Lack of Hemodynamic Effects After Extended Heme Synthesis Inhibition by Succinylacetone in Rats

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Received October 23, 2009; accepted January 12, 2010

ABSTRACT

Hypertyrosinemia (HT) is a life-threatening condition caused in large part by the buildup of tyrosine metabolites and their derivatives. One such metabolite is succinylacetone (SA), a potent irreversible inhibitor of heme biosynthesis. Heme is a key component of numerous enzymes involved in arterial blood pressure (BP) regulation, including nitric-oxide synthase (NOS) and its downstream mediator soluble guanylyl cyclase (sGC). Because NOS and sGC are important regulators of cardiovascular function, we hypothesized that inhibition of heme supply to these enzymes by SA would result in the induction of a measurable hypertensive response. Male Sprague-Dawley rats were treated with SA (80 mg·kg⁻¹·day⁻¹ i.p.) for 14 days, resulting in a marked increase in urinary SA and δ-aminolevulinic acid (P < 0.001 for both parameters) and decreased heme concentrations in kidney, liver, spleen, and vascular tissues (P < 0.05 for all parameters). After SA treatment, systemic nitrite/nitrate excretion was reduced by 72% (P < 0.001), and renal NOS and sGC activities were decreased by 32% (P < 0.05) and 38% (P < 0.01), respectively. SA administration also compromised the ex vivo sensitivity of aorta to endothelium-dependent and -independent vasodilation. Despite these effects, SA treatment failed to induce any changes in BP, as assessed by radiotelemetry. Moreover, BP profiles in the SA-treated animals were less responsive to altered sodium intake. The present results demonstrate that extended inhibition of heme synthesis with SA affects hemoenzyme function, albeit without consequent effects on BP regulation and sodium excretion.
prises a major dilatory mechanism for blood vessels (Moncada and Higgs, 2006). Moreover, renal NOS has been shown to be an important regulator of blood volume, and hence BP, by influencing sodium and fluid excretion (Zou and Cowley, 1999). NOS uses heme at its active site, where it converts l-arginine to l-citrulline, releasing nitric oxide (NO) as a coproduct. sGC contains a heme moiety as a regulatory prosthetic group, which is activated by NO binding.

On the basis of these underlying mechanisms, we hypothesized that conditions of heme insufficiency could lead to a hypertensive tendency. Indeed, it is well established that certain clinical conditions in which heme synthesis is disturbed are associated with elevated BP. For example, more than half of patients who have porphyria, a genetic disease in which one or more enzymes required for heme biosynthesis are impaired, are also hypertensive (Church et al., 1992; Andersson and Lithner, 1994). Similarly, in cases of lead poisoning, wherein lead toxicity disrupts heme biosynthesis, hypertension is a common outcome (Batuman et al., 1983; Pirkle et al., 1985).

Hypertension is a recognized outcome in HT, even in pediatric subjects (Strife et al., 1977; Gibbs et al., 1993; Kluger et al., 1997; Schlump et al., 2008), and remains an important diagnostic feature of the disease (Bonkovsky, 2005). We hypothesized that the accumulation of SA in type 1 HT may lead to cardiovascular hemoenzyme deficiency, thereby precipitating a hypertensive phenotype. Given the importance of renal mechanisms in establishing the long-term set point of BP (Cowley and Roman, 1996), we chose to focus on the kidney as a potential site of action for SA. The purpose of the present study was to examine whether extended heme depletion by SA impairs hemoenzyme function and alters BP regulation in vivo. We investigated the effects of a ≥14-day treatment with SA on 1) heme levels and hemoenzyme function in organs with a particular emphasis on the kidney and 2) BP and its responsiveness to alterations in sodium intake and NOS inhibition.

Methods and Materials

Animals and Treatments

Study 1: Heme Content, Hemoenzyme Activity, and Ex Vivo Assessment of Vascular Function Studies. Fourteen adolescent male Sprague-Dawley rats (150–175 g) were obtained from Charles River Canada (St-Constant, QC, Canada) and housed in Queen’s University Animal Care Facility, which maintained a 12-h/12-h light/dark cycle (transition at 7:00 AM and 7:00 PM). Rats had ad libitum access to water. Rats were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care, and the experimental protocols were approved by the Queen’s University Animal Care Committee. After 1 week of acclimatization, rats were injected twice daily with SA (40 mg · kg⁻¹ · i.p., at 10:00 AM and 10:00 PM) (n = 7) or saline (n = 7) for 14 days. On day 15, rats were anesthetized with sodium pentobarbital (60 mg · kg⁻¹ · i.p.), and blood was collected from the lower vena cava into Vacutainer tubes coated with EDTA or clot activator (BD Medical, Franklin Lakes, NJ), for hematological assessments (at Kingston General Hospital, Kingston, ON, Canada). Rats were then heparinized and perfused with ice-cold saline through the abdominal aorta until livers and kidneys were blanched. Kidneys, liver, spleen, and mesenteric vessels were excised, cleaned of extraneous connective tissue, weighed, frozen in liquid nitrogen, and stored at −80°C until processed. Aortas were also removed and used immediately to assess vascular function, as described below.

For urinary metabolites analysis, rats were placed in metabolic cages after 14 days of SA treatment, and urine was collected for 24 h. Urine samples were centrifuged at 1350g, and the supernatants were treated with 2-propanol (6.5% v/v) to prevent bacterial growth, frozen in liquid nitrogen, and stored at −80°C.

Study 2: Arterial Pressure Responsiveness to Altered Sodium Intake and NOS Inhibition. A separate cohort of 14 male Sprague-Dawley rats (150–175 g), after acclimatization, were surgically instrumented with radiotelemetric pressure transducers (TA11PA-C40; Data Sciences International, St. Paul, MN), as described previously (Bourque et al., 2008). Rats were then given 10 days to recover, after which time hemodynamic parameters were continuously monitored for 15 a every 5 min. After an initial 4-day baseline recording period, SA was administered twice daily (40 mg · kg⁻¹ · i.p., at 10:00 AM and 10:00 PM) via an indwelling catheter that was placed in the intraperitoneal cavity (to reduce stress responses associated with injections). SA treatment began when the rats were approximately 10 weeks of age. After the first 14 days of treatment with SA, manipulations of sodium intake began, consisting of a 4-day low-sodium treatment regimen, followed by a 5-day high-sodium treatment regimen. For the low-sodium regimen, rats had ad libitum access to a low-sodium (0.04% Na⁺) purified diet (Research Diets Inc., New Brunswick, NJ) based on the ALN-76A rodent diet and tap water. For the high-sodium treatment regimen, rats had ad libitum access to the standard grain-based rodent diet described above (0.4% Na⁺) and drinking water supplemented with 1% NaCl (w/v). After 5 days of high-sodium intake, the drinking water was supplemented with N-nitro-l-arginine methyl ester (l-NAM) in addition to the 1% NaCl, such that the animals ingested l-NAM at approximately 1 mg · kg⁻¹ · day⁻¹ for 3 days, 3 mg · kg⁻¹ · day⁻¹ for 3 days, and 30 mg · kg⁻¹ · day⁻¹ for 2 days. Body weights and food and water intake were monitored daily throughout these treatment protocols. Upon completion of the telemetry studies, rats were euthanized, and the radiotelemetric transducers were removed and verified for accuracy by using a sphygmomanometer.

Measurement of Heme Depletion and Hemoenzyme Activity

Rat tissues were homogenized in ice-cold 20 mM phosphate buffer (pH 7.4) with a Complete Mini, EDTA-free protease inhibitor tablet (Roche Diagnostics, Laval, QC, Canada). Samples were centrifuged for 10 min at 1350g to remove cellular debris, frozen in liquid nitrogen, and stored at −80°C until analyzed. Heme content in the liver, spleen, kidney, and mesenteric homogenates were assessed by using the fluorometric method of Morrison (1965).

Optimized in vitro activities of NOS were assessed in kidney homogenates by the radiometric assay of Kimura et al. (1996). sGC activity was assessed in kidney cytosolic fractions as described by Kinobe et al. (2006) using an enzyme immunoassay cGMP detection kit (Cayman Chemical, Ann Arbor, MI). For sodium nitroprusside (SNP)-induced sGC activity, kidney cytosolic fractions were preincubated with 100 μM SNP for 10 min. Urine nitrate levels were quantified by a nonenzymatic colorimetric NO assay kit (NB-88, Oxford Biomedical Research, Oxford, MI), which measures total nitrite content after converting nitrate to nitrite by metallic cadmium. Urinary 8-isoprostanes were measured by enzyme immunoassay using a kit (Cayman Chemical). Urinary SA was analyzed at the University of California at San Diego Biochemical Genetics Laboratory by gas chromatography-mass spectrometry. Urinary 5-ALA was analyzed at the Ottawa Hospital (Ottawa, ON, Canada) by colorimetric assay.

Ex Vivo Assessment of Vascular Function

Isolated rat aortas obtained from study 1 were used to evaluate concentration-dependent effects of phenylephrine (PE), acetylcholine (ACH), and methylamine hexamethylene methyamine NONOate
(MAHMA-NONOate). Thoracic aortas were cut into rings 4 mm in width and mounted on pressure transducers in 10-ml tissue baths containing Krebs bicarbonate solution aerated with 95% O₂-5% CO₂ at 37°C. Data were collected with a Powerlab data acquisition system (ADInstruments Ltd., Chalgrove, Oxfordshire, UK) and displayed by using Chart (version 5) software (ADInstruments Ltd.). Tissues were allowed to equilibrate at a resting tension of 9.5 mN for 1 h, with rinses every 15 min, before testing. PE concentration-response relationships were obtained by adding increasing doses of PE to the bath (100 pM to 30 μM, in approximately 3-fold increments). Aortic rings were then rinsed until they returned to baseline. For concentration-response relationships for ACh and MAHMA-NONOate, aortic rings were submaximally contracted (50–80%) with PE before administration of vasodilators. After steady precontraction, increasing concentrations (100 pM to 30 μM for MAHMA-NONOate, in approximate 3-fold increments) were then added to the tissue baths. Tissues were rinsed and allowed to return to baseline over a 30-min period between ACh and MAHMA NONOate testing.

Statistical Analyses

Biochemical data between control and SA-treated rats were analyzed by unpaired Student’s t test. Because urinary SA levels in the control group were below the limits of detection, values were obtained by imputation of the lowest limit of detection (10 mmol · mol⁻¹ creatinine). Changes in BP caused by sodium manipulations were also analyzed by Student’s t test for each treatment. For ex vivo assessment of vascular function, exclusion criteria were established before experimentation to ensure tissue viability; specifically, aortic rings that did not achieve an initial contraction of at least 19.6 mN or a minimum of 50% relaxation of the submaximal precontraction with ACh were deemed to be compromised and were therefore excluded from all analyses. Telemetry data were analyzed using a repeated measures two-way analysis of variance; where significant differences were found, Student’s t test with Bonferroni correction was conducted on data sets, as appropriate. Mean arterial pressure (MAP) profiles assessed by radiotelemetry are presented as the difference from the last day of baseline (before SA or vehicle treatment) to account for differences in baseline recordings between animals. Telemetry data presented herein were obtained between 10:00 PM and 4:00 AM, which corresponds to the night phase of the light cycle in the Queen’s Animal Care Facility and the period of greatest activity in rats. However, similar results were obtained for daytime recordings and when data were assessed as 24-h mean values (data not shown).

High- and low-sodium MAP values represent the highest and lowest 1-day average MAP measurement within the corresponding treatment period, respectively. Data points on the renal function curve were derived by plotting the maximum changes in MAP versus sodium consumed on high-sodium (upper point) and low-sodium (lower point) treatments; a separate slope was obtained for each animal and pooled to calculate group means. All data are presented as mean ± S.E.M. P < 0.05 was considered statistically significant.

### TABLE 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>SA-Treated</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine volume (ml 24 h⁻¹)</td>
<td>25.8 ± 1.7</td>
<td>19.9 ± 1.4</td>
<td>0.02</td>
</tr>
<tr>
<td>Urinary SA (mmol · mol⁻¹ creatinine)</td>
<td>N.D.</td>
<td>427.3 ± 72.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urinary d-ALA (mmol · mol⁻¹ creatinine)</td>
<td>5.7 ± 0.2</td>
<td>74.4 ± 17.4</td>
<td>0.002</td>
</tr>
<tr>
<td>Urinary 8-isoprostanes (μmol · mol⁻¹ creatinine)</td>
<td>0.65 ± 0.07</td>
<td>0.61 ± 0.03</td>
<td>0.56</td>
</tr>
<tr>
<td>Liver heme (mg · g⁻¹)</td>
<td>0.51 ± 0.11</td>
<td>0.19 ± 0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>Spleen heme (mg · g⁻¹)</td>
<td>23.5 ± 4.5</td>
<td>12.5 ± 1.8</td>
<td>0.04</td>
</tr>
<tr>
<td>Kidney heme (mg · g⁻¹)</td>
<td>0.45 ± 0.05</td>
<td>0.19 ± 0.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mesenteric vasculature heme (mg · g⁻¹)</td>
<td>0.029 ± 0.002</td>
<td>0.020 ± 0.001</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Control: n = 7; SA-treated: n = 7. N.D., not detectable.

### Results

**Depletion of Heme by SA (Study 1).** Fourteen-day treatment with 80 mg · kg⁻¹ · day⁻¹ SA resulted in an enhanced excretion of SA in the urine, as expected (P < 0.001; Table 1). To assess whether there was a functional effect of SA treatment, urinary d-ALA levels were analyzed and found to be 13-fold higher in the SA-treated group than controls (P < 0.01; Table 1). SA treatment also caused a significant decrease in heme content in all tissues analyzed; heme concentration was diminished in liver by 63% (P < 0.05), spleen by 47% (P < 0.05), kidney by 58% (P < 0.001), and mesenteric vessels by 31% (P < 0.05). SA treatment also caused 14, 16, and 7.5% decreases in hematocrit values, hemoglobin levels, and mean corpuscular volume, respectively (P < 0.001 for all parameters). All other hematological parameters assessed, including counts for erythrocytes, leukocytes, and platelets and indices of circulating iron levels, were not significantly different between SA- and vehicle-treated controls (Table 2).

SA-treated rats had 20% reduced urinary output over a 24-h period spent in metabolic cages (P < 0.05). Parameters of renal excretory function, notably serum urea and creatinine, were not affected by SA treatment (Table 2). Serum Na⁺ levels were not affected by treatment, although serum K⁺ levels were elevated in SA-treated rats compared with controls (P < 0.01); however, it is noteworthy that there was a minimal degree of hemolysis in some samples. SA-treated rats also had significantly reduced body weights at the end of the 14-day treatment period (control: 324.1 ± 3.9 g versus SA treated: 300.6 ± 8.2 g; P < 0.05).

After 7 days of SA treatment, urinary output of nitrite/nitrate was significantly lower than control values (P < 0.01) (Fig. 1A) and was 72% lower than control values by 14 days of treatment (P < 0.001). Similarly, NOS activity as assessed in kidney homogenates was 32% lower in rats treated with SA for 14 days compared with saline-treated controls (P < 0.05) (Fig. 1B). Basal and SNP-induced cGMP activity in cytosolic fractions of SA-treated animals were 38% (P < 0.01) and 47% (P < 0.001) lower than controls, respectively (Fig. 1C). In both SA-treated and control samples, production of cGMP after stimulation with SNP was approximately 5-fold higher than basal production levels (P < 0.001).

SA treatment had no effect on the sensitivity of the rat aorta to PE based on the observation that the two concentration response curves were superimposable (Fig. 2A). In contrast, blood vessels of SA-treated animals showed diminished sensitivity to ACh, as indicated by an approximate 12-fold rightward shift in EC₅₀ (P < 0.001) (Fig. 2B). Similarly, the...
compared with control animals (MAHMA-NONOate, as shown by a 3.5-fold increase in EC₅₀ aortas of SA-treated animals had decreased sensitivity to MAHMA-NONOate, as shown by a 3.5-fold increase in EC₅₀

**TABLE 2**

Effect of 14-day SA treatment on serum and blood parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>SA-Treated</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine (mmol·l⁻¹)</td>
<td>6.8 ± 0.7</td>
<td>8.6 ± 0.7</td>
<td>0.09</td>
</tr>
<tr>
<td>Serum urea (mmol·l⁻¹)</td>
<td>4.7 ± 0.2</td>
<td>4.9 ± 0.2</td>
<td>0.64</td>
</tr>
<tr>
<td>Serum bilirubin (µmol·l⁻¹)</td>
<td>10.1 ± 0.7</td>
<td>8.0 ± 0.8</td>
<td>0.07</td>
</tr>
<tr>
<td>Serum Na⁺ (mmol·l⁻¹)</td>
<td>140.9 ± 0.4</td>
<td>141.1 ± 0.7</td>
<td>0.72</td>
</tr>
<tr>
<td>Serum K⁺ (mmol·l⁻¹)</td>
<td>5.6 ± 0.3</td>
<td>7.1 ± 0.4</td>
<td>0.005</td>
</tr>
<tr>
<td>Serum CO₂ (mmol·l⁻¹)</td>
<td>32.9 ± 0.9</td>
<td>32.3 ± 1.4</td>
<td>0.45</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.439 ± 0.009</td>
<td>0.376 ± 0.011</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hemoglobin (g·dl⁻¹)</td>
<td>15.0 ± 0.3</td>
<td>12.6 ± 0.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mean corpuscular volume (FL)</td>
<td>61.7 ± 1.5</td>
<td>57.1 ± 0.8</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Erythrocytes (×10¹²·l⁻¹)</td>
<td>7.1 ± 0.2</td>
<td>6.6 ± 0.2</td>
<td>0.08</td>
</tr>
<tr>
<td>Leukocytes (×10⁹·l⁻¹)</td>
<td>10.9 ± 1.7</td>
<td>7.8 ± 2.4</td>
<td>0.29</td>
</tr>
<tr>
<td>Platelets (×10¹²·l⁻¹)</td>
<td>905 ± 55</td>
<td>902 ± 100</td>
<td>0.81</td>
</tr>
<tr>
<td>Serum iron (mol·l⁻¹)</td>
<td>31.6 ± 4.7</td>
<td>38.6 ± 3.1</td>
<td>0.23</td>
</tr>
<tr>
<td>Total iron binding capacity (mol·l⁻¹)</td>
<td>80.8 ± 3.0</td>
<td>79.9 ± 2.5</td>
<td>0.82</td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>39.8 ± 6.8</td>
<td>49.0 ± 5.5</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Control: n = 7; SA treated: n = 7.

**Hemodynamic Assessments (Study 2).** The effect of SA treatment on MAP, obtained by radiotelemetry, is presented in Fig. 3. SA treatment did not affect MAP levels during the initial 14-day treatment period. Low-sodium treatment caused a significant decrease in MAP (compared with the net change from baseline on days 11 to 14 of SA or vehicle treatment) in both the SA-treated and control groups (P = 0.025; α = 0.025). High-sodium treatment caused a significant increase in MAP in both treatment groups (P < 0.01 for both groups; α = 0.025). However, there were no significant differences in MAP responses with low- or high-salt intake between treatment groups (Fig. 4A). Interestingly, SA-treated animals had a trend for diminished MAP responses with increased sodium intake (P = 0.08) and the total MAP change from low sodium to high sodium (P = 0.08). The resultant in vivo renal function curve derived from these data, which takes into consideration the amount of sodium consumed, revealed a significantly steeper slope in the SA-treated animals compared with controls (P < 0.05) (Fig. 4B). After cotreatment with high sodium and l-NAME, only 30 mg·kg⁻¹·l-NAME treatment caused a significant increase in MAP (compared with the net change from high-sodium treatment) in both treatment groups (P < 0.001), although there were no differences between treatment groups (Fig. 5).

In addition to MAP, systolic and diastolic blood pressure, heart rate, and pulse pressure were assessed. Diastolic and systolic blood pressure were elevated because of the high-sodium treatment (P < 0.01) and increasing doses of l-NAME in the drinking water (P < 0.001) (data not shown), whereas heart rate was decreased because of these treatments (P < 0.01). There was also an increase in pulse pressure caused by 30 mg/kg l-NAME in both the SA-treated and control groups (P < 0.001). SA treatment did not affect these hemodynamic parameters, either under normal conditions, when challenged with altered sodium intake, or with cotreatment with high sodium and l-NAME (data not shown).

**Discussion**

In the present study, rats were treated with SA to examine the effect of heme synthesis inhibition on cardiovascular function that accompanies the buildup of this toxic tyrosine metabolite in type I HT. This study tested the hypothesis that extended heme depletion caused by SA would impair hemoenzyme function and alter BP regulation in vivo. The

Fig. 1. SA treatment decreased NOS and sGC activity. Rats were treated twice daily with SA (80 mg·kg⁻¹, i.p.) for 14 days. A, urine nitrite/nitrate excretion was measured at 4, 7, 10, and 14 days. B, NOS activity in kidney homogenates was measured after day 14. C, sGC activity in kidney cytosolic samples was assessed after day 14. Control: n = 7; SA treated: n = 7. *, P < 0.05; **, P < 0.01; ***. P < 0.001 versus controls. §, P < 0.01 versus value at day 4 in A.
The major findings of this study were that SA treatment for ≥14 days resulted in 1) reduction in tissue and circulating heme concentrations, 2) impairment of systemic and renal NOS and sGC activity, and 3) reduced ex vivo sensitivity of blood vessels to NOS-dependent and -independent vasodilators. Despite the impact of SA treatment on systemic and intra-renal hemoenzyme function, no overt cardiovascular dysregulation was observed, either under normal circumstances, when challenged with low or high sodium intake, or when challenged with high-sodium and l-NAME treatment. Taken together, these results indicate that the hypertensive phenotype frequently observed in patients with HT cannot be ascribed to the effects of SA buildup alone.

There are obvious difficulties in developing a suitable rodent model of heme synthesis inhibition to study HT, because data relating to tissue heme levels and hemoenzyme activities in humans with this disorder have, to our knowledge, never been reported. The dosing regimen of SA used in the present study was intended to produce a state of heme deficiency similar to that in patients with HT, to assess whether reductions in tissue heme content could affect cardiovascular function. The dose of SA was chosen because it had previously been shown to inhibit hepatic δ-ALA dehydratase ac-
tivity by more than 90% in rats (Tschudy et al., 1981) and effectively lower heme content in tissues. In SA-treated rats, we observed marked increases in both urinary SA and δ-ALA levels, similar to those levels observed in HT patients (Christensen et al., 1981; Sassa and Kappas, 1982; Tuchman et al., 1987; Schierbeek et al., 1993). Treatment with SA also caused markedly decreased heme levels in all tissues studied, without influencing renal function, iron status, or leukocyte counts. Although tissue levels of heme appear to have been less affected by 14 days of treatment with SA compared with 4 days of treatment (Soong et al., 2008), it is noteworthy that in the present experiments rats were heparinized and perfused sistemically with ice-cold saline, which completely removed any residual blood from these organs. Consequently, the disparity in heme assessments between these studies may reflect the different methodologies used to remove blood contamination. Indeed, the finding that NOS and sGC activity were more profoundly affected by 14-day treatment compared with 4-day SA treatment (Soong et al., 2008) suggests that the state of heme depletion was more severe in the present study.

The hypothesis presented herein predicted that blood vessels from SA-treated rats would be less sensitive to heme-dependent vasodilators. The blunted sensitivity of aortic rings from SA-treated rats to a NOS-dependent vasodilator (ACh) is consistent with the notion that there was reduced NOS and/or sGC activity in these tissues. Moreover, the reduced sensitivity to the NO-like donor MAHMA-NONOate, which acts via direct activation of sGC, suggests reduced sGC activity caused by SA treatment. The greater effect of SA treatment on ACh sensitivity compared with MAHMA-NONOate sensitivity follows because ACh-induced responses require two heme-containing enzymes, whereas MAHMA-NONOate requires only one. These observations are taken as further indication of altered hemoenzyme function induced by SA treatment.

The resultant impact of SA treatment on renal and vascular hemoenzyme function was anticipated to cause altered cardiovascular regulation. Chronic administration of L-NAME has been shown to cause dramatic, sustained elevations in BP (Banting et al., 1997; Newcomer et al., 2008). Moreover, renal hemoenzyme activity has been shown to be crucial in modulating the intrinsic pressure-natriuresis mechanism. Indeed, administration of even subconstrictor doses of NOS inhibitors into the medulla has been shown to influence urinary output and BP (Zou and Cowley, 1999). It was therefore surprising that SA did not affect BP regulation, despite the observation that SA treatment reduced urine output (Table 1). We also investigated cardiovascular responses to high and low sodium intake, because diminished intrarenal NOS activity has been linked to salt sensitivity, even in the absence of a spontaneous hypertensive phenotype [e.g., Dahl salt-sensitive rat (Majid and Navar, 1997), Sabra rat (Rees et al., 1996)]. In these animals, the deficits in NOS signaling, at least in part, contribute to the blunting of the pressure-natriuresis relationship (Salom et al., 1992), such that exaggerated changes in BP are required to maintain sodium balance when challenged with either high or low sodium intake. It was therefore hypothesized that the impact of SA treatment would become manifest with diminished or excess sodium intake. Contrary to our hypothesis, SA-treated rats were associated with a steeper in vivo renal function curve, indicating these animals require less change in MAP to regain sodium balance when challenged with a low- or high-salt diet. We sought to determine whether NOS redundancy could explain the lack of hemodynamic changes by administering stepwise increases in L-NAME along with high salt, because NO signaling is necessary for adequate sodium excretion in normal animals (Salom et al., 1992). We reasoned that if rats had reduced NO function, albeit sufficient for normal excretory function, stepwise NOS inhibition with L-NAME would reveal these differences. However, coadministration of L-NAME with high salt revealed no differences between treatment groups, suggesting that the cardiovascular reliance on NO signaling is similar in both groups.

The lack of cardiovascular responses observed in SA-treated rats supports the notion that there is considerable functional redundancy in cardiovascular regulation. BP was not elevated, and responsiveness to high sodium intake was even improved, despite 32 and 38% reductions in renal NOS and sGC activity, respectively. Thus, it may be that even greater deficits in hemoenzyme function by heme synthesis inhibition are required to cause overt cardiovascular dysregulation. Indeed, Newcomer et al. (2008) reported that arterioles have a great capacity to functionally compensate for the loss of NO activity. The data presented herein may indicate that the kidney also has this ability. Although a higher dose of SA would be expected to generate a more severe degree of heme depletion to examine this redundancy, higher doses would also be expected to interfere with erythropoiesis, resulting in anemia, which has been associated with increased activity of vascular and renal NOS (Ni et al., 1997). Given that SA-treated animals were beginning to manifest early signs of anemia, it is likely that a more severe heme depletion would be associated with confounding effects of increased NOS signaling.

Another possibility is that the cardiovascular effects of SA have been mitigated by secondary effects of SA treatment; that is, functionally antagonistic effects of SA treatment oppose the changes induced by the reduction of intrarenal and vascular NOS and sGC. For example, circulating heme has been reported to be highly cytotoxic (Nath et al., 2000; Jeney et al., 2002) and may contribute to oxidative stress in these organs, which has been shown to impair vascular relaxation (Rosen et al., 1995; Miller et al., 1998). It may be that the reduction in renal and vascular heme content by SA treatment was associated with reduced oxidative stress and im-
proved vascular function, which would be hypothesized to attenuate the functional consequences of reduced hemo-
zyrne function in the kidney and vasculature. In contrast, the
accumulation of δ-ALA caused by the inhibition of heme
synthesis by SA is expected to contribute to oxidative stress (Karbowiak and Reiter, 2002) and thus contribute to the
pathogenesis of hypertension. Although the balance between
pro-oxidant and antioxidant species is beyond the scope of
the present study, we observed no differences in urinary
excretion of 8-isoprostanones, suggesting that this balance is
not markedly upset in our model. Further investigations into
these mechanisms will provide valuable insights into the
pathophysiology of various disorders of heme synthesis.

In summary, the present studies suggest that the accumu-
lation of SA does not appear to be directly responsible for
the cardiovascular dysregulation associated with HT, despite its
impact on hemoenzyme function. However, these results do
not necessarily preclude the involvement of a state of heme
insufficiency as an etiological factor in the development of
hypertension or salt sensitivity, which may need to be severe
before cardiovascular effects manifest. As mentioned above,
other clinical cases of more severe and prolonged heme dis-
rupution, such as the porphyrias and lead intoxication, are
often associated with altered regulation of blood pressure
and compromised vascular function (Thachil 2008; Nem-
sadze et al., 2009). Moreover, it has been reported that treat-
ment of spontaneously hypertensive rats with hemin pro-
duces substantial decreases in blood pressure (Wang et al.,
2006). This notion is further supported by clinical studies in
which hypertensive patients administered δ-ALA, a precur-
sor to heme, show dramatic arterial pressure lowering (Her-
man et al., 1998). These independent lines of evidence sug-
gest that heme deficiency may be an important factor in the
development of certain cases of hypertension. However, the
evidence obtained in the present study indicates that the
mechanisms involved in the hypertension commonly associ-
ated with HT are complex and likely multifaceted.

Acknowledgments
We thank Corry Smallage for technical assistance.

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