Increasing oxidative stress with molsidomine increases blood pressure in genetically hypertensive rats but not normotensive controls

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Fortepiani, Lourdes A., and Jane F. Reckelhoff. Increasing oxidative stress with molsidomine increases blood pressure in genetically hypertensive rats but not normotensive controls. Am J Physiol Regul Integr Comp Physiol 289: R763–R770, 2005. First published May 19, 2005; doi:10.1152/ajpregu.00526.2004.—Spontaneously hypertensive rats (SHR) have a higher level of oxidative stress and exhibit a greater depressor response to a superoxide scavenger, tem- pol, than normotensive Wistar-Kyoto rats (WKY). This study determined whether an increase in oxidative stress with a superoxide/NO donor, molsidomine, would amplify the blood pressure in SHR. Male SHR and WKY were given molsidomine (30 mg·kg\(^{-1}\)·day\(^{-1}\)) or vehicle (0.01% ethanol) for 1 wk, and blood pressure, renal hemodynamics, nitrate and nitrite excretion (NO\(_x\)), renal superoxide production, and expression of renal antioxidant enzymes, Mn- and Cu,Zn-SOD, catalase, and glutathione peroxidase (Gpx), were measured. Renal superoxide and NO\(_x\) were higher in control SHR than in WKY. Molsidomine increased superoxide by ~35% and NO\(_x\) by 250% in both SHR and WKY. Mean arterial blood pressure (MAP) was also higher in control SHR than WKY. Molsidomine increased MAP by 14% and caused renal vasoconstriction in SHR but reduced MAP by 16%, with no effect on renal hemodynamics, in WKY. Renal expres- sion of Mn- and Cu,Zn-SOD was not different between SHR and WKY, but expression of catalase and Gpx were ~30% lower in kidney of SHR than WKY. The levels of Mn- and Cu,Zn-SOD were not increased with molsidomine in either WKY or SHR. Renal catalase and Gpx expression was increased by 300–400% with molsidomine in WKY, but there was no effect in SHR. Increasing oxidative stress elevated blood pressure further in SHR but not WKY. WKY are likely protected because of higher bioavailable levels of NO and the ability to upregulate catalase and Gpx.

Asexual dimorphism; glomerular filtration rate; catalase; glutathione peroxidase

In human studies, higher blood pressure has been shown to be associated with increased oxidative stress. Lacy and colleagues (14) reported that hydrogen peroxide was increased in plasma of hypertensive individuals compared with age-matched control subjects. However, treatment of hypertensive individuals with antioxidants has not been entirely successful in reducing blood pressure (6, 8, 13).

The spontaneously hypertensive rat (SHR) is a genetic model of essential hypertension that exhibits elevated oxidative stress that plays a role in the development and maintenance of hypertension (25). Renal and urinary F\(_2\)-isoprostanes, an indicator of oxidative stress, are higher in SHR than in Wistar-Kyoto rats (WKY), and we (4, 5) and others (25) have shown that antioxidant treatment of SHR reduces their blood pressure.

Because we showed previously that reducing oxidative stress with a SOD mimetic lowers blood pressure in SHR (4), we wanted to further evaluate the impact of increasing oxidative stress in models of both hypertensive and normotensive rats. In the present study, the hypothesis was tested that an additional increase in oxidative stress would further increase blood pressure to higher levels than found in control SHR. In contrast, we anticipated that an increase in oxidative stress in WKY would not adversely affect the blood pressure because of compensatory mechanisms that would be able to metabolize the reactive oxygen species (ROS). This study also allowed us to determine the effect of increasing ROS on expression of downstream antioxidant enzymes, Mn- and Cu,Zn-SOD, cata- lase, and glutathione peroxidase. To test these hypotheses, male SHR and WKY were given molsidomine for 1 wk, and blood pressure and renal hemodynamics were measured. Molsidomine is converted to 3-morpholinosydnonimine (SIN-1) on passage through the liver and degenerates into both superoxide and NO and perhaps peroxynitrite (26). To verify that the drug had similar effects on oxidative stress and NO production in both SHR and WKY, renal superoxide production and urinary excretion of the NO metabolites nitrate and nitrite were measured. In addition, the effect of molsidomine on nitrotyrosi- nated proteins in the kidney was evaluated.

METHODS

Rats. Male SHR and WKY were obtained from Taconic Farms (Germantown, NY). The rats were maintained on standard rat chow (Teklad, Harlan SD, Indianapolis, IN) and tap water unless otherwise stated, in an environment with a 12:12-h light-dark cycle. The rats were studied at 17–19 wk of age. The protocols complied with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Missis- sippi Medical Center.

Treatment of rats with molsidomine. Molsidomine was given in drinking water containing 0.01% ethanol for 1 wk. Control rats received the vehicle. Rats were weighed daily, and the dose was adjusted so that the rats received 30 mg·kg\(^{-1}\)·day\(^{-1}\).

Measurement of anesthetized arterial blood pressure and renal hemodynamics. SHR and WKY (\(n = 8–10\) group) were anesthetized with the thiobarbiturate Inactin (110 mg·kg\(^{-1}\); RBI, Natick, MA) and placed on a temperature-regulated surgery table. Tracheostomy was performed, and catheters were placed: femoral arterial for continuous monitoring of blood pressure and blood sampling, femoral venous for infusion of isoncotic artificial rat plasma (2.5 g/dl bovine immunoglobulin, 2.5 g/dl bovine serum albumin in Ringer solution) at 12.5 ml·kg\(^{-1}\)·h\(^{-1}\) for 45 min during the preparatory surgery and thereafter

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at 1.5 ml·kg⁻¹·h⁻¹ throughout the experiment to maintain a euvolmic preparation (11), and left jugular venous (for infusion of 0.9% saline with or without [³H]inulin; 15–20 μCi/ml at 1.5 ml/h; New England Nuclear, Wilmington, DE).

After the 50-min equilibration period for the [³H]inulin, two 30-min urine collections with midpoint femoral arterial blood samples were taken. After the final period, before the [³H]inulin was stopped, a sample of femoral arterial blood was taken, and after a midline abdominal incision, the left renal vein was cannulated in the retrograde position with a 23-gauge needle connected to PE-50 tubing for renal venous blood sampling, to be used for calculation of renal plasma flow, as previously described (21, 23). The kidneys were perfused free of blood in situ with PBS containing 2% heparin and were removed, weighed, and either snap-frozen in liquid nitrogen for Western blot analyses or used to measure renal superoxide.

Measurement of conscious blood pressure in SHR. To verify the pressor effect we observed with molsidomine in anesthetized rats, we treated male SHR (n = 5/group) with molsidomine or vehicle for 1 wk, as described above. On day 4 of the treatment, a catheter was placed in the femoral artery. On day 7, blood pressure was measured by Power Lab (ADInstruments) and averaged over 4 h in conscious, freely moving, chronically instrumented rats that were resting quietly in their home cages.

Measurement of urinary nitrate/nitrite. Nitrate and nitrite concentrations in 24-h urine samples were measured by the Griess reagent method, using *Escherichia coli* as the nitrate reductase enzyme to convert nitrate to nitrite, as we described previously (22). The data are presented as nitrate/nitrite excreted (NOx) per day per kilogram of body weight because WKY are larger than SHR.

Measurement of superoxide in kidney homogenates by lucigenin chemiluminescence. Superoxide was measured by a method modified from Park et al. (20). Because the kidney is known to be involved in the control of blood pressure (9), superoxide production was measured in kidney tissue of the rats as an index of basal superoxide production in controls and as molsidomine-stimulated production of superoxide in the experimental groups. Briefly, kidneys were homogenized (1:8 wt/vol) in RIPA buffer [PBS, 1% Nonidet P-40 or Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail (Sigma)] with a Polytron (PT10–35). The samples were centrifuged at 1,800 rpm for 10 min at 4°C. For measurement of superoxide, the assay included supernatant (100 μl; ~1.5 mg protein) added to PBS (350 μl) plus lucigenin (50 μl; final concentration 5 μM). The samples were allowed to equilibrate for 3–5 min in the dark, and then luminescence was measured every 1.8 s for 5 min with a luminometer (Berthold, Bad Wildbad, Germany). Luminescence was recorded as relative light units (RLU) per second. A buffer blank containing lucigenin alone was subtracted, and the results are expressed as RLU per second per milligram of protein in the kidney homogenate. Protein concentrations in the kidney homogenates were determined by the method of Lowry et al. (16).

Western blot analyses of Mn-, and Cu,Zn-SOD, glutathione peroxidase, catalase, and nitrotyrosinated proteins. Proteins in kidney homogenates (25–125 μg) from control and molsidomine-treated rats were separated by SDS-PAGE and electrophoretically transferred to PVDF membranes. Membranes were probed with antibodies to anti-Mn-SOD, anti-Cu,Zn-SOD, anti-glutathione peroxidase, or anti-catalase antibodies (BioDesign) and detected with rabbit anti-sheep secondary antibodies. For nitrotyrosinated protein detection, 100 μg of kidney homogenate was separated on SDS-PAGE and electrophoretically transferred to PVDF membranes. Membranes were probed with rabbit anti-nitrotyrosine polyclonal antibody (Cayman Chemical) and detected with goat anti-rabbit secondary antibodies. Bands were detected by ECL Plus (Amersham) and quantified by densitometry. Membranes were stripped and reprobed with primary antibodies. Membranes were then electrophoretically blotted onto polyvinylidene difluoride (PVDF) membranes. Membranes were probed with primary antibodies and detected with secondary antibodies. Bands were detected by ECL Plus (Amersham) and quantified by densitometry.

Statistical analyses. Statistical differences for all data were determined by ANOVA and Dunnett’s test (3). Data are expressed as means ± SE.

RESULTS

Male SHR were considerably smaller than age-matched male WKY (SHR: control 334.9 ± 6.1 g, molsidomine 321.9 ± 6.1 g; WKY: control 498.3 ± 13.3 g, molsidomine: 494.4 ± 10.6 g; P < 0.01 SHR vs. WKY for either treatment group). Kidney weights were also lower in SHR than in WKY (SHR: control 13.3 ± 0.11 g; molsidomine 13.3 ± 0.11 g; P < 0.01 SHR vs. WKY for either treatment group).

Effect of molsidomine on renal superoxide production. Molsidomine can dissociate into both superoxide and NO. Therefore, we measured renal superoxide, using a lucigenin chemiluminescence assay. As shown in Fig. 1, renal superoxide was 24% higher in control SHR than WKY, and molsidomine

![Fig. 1. Molsidomine increased renal superoxide in male spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY). Rats were given molsidomine for 1 wk as described in METHODS. Superoxide production was measured by lucigenin chemiluminescence. Data are presented as relative light units (RLU)/mg protein in kidney homogenate. *P < 0.05 compared with control WKY; †P < 0.01 compared with control of same strain; ‡ P < 0.01 compared with molsidomine-treated WKY.](http://ajpregu.physiology.org/)
increased superoxide similarly in male SHR and WKY, suggesting that the increase in renal superoxide produced with molsidomine was similar between the groups.

Effect of molsidomine on NOx. As shown in Fig. 2, NOx was slightly higher in control SHR than WKY, and molsidomine increased NOx in both groups by similar amounts (2.4- to 2.7-fold), suggesting that the level of NO produced by molsidomine was similar between the groups.

Effect of molsidomine on mean arterial blood pressure. Mean arterial blood pressure (MAP) in anesthetized rats was significantly higher in control SHR than WKY (Fig. 3). With molsidomine treatment, MAP increased by 14% in SHR but was reduced by 16% in WKY. To verify the effects of molsidomine on blood pressure that we obtained in anesthetized SHR, blood pressure was also measured in conscious, chronically catheterized SHR. In conscious SHR, MAP was 166 ± 3 mmHg in controls, and with molsidomine MAP increased to 184 ± 6 mmHg (P < 0.01), an 11% increase.

Effect of molsidomine on renal hemodynamics. As shown in Fig. 4, A–C, respectively, glomerular filtration rate (GFR), renal plasma flow (RPF), and renal vascular resistance (RVR) were similar between control SHR and WKY. Molsidomine reduced GFR and RPF and increased RVR in SHR compared with controls. In contrast, molsidomine had no effect on renal hemodynamics in WKY.

Effect of molsidomine on renal expression of antioxidant enzymes. Expression of Mn- and Cu,Zn-SOD, catalase, and glutathione peroxidase in kidney tissue was measured by Western blot analyses. There was no difference in the basal levels of renal Mn-SOD or Cu,Zn-SOD expression between WKY and SHR, and molsidomine had no effect in either strain (see Fig. 5). Expression of both catalase and glutathione peroxidase was higher in kidneys of WKY than SHR, as shown by the differences in scales of the densitometric units in Fig. 6, A and B. With molsidomine treatment, catalase and glutathione peroxidase expression increased by three- to fourfold in kidneys of WKY rats, but molsidomine had no effect on expression of the enzymes in kidneys of SHR.

Effect of molsidomine on renal expression of nitrotyrosinated proteins. Nitrotyrosinated proteins were identified by Western blot analyses using a polyclonal antibody. Six rats from each group were compared. As can be seen in Fig. 7, the
major nitrotyrosinated band in control WKY and SHR occurred at 84 kDa. The expression of the 84-kDa band tended to be higher in SHR but not statistically significantly (see Fig. 7B). With molsidomine, there was a tendency for the expression to increase in both WKY and SHR, but again, not statistically significantly. There was a second band at 75 kDa whose expression was increased by threefold in control SHR compared with WKY, and molsidomine caused 8-fold and 10-fold increases in expression in WKY and SHR, respectively.

**DISCUSSION**

In the present study, a 30% increase in superoxide produced by treatment with molsidomine caused an increase in blood
pressure and renal vasoconstriction in male SHR. In contrast, a similar increase in superoxide in WKY with molsidomine reduced MAP in WKY and had no effect on renal hemodynamics. The data support our hypothesis that increasing oxidative stress in male SHR causes a further increase in blood pressure compared with controls. These data also support the contention that blood pressure in SHR can be modulated by the level of oxidative stress.

In the present study we found that the ability of the kidney of SHR to produce superoxide in the basal state was higher than WKY but increased by a similar percentage (31–37%) with molsidomine in both strains. However, we found that the expression of Mn-SOD and Cu,Zn-SOD was similar in control WKY and SHR, as shown recently by Adler and Huang (1), and was not changed with molsidomine. This is not unexpected because not all enzymes upregulate their expression in response to an increase in substrate. However, Gomez and colleagues (7) reported that increasing oxidative stress with aluminum treatment caused an upregulation of Cu,Zn-SOD in the hippocampus of normotensive rats. It is not clear then why renal Cu,Zn-SOD was not upregulated in molsidomine-treated SHR and WKY in our study.

In contrast to the SODs, the expression of glutathione peroxidase and catalase were 30% lower in SHR than WKY in the basal state, which suggests that the SHR were already at an antioxidant disadvantage compared with WKY in handling ROS in the control setting. With the increase in oxidative stress due to molsidomine, the protein expression of glutathione peroxidase and catalase enzymes in the kidney was upregulated further in WKY, but there was no change in renal expression of the antioxidant enzymes in SHR. These data suggest that the upregulation of the antioxidant enzymes could have been a compensatory mechanism for the increased superoxide and subsequent hydrogen peroxide produced by molsidomine in WKY. Gomez and colleagues (7) also found that catalase and glutathione peroxidase were upregulated with increased oxidative stress in normotensive rats. The upregulation of the antioxidant enzymes glutathione peroxidase and catalase likely played a role in protecting the WKY from increases in blood pressure. In support of this contention, Makino and colleagues (18) reported that blockade of SOD in the medulla of normotensive rats was able to increase their blood pressure. Treatment with the superoxide scavenger tempol reduced the blood pressure somewhat, but only when catalase was given in addition to tempol was the blood pressure normalized.

It was shown previously that tempol, a superoxide scavenger, reduces blood pressure in male SHR but has no effect in WKY (25). These data suggested that there is a basal level of superoxide present in WKY that does not impact their blood pressure. In the present study, despite the fact that molsidomine was able to increase superoxide production in the kidney in WKY to levels similar to the basal levels in SHR, there was no increase in their blood pressure, suggesting that the increase in oxidative stress was not sufficient to cause an increase in blood pressure. The WKY is thought to be a model of higher NO production compared with the SHR. It is likely then that the increase in superoxide with molsidomine was not able to overwhelm the NO production in the WKY and lead to increases in blood pressure. However, as Makino and colleagues were able to produce hypertension in normotensive rats with inhibition of SOD, these data suggest that there is a threshold of ROS that must be exceeded to increase blood pressure. This hypothesis is consistent with studies by Kristek and colleagues (12), who found that high doses of molsidomine for 6 wk did cause an increase in blood pressure in WKY.

The mechanism(s) by which oxidative stress increases blood pressure in SHR has not been completely elucidated. In the present study, we also measured nitrotyrosinated proteins in kidney homogenates from control and molsidomine-treated rats and found not only that the concentrations of nitrotyrosinated proteins were higher in SHR but that different proteins were nitrotyrosinated in molsidomine-treated SHRs and WKY. Nitrotyrosination of enzymes can lead to either activation or inactivation of the proteins. It is possible that in the SHR, different proteins, such as enzymes necessary for synthesis of vasoconstrictors and vasodilators, are activated and inactivated differently than in WKY (24), leading to the differential pressor responses to molsidomine. In addition, the fact that control SHR had a higher level of nitrotyrosinated proteins suggests that what NO is produced in the kidney is coupled to

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**Fig. 5.** There were no differences in renal expression of Mn-SOD (A) or Cu,Zn-SOD (B) in control WKY and SHR, nor did molsidomine change expression in either rat strain. +, Positive control. Data are expressed as densitometric units factored for expression of actin as control.
superoxide to produce peroxynitrite and thus loss of bioavailable NO contributes to the increase in blood pressure.

In the past, molsidomine, or its metabolite SIN-1, has been given as a vasodilator to patients with stable angina or congestive heart failure (15, 19). Molsidomine also causes flow-dependent vasodilation in brachial arteries of individuals with coronary artery disease (2). When molsidomine was given by mouth to normal male subjects aged 26–35 yr, it caused a significant reduction in blood pressure similar to isosorbide dinitrate and was more efficacious in lowering blood pressure than L-arginine given intravenously (17). In all of these studies, molsidomine or SIN-1 was given acutely. In one chronic study, molsidomine was given for 6 mo to patients who had undergone angioplasty to evaluate whether molsidomine could prevent restenosis, which it did not (27). However, in this study, the blood pressure of the patients was not recorded. Taking into consideration our present data in which molsidomine further increased blood pressure in hypertensive rats, the chronic use of molsidomine as a vasodilator in individuals who have elevated blood pressure associated with increased oxidative stress should be reevaluated.

In summary, chronic treatment for 1 wk with molsidomine, which increases oxidative stress, resulted in an increase in blood pressure and renal vasoconstriction in SHR but caused a reduction in blood pressure and no change in renal hemodynamics in WKY. Mn- and Cu,Zn-SOD expression were similar in control SHR and WKY, but glutathione peroxidase and catalase expression were 30% lower in SHR than WKY. In

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**Fig. 6.** Effect of molsidomine on renal expression of catalase and glutathione peroxidase (GPx) in WKY (A) and SHR (B). Data are expressed as densitometric units factored for expression of actin as control. *P < 0.01 compared with control.
response to molsidomine, renal expression of Mn- and Cu,Zn-SOD was not changed in either WKY or SHR, but expression of glutathione peroxidase and catalase was increased in WKY but not SHR. These data suggest that there is a compensatory upregulation of the antioxidant enzymes in response to increased ROS, and this may have played a role in protecting WKY from the oxidative stress that increased blood pressure in SHR. Furthermore, it is likely that there was more bioavailable NO in WKY than in SHR in control conditions and in response to molsidomine that also contributed to their respective blood pressure responses.

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