Title: Lack of hemodynamic effects after extended heme synthesis inhibition by succinylacetone in rats.

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Running Title: Succinylacetone and blood pressure

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Non-standard abbreviations: BP, arterial blood pressure; ALA, δ-aminolevulinic acid; ALA-D, δ-aminolevulinic acid dehydratase; FAH, fumarylacetoacetate hydroxylase; HT, hypertyrosinemia; NO, nitric oxide; NOS, nitric oxide synthase; SA, succinylacetone; sGC, soluble guanylyl cyclase.

Section assignment: Cardiovascular
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). As 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-ALA (P<0.001 for both parameters), as well as 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 impacts hemoenzyme function, albeit without consequent effects on BP regulation and sodium excretion.
Introduction

Hypertyrosinemia (HT) encompasses several disease entities which are caused by inborn errors of tyrosine catabolism. Type I HT (or hepatorenal tyrosinemia), is the most clinically severe form of the disease, and results from a defect in the enzyme fumarylacetoacetate hydroxylase (Russo et al., 2001), the fifth and final enzyme in the catabolic pathway that ultimately converts tyrosine to fumarate and acetoacetate. The worldwide incidence of Type 1 HT is 1 in 100,000 live births, though the disease occurs much more frequently in French Canadians living in the Saguenay Lac St. Jean area, where the incidence is approximately 1 in 1846 live births; 1 in 22 is believed to be a carrier in this region (De Braekeleer and Larochelle, 1990). The pathological manifestations of Type I HT include early severe hepatopathology, chronic liver disease, rickets and porphyria, as well as progressive kidney, bone, and peripheral nerve damage (Nakamura et al., 2007). The clinical severity of HT stems, in large part, from a buildup of tyrosine breakdown metabolites maleylacetoacetate and fumarylacetoacetate and their derivatives, such as succinylacetone (SA, 4,6-dioxoheptanoic acid) (Russo et al., 2001).

SA is an irreversible inhibitor of δ-aminolevulinic acid dehydratase (δ-ALA-D), the second enzyme in the heme biosynthetic pathway, which converts δ-ALA to porphobilinogen. Heme, in turn, is an important component of many enzymes, several of which are involved in controlling arterial blood pressure (BP). These include, for example, nitric oxide synthase (NOS), soluble guanylyl cyclase (sGC), heme oxygenase, and cytochromes. In the context of hypertension, the NOS/sGC system is particularly interesting because it comprises 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 employs heme at its active site, where it converts L-arginine to L-citrulline, releasing nitric oxide (NO) as a co-product. 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 suffer from porphyria, a genetic disease in which one or more enzymes required for heme biosynthesis are impaired, are also hypertensive (Andersson and Lithner, 1994; Church et al., 1992). 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 (Gibbs et al., 1993; Klujber et al., 1997; Schlump et al., 2008; Strife et al., 1977), 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 setpoint 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
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≥14-day treatment with SA on (i) heme levels and hemoenzyme function in organs with a particular emphasis on the kidney, (ii) 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), and housed in Queen’s University Animal Care Facility, which maintained a 12 h/12 h light/dark cycle (transition at 07:00 h and 19:00 h). Rats had ad libitum access to a standard rodent diet (Lab Diet, St-Louis, MO) and 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 one week of acclimatization, rats were injected twice daily with SA (4,6-dioxoheptanoic acid; 40 mg·kg⁻¹, i.p., @ 10:00 h and 22:00 h) (n = 7) or saline (n = 7) for 14 days. On day 15, rats were anaesthetized 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 (see below). 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, and 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 1,350 x g, 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 fourteen 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 previously described (Bourque et al., 2008). Rats were then given 10 days to recover, after which time hemodynamic parameters were continuously monitored for 15 seconds every 5 minutes. After an initial four day baseline recording period, SA was administered twice daily (40 mg·kg⁻¹, i.p., q12 h @ 10:00 and 22:00) 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 AIN-76A rodent diet, as well as 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⁺), as well as drinking water supplemented with 1% NaCl (w/v). After five days of high-sodium...
intake, the drinking water was supplemented with N-nitro-L-arginine methyl ester (L-NAME) in addition to the 1% NaCl, such that the animals ingested L-NAME 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, as well as 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 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). Samples were centrifuged for 10 min at 1350 x g 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 using the fluorometric method of Morrison *et al.* (Morrison, 1965). Hematocrits were assessed by collecting a small sample of blood (70 μL) into a heparinized microcapillary tube, followed by centrifugation at 1350 x g, for determination of packed cell volume.

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

*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 methylamine 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 using a Powerlab® data acquisition system (ADInstruments) and displayed using Chart (version 5) software. Tissues were allowed to equilibrate at a resting tension of 9.8 mN for one hour, with rinses every 15 minutes, prior to testing. PE concentration-response relationships were obtained by adding increasing doses of PE to the bath (100 pM to 30 μM, in approximate 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 sub-maximally contracted (50-80%) with PE prior to administration of
vasodilators. After steady pre-contraction, increasing concentrations of vasodilators (100
pM to 300 μM for ACh, and 1 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 minute 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. Since 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 due to sodium manipulations were also analyzed
by Student’s t test for each treatment. For ex vivo assessment of vascular function,
exclusion criteria were established prior to experimentation to ensure tissue viability;
specifically, aortic rings that did not achieve an initial contraction of at least 19.6 mN or
did not achieve a minimum of 50% relaxation of the submaximal pre-contraction with
ACh were deemed to be compromised, and were therefore excluded from all analyses.
Telemetry data were analyzed using a repeated measures 2-way analysis of variance
(ANOVA); where significant differences were found, Student’s t test with Bonferonni
correction was conducted on data sets, as appropriate. MAP profiles assessed by
radiotelemetry are presented as the difference from the last day of baseline (prior to SA
or vehicle treatment), to account for differences in baseline recordings between animals.
Telemetry data presented herein were obtained between the hours of 22:00 and 04:00,
which corresponds to the night phase of the light cycle in the Queen’s Animal Care Facility, and hence the period of greatest activity in rats. However, similar results were obtained for daytime recordings, as well as when data were assessed as 24 h mean values (data not shown). High and low sodium MAP values represent 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 (upper point) and low (lower point) sodium treatments; a separate slope was obtained for each animal, and pooled to calculate group means. All data are presented as mean ± SEM. P ≤ 0.05 was considered statistically significant.
Results

Depletion of heme by succinylacetone (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 δ-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, 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, as well as indices of circulating iron levels, were not significantly different between SA-treated and vehicle-treated controls (Table 2).

SA-treated rats had 20% reduced urinary output over a 24h 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 to 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 vs. SA-treated: 300.6 ± 8.2 g; P < 0.05).
After seven days of SA treatment, urinary output of nitrite/nitrate was significantly lower than control values (P<0.01) (Figure 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 to saline treated controls (P < 0.05) (Figure 1B). Basal and SNP-induced sGC activity in cytosolic fractions of SA-treated animals were 38% (P < 0.01) and 47% (P < 0.001) lower than controls, respectively (Figure 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 as the two concentration response curves were superimposable (Figure 2A). In contrast, blood vessels of SA-treated animals showed diminished sensitivity to ACh, as indicated by an approximate 12-fold rightward shift in EC$_{50}$ (P < 0.001) (Figure 2B). Similarly, the aortas of SA-treated animals had decreased sensitivity to MAHMA-NONOate, as shown by a 3.5-fold increase in EC$_{50}$ compared to control animals (P < 0.001) (Figure 2C).

**Hemodynamic assessments (study 2)**

The effect of SA treatment on MAP, obtained by radiotelemetry, is presented in Figure 3. SA treatment did not impact MAP levels during the initial 14-day treatment period. Low sodium treatment caused a significant decrease in MAP (compared to the net change from baseline on days 11-14 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 salt or high salt intake between treatment groups (Figure 4A). Interestingly, SA-treated animals had a trend for diminished MAP responses with increased sodium intake (P = 0.08), as well as 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 to controls (P < 0.05) (Figure 4B). After co-treatment with high sodium and L-NAME, only 30 mg·kg\(^{-1}\) L-NAME treatment caused a significant increase in MAP (compared to the net change from high sodium treatment) in both treatment groups (P < 0.001), although there were no differences between treatment groups (Figure 5).

In addition to MAP, systolic (SBP) and diastolic BP (DBP), heart rate and pulse pressure were also assessed. DBP, and SBP were elevated due to 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 due to these treatments (P<0.01). There was also an increase in pulse pressure due to 30 mg/kg L-NAME in both the SA treated and control groups (P<0.001). SA-treatment did not impact these hemodynamic parameters, either under normal conditions, or when challenged with altered sodium intake, or co-treatment with high sodium and L-NAME (data not shown).
Discussion

In the present study, rats were treated with SA in order 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 major findings of this study were that SA treatment for ≥14 days resulted in (i) reduction in tissue and circulating heme concentrations, (ii) impairment of systemic and renal NOS and sGC activity and (iii) reduced ex vivo sensitivity of blood vessels to NOS-dependent and -independent vasodilators. Despite the impact of SA treatment on systemic and intrarenal 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, since 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 impact cardiovascular function. The dose of SA was chosen because it has previously been shown to inhibit hepatic ALA-D activity 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; Schierbeek et al., 1993; Tuchman et al., 1987). 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 may appear to have been less affected compared to 4-day treatment with SA (Soong et al., 2008), it is noteworthy that in the present experiments, rats were heparinized and perfused systemically 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 to 4-day SA treatment (Soong et al., 2008) suggests 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 were 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 due to SA treatment. The greater effect of SA treatment on ACh sensitivity compared to MAHMA-NONOate sensitivity follows since ACh-induced responses require two heme-containing enzymes, while 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 as well as BP (Zou and Cowley, 1999). It was therefore surprising that SA did not impact BP regulation, despite the observation that SA-treatment reduced urine output (Table 1). We also investigated cardiovascular responses to high and low sodium intake, since 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, since 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, co-administration 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. reported that arterioles have a great capacity to functionally compensate for the loss of NO activity (Newcomer et al., 2008). 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 (Jeney et al., 2002; Nath et al., 2000), and may contribute to oxidative stress in these organs, which has been shown to impair vascular relaxation (Miller et al., 1998; Rosen et al., 1995). It may be that the reduction in renal and vascular heme content by SA treatment was associated with reduced oxidative stress, and improved vascular function, which would be hypothesized to attenuate the functional consequences of reduced hemoenzyme function in the kidney and vasculature. In contrast, the accumulation of δ-ALA due to inhibition of heme synthesis by SA is expected to contribute to oxidative stress (Karbownik and Reiter, 2002), and thus contribute to the pathogenesis of hypertension. Although the balance between pro and anti-oxidant species is beyond the scope of the present study, we observed no differences in urinary excretion of 8-isoprostanes, suggesting 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 accumulation 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 disruption, such as the porphyrias and lead intoxication, are often associated with altered regulation of blood pressure and compromised vascular function

{{2881 Nemsadze,K. 2009;2882 Thachil,J. 2008;}}. Moreover, it has been reported that treatment of spontaneously hypertensive rats with hemin produces substantial decreases in blood pressure (Wang et al., 2006). Similar results have been demonstrated in the clinical setting, wherein hypertensive patients administered δ-ALA, a precursor to heme, show dramatic arterial pressure lowering (Herman et al., 1998). These independent lines of evidence suggest 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 associated with HT is complex, and likely multifaceted.
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References


Footnotes
a) This study was supported by the Canadian Institutes of Health Research [MOP-68993, MOP-74521]. S.L.B. is a recipient of the Canadian Hypertension Society/Pfizer/Canadian Institutes of Health Research Research and Development Doctoral Award. C.D.B. is a recipient of the Canadian Institutes of Health Research - Gasotransmitter Research And Training Master’s Award.

b) This study was presented, in part, at the 19th Annual Scientific Meeting of the Ontario Hypertension Society. Parts of this study were included in the PhD thesis of S.L.B., and the MSc. Thesis of C.D.B.

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Legends for Figures

**Figure 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) nitric oxide synthase activity in kidney homogenates was measured after day 14; (C) soluble guanylyl cyclase activity in kidney cytosolic samples was assessed after day 14. Control: n = 7; SA-treated: n = 7. *P < 0.05, ***P < 0.001 vs. controls.

**Figure 2.** Decreased sensitivity of rat aorta to ACh and a NO donor after SA treatment (80 mg·kg⁻¹, i.p.). Rats were treated with SA for 14 days, and aortas were tested *ex vivo* for sensitivity to (A) phenylephrine (PE), (B) acetylcholine (ACh) and (C) methylamine hexamethylene methylamine NONOate (MAHMA-NONOate). Controls received saline vehicle. For details on protocol, see the methods section. Control: n = 7; SA-treated: n = 7. *P < 0.05, ***P < 0.001 vs. controls.

**Figure 3.** Lack of effect of 14-day succinylacetone (SA) treatment (80 mg/kg, i.p.) or saline control on mean arterial pressure (MAP), and responses to changes in sodium intake and nitric oxide synthase inhibition, as assessed by radiotelemetry. Data represent MAP differences from baseline. SA, succinylacetone treatment phase; LS, SA treatment with low sodium; SA + HS, SA treatment with high sodium; +1L, SA treatment with high sodium, and 1 mg/kg L-NAME; +3L, SA treatment with high sodium and 3 mg/kg L-NAME; +30L, SA treatment with high sodium and 30 mg/kg L-NAME. Note that controls were treated with saline instead of SA during all periods. Control: n = 7; SA-treated: n = 7.
Figure 4. The effect of 14-day succinylacetone (SA) treatment (80 mg·kg⁻¹, i.p.) on (A) mean arterial pressure (MAP) responses to changes sodium intake, and (B) the resultant in vivo renal function curves derived from these data. Data represent changes in hemodynamic parameters assessed at night (see methods for details). All bars in (A) are significantly non-zero (i.e. changes with sodium intake are different from baseline) *P < 0.05 compared to control slope. ‘Total’ representing the MAP change from low to high sodium treatments; low sodium (LS) representing the MAP change from normal to low sodium treatments; high sodium (HS) representing the MAP change from normal to high sodium treatments. †P < 0.05, ‡P < 0.01 compared to baseline value. Control: n=5; SA-treated: n=6.

Figure 5. Mean arterial pressure (MAP) responses induced by L-NAME are not affected by 14-day SA treatment (80 mg·kg⁻¹, i.p.). Data represent MAP differences from baseline. Control: n=5; SA-treated: n=6. HS, high sodium; HS+1, high sodium + 1mg/kg L-NAME; HS+3, high sodium + 3mg/kg L-NAME; HS+30, high sodium + 30mg/kg L-NAME.
Table 1. Effect of 14-day succinylacetone (SA) treatment on urine and tissue parameters in rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>SA Treated</th>
<th>P Value</th>
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<tr>
<td>Urine Volume (mL 24h⁻¹)</td>
<td>25.8 ± 1.7</td>
<td>19.9 ± 1.4</td>
<td>0.03</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 δ-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.
Table 2. Effect of 14-day succinylacetone (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 CO(_2)</td>
<td>32.0 ± 0.9</td>
<td>33.3 ± 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 ± 0.31</td>
<td>12.6 ± 0.32</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 (x10(^{12})·L(^{-1}))</td>
<td>7.1 ± 0.2</td>
<td>6.6 ± 0.2</td>
<td>0.08</td>
</tr>
<tr>
<td>Leukocytes (x10(^{9})·L(^{-1}))</td>
<td>10.9 ± 1.7</td>
<td>7.8 ± 2.4</td>
<td>0.29</td>
</tr>
<tr>
<td>Platelets (x10(^{9})·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 ± 5.47</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Control: n=7; SA treated: n=7.
Fig. 1

A. Nitrate (μmol/g/day) over days 4 to 14 for Control and SA-treated groups. The y-axis shows nitrate concentration, and the x-axis shows days. The graph includes error bars and indicates a statistically significant difference between the groups.

B. Basal and SNP-induced pmol NO/mg/hr for Control and SA-treated groups. The y-axis represents pmol NO/mg/hr, and the x-axis represents the groups. The graph shows a statistically significant increase in SNP-induced NO for the SA-treated group compared to the Control group.

C. Treatment-induced pmol cGMP/mg/hr for Basal and SNP-induced conditions. The x-axis shows treatment types, and the y-axis represents pmol cGMP/mg/hr. The graph includes error bars and shows a statistically significant increase in SNP-induced cGMP for the SA-treated group compared to the Control group.
Fig. 2

A. 

% Constriction

\[ \text{LogEC}_{50} = -7.47 \pm 0.04 \] for Control, and
\[ \text{LogEC}_{50} = -7.48 \pm 0.04 \] for SA.

B. 

% Relaxation

\[ \text{LogEC}_{50} = -8.57 \pm 0.26 \] for Control, and
\[ \text{LogEC}_{50} = -7.50 \pm 0.08 \text{***} \] for SA.

C. 

% Relaxation

\[ \text{LogEC}_{50} = -8.65 \pm 0.05 \] for Control, and
\[ \text{LogEC}_{50} = -8.12 \pm 0.05 \text{***} \] for SA.
Fig. 3

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Fig. 5

The graph shows the change in MAP (Δ MAP) for different treatments. The treatments include Control, HS, HS+1, HS+3, and HS+30. The dark bars represent Control, and the light bars represent SA. The y-axis represents Δ MAP (mmHg) ranging from 0 to 30, and the x-axis represents the Treatment categories.