ± 12	233 ± 37
sl/sl + Tempol	193 ± 14	49 ± 10	135 ± 23	220 ± 13	115 ± 20	246 ± 52
H_2O_2 excretion (nmol/day)						
wt	b/d	44 ± 7	103 ± 20[b]	b/d	84 ± 19[b]	67 ± 14[b]
wt + Tempol	b/d	54 ± 11	67 ± 6[b]	b/d	67 ± 4[b]	57 ± 10[b]
sl/sl	b/d	110 ± 20[b,c]	81 ± 25[b]	5 ± 2	59 ± 10[b]	59 ± 17[b]
sl/sl + Tempol	b/d	37 ± 8	52 ± 12	7 ± 2	46 ± 13	41 ± 11

[a] Values are means ± SEM. b/d, below detection.
[b] Indicates significant difference from Day 0.
[c] Indicates significant difference from wt on the same day.

account for these differences. It would appear that a HS diet stimulates more ET-1 production in females compared with males, although the mechanism of how a HS diet increases ET-1 production or activity is not known. Following tempol treatment, plasma ET-1 levels in female sl/sl rats are decreased to the level seen in male sl/sl rats, regardless of tempol treatment. Although oxidative stress is implicated in the rise in plasma ET-1 in female sl/sl rats, the mechanism by which this is occurring is unknown.

Finally, male and female sl/sl rats, but not wt rats, had elevated microalbumin excretion when placed on a HS diet, indicating some degree of hypertension-induced renal injury. Tempol prevented microalbuminuria only in the male rats, indicating that the injury in female rats may not be dependent on superoxide. However, it is possible that a more severe degree of oxidative stress–induced injury is more difficult to overcome with tempol treatment in the female rats. A more thorough investigation of renal injury in this model is warranted.

In conclusion, results from our study indicate that the early stages of salt-dependent hypertension produced by ET_B receptor deficiency are dependent on superoxide. The elevated pressure in the female rats may be due to elevated circulating levels of ET-1.

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