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Anomalous Renal Effects of Tin Protoporphyrin in a Murine Model of Sick Cell Disease

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In human and murine models of sickle cell disease (SCD), heme oxygenase-1 (HO-1) is induced in the kidney, an organ commonly involved in SCD. The present study assessed the role of HO-1 by using a competitive inhibitor of HO activity, tin protoporphyrin (SnPP), in protocols affording a composite, clinically relevant analysis of the kidney in SCD under unstressed and stressed conditions. Whereas short-term administration of SnPP exerted comparable renal hemodynamic effects in wild-type and sickle mice, chronic administration of SnPP exerted divergent effects: SnPP provoked tubulointerstitial inflammation and up-regulation of injury-related genes in wild-type mice, whereas in sickle mice SnPP reduced expression of injury-related genes and vascular congestion without provoking tubulointerstitial inflammation. SnPP also protected against the heightened sensitivity to renal ischemia observed in sickle mice, preventing ischemia-induced worsening of renal injury in sickle mice above that observed in wild-type mice. Effective and comparable inhibition of HO activity by SnPP in wild-type and sickle mice was confirmed. These findings suggest that induction of HO-1, at least as assessed by this approach, may contribute to renal injury in this murine model of SCD and uncover an experimental maneuver that protects the kidney in murine SCD. (*Am J Pathol* 2006, 169:21–31; DOI: 10.2353/ajpath.2006.051195)

Kidney disease is a cardinal complication of sickle cell disease (SCD) and contributes quite substantially to the

morbidity and increased mortality that occur in patients with SCD.^{1–4} SCD afflicts the kidney in numerous ways, including medullary congestion and papillary necrosis, chronic glomerulopathy and tubulointerstitial disease, progressive and endstage kidney disease, and acute renal dysfunction attended by acute vaso-occlusion and tubular necrosis.^{5–8} The mechanisms accounting for such disease processes in the kidney are heterogeneous, incompletely defined, and involve diverse forms of stress, including those that are hemodynamic, oxidative, nitrosative, and inflammatory in origin.^{1–4}

Stress elicits assorted cellular responses that may influence attendant tissue injury. In this regard, we originally demonstrated that the stress-inducible gene heme oxygenase-1 (HO-1) is up-regulated in the kidney and vasculature in human and murine SCD.⁹ HO is the rate-limiting enzyme in the degradation of heme, converting heme to biliverdin, and facilitating the release of iron and CO; biliverdin is then reduced to bilirubin.^{10–13} HO-1 is the isoform induced by heme, oxidants, cytokines, ischemia, hypoxia, and other stressors; HO-2 is the constitutive isoform that is basally expressed, and abundantly so, in the unstressed kidney.^{10–13} Our findings regarding the induction of HO-1 in SCD are supported by studies demonstrating increased CO production in human SCD^{14,15} and by more recent findings demonstrating increased mRNA expression for HO-1 and biliverdin reductase in human SCD along with increased generation of CO and bilirubin.¹⁶

Interest in HO-1 in SCD, as in other conditions in which it is expressed, is engendered by the capacity of HO-1 to protect against diverse insults, including heme protein-induced injury,^{17–21} to the kidney and other organs and tissues. The basis for the protective effects of HO-1 is manifold and includes the following: HO-1 reduces oxidative stress by degrading heme (a lipophilic pro-oxidant)¹⁹ and by facilitating cellular storage and export of iron^{17,22,23,24}; the products of HO, bile pigments and CO,

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are cytoprotective, anti-inflammatory metabolites^{25,26}; additionally, bile pigments exert potent antioxidant effects²⁷ while CO possesses anti-apoptotic and vasorelaxant properties.^{26,28} HO-1 may also protect against tissue injury by inducing the expression of cytoprotective molecules such as the cyclin-dependent kinase inhibitor, p21.^{29,30}

The present study examined the extent to which HO activity, emanating essentially from the combined contributions from HO-1 and HO-2, influences the phenotype of the kidney in SCD in the unstressed state and following clinically relevant stress. In this regard, we used a transgenic murine model of SCD in three separate protocols that, in aggregate, afforded a clinically relevant, composite analysis of the kidney in SCD under basal and stress conditions. The first protocol examined the dependency of renal hemodynamics on basal HO activity, whereas the second protocol examined the extent to which HO activity influences the evolution of chronic kidney injury in SCD. The third protocol continued this investigative line by examining the influence of HO activity in SCD under conditions of stress that simulate acute vasoocclusive crisis and draw on our prior studies demonstrating heightened sensitivity of the murine kidney in SCD to acute ischemic insults.³¹

The approach adopted in the present study used the competitive inhibitor of HO activity, tin protoporphyrin (SnPP). The basic experimental design in all three protocols involved wild-type and sickle mice treated with vehicle or SnPP. SnPP and other metalloporphyrins are highly effective inhibitors of HO activity and provide a well established and accepted method for elucidating the role of HO activity in influencing tissue responses and behavior in health and disease.^{10–13} For example, the use of SnPP *in vivo* revealed the marked protective effects of induced HO activity in acute heme protein-mediated nephrotoxicity,¹⁷ the immunoprotective effects of HO in skin,³² and the role of HO in mediating the inhibitory effects of IL-10 on neointimal proliferation in vascular allografts.³³

Materials and Methods

The transgenic murine model of SCD, used in the current study and our previous publications, is on a C57BL/6 background, is homozygous for the murine β -globin deletion, and carries the two transgenes $\alpha^H\beta^S$ and $\alpha^H\beta^{S-}$ Antilles.^{9,31,34} Studies were conducted in age-matched wild-type and sickle mice 5 to 18 months old with similar numbers of males and females in each group in all protocols. Mice were provided with free access to standard mouse chow and tap water. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the Mayo Clinic.

Protocol 1: Effect of SnPP on Renal Hemodynamics in Wild-Type and Sickle Mice

To examine the role of HO in maintaining renal hemodynamics in wild-type and sickle mice, SnPP (50 μ mol/kg of

body weight, intraperitoneal [i.p.]), or vehicle (0.9% sodium chloride) was administered 24 hours before the assessment of renal hemodynamics.³⁵ Mice were anesthetized with sodium pentobarbital (60 mg/kg of body weight, i.p.) and placed on a temperature-regulated table to maintain body temperature at 37°C throughout these studies. Following the placement of a tracheostomy with PE-160 tubing, the carotid artery, the jugular vein, and the urinary bladder were cannulated with PE-10 tubing for the monitoring of mean arterial pressure, the infusion of inulin and fluids, and the collection of timed urinary samples, respectively; mean arterial pressure was continuously recorded throughout the study. To determine glomerular filtration rate (GFR) and to maintain euvoemia, a composite solution of 0.9% saline containing 2.25% of bovine serum albumin and 0.75% of fluorescein isothiocyanate-inulin (FITC-I; Sigma-Aldrich, St. Louis, MO) was infused into the jugular vein at a rate of 0.25 μ l/min/g body weight.³⁶ To measure total renal blood flow (RBF), the left kidney was exposed via a midline incision and an electromagnetic flow probe (Transonic Systems Inc., Ithaca, NY) was placed around the left renal artery; RBF was continuously recorded throughout the study using a small animal blood flow meter (T206; Transonic Systems Inc.).³⁷ After completing this surgical procedure, the abdomen was covered with parafilm to minimize evaporation, and 45 minutes were allowed for equilibration. After the equilibration period, measurement of the clearance of FITC-I was used to determine GFR³⁶; the concentrations of FITC-I in urine and blood were measured using a fluorescence reader (FL 600; BIO-TEK, Winooski, VT). Renal vascular resistances were determined as the ratio of mean arterial pressure/RBF. Of the 15 wild-type mice and 15 sickle mice subjected to renal hemodynamic measurements (GFR and RBF), RBF was not measurable because of difficulty with placement of the flow probe in three wild-type mice and one sickle mouse, whereas GFR was not measurable due to blockage of the bladder catheter in one wild-type mouse and two sickle mice. At the end of the experimental procedures, the kidneys were removed and weighed, and HO activity was determined, as previously described, by measuring the rate of generation of bilirubin by renal microsomes.^{9,17}

Protocol II: Renal Effect of Chronic Administration of SnPP in Wild-Type and Sickle Mice

Wild-type and sickle mice were chronically treated with either SnPP (30 μ mol/kg of body weight, i.p.) or vehicle (0.9% saline) every other day for 25 days,³⁸ after which mice were sacrificed, and kidney tissue was either fixed with 10% formalin for histological assessment or snap frozen for subsequent studies of gene expression by RT-PCR.

Total RNA was extracted from mouse kidneys using the TRIzol method (Invitrogen, Carlsbad, CA) and further purified using a RNeasy Mini Kit (Qiagen, Valencia, CA) according to each manufacturer's protocol. Reverse transcription was performed on 100 ng of each purified RNA

Table 1. Primers and Probes Used for Quantitative Real-Time RT-PCR

Gene	Primer/probe	Sequence
<i>IL-6</i>	Forward	5'-CCAGAAACCGCTATGAAGTTCCT-3'
	Reverse	5'-CACCAGCATCAGTCCCAAGA-3'
	TaqMan	5'-(FAM) TCTGCAAGAGACTTCCATCCAGTTGCC (TAMRA)-3'
<i>TGF-β1</i>	Forward	5'-GCCTGAGTGGCTGTCTTTGA-3'
	Reverse	5'-TGTATTCCGTCTCCTTGGTTCA-3'
	TaqMan	5'-(FAM) TCACTGGAGTTGTACGGCAGTGG (TAMRA)-3'
<i>Col III</i>	Forward	5'-TGTCCAACTGGTGGCCAAA-3'
	Reverse	5'-CCATTCTCTCCACTCCAGACT-3'
	TaqMan	5'-(FAM) TATTCTCCCAATTTCGACTCATATG (TAMRA)-3'
<i>Col IV</i>	Forward	5'-ACAACATCCGGCCCTTCA-3'
	Reverse	5'-ACTGTGTACCGCCATCACCAT-3'
	TaqMan	5'-(FAM) TAGCAGGTGTGCGGTTTGTGAAGCA (TAMRA)-3'
<i>18S</i>	Forward	5'-TGTCTCAAAGATTAAGCCATGCAT-3'
	Reverse	5'-AACCATAACTGATTTAATGAGCCATTTC-3'
	TaqMan	5'-(VIC) TACGCACGGCCGGTACAGTGAAGT (TAMRA)-3'

sample using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN) using random hexamers. Subsequently, 5 μ l of diluted cDNA product were used in duplicate 25- μ l quantitative real-time PCR reactions performed on an ABI Prism 7000 using TaqMan Universal PCR Master Mix reagent (Applied Biosystems, Foster City, CA) as previously described.³⁹ Primers and probes (Table 1) for the amplification and detection were designed using Primer Express software (Applied Biosystems), and their concentrations in the reactions were 300 and 200 nmol/L, respectively. Conditions for the real-time PCR detection were as follows: 2 minutes at 48°C and 10 minutes at 95°C (preheating) followed by 40 cycles of amplification consisting of 15 seconds at 95°C (denaturation) and 1 minute at 60°C (annealing and extension). Standard curves were generated for both 18S and target mRNAs using 10-fold serial dilutions of cDNA as described previously.³⁹ Results are expressed as a ratio of target mRNA expression to 18S rRNA expression.

Qualitative and semiquantitative analyses of kidney tissue were performed by light microscopy on paraffin-embedded sections stained with hematoxylin and eosin. Semiquantitative analyses were performed by a renal pathologist (J.P. Grande) in a blinded fashion and based on the following scoring criteria. Congestion of interstitial vessels and glomerular capillary loops was semiquantitatively evaluated as absent (0), mild (1), moderate (2), and severe (3), based on the extent of capillary dilation and number of vessels involved. Both cortical and medullary vessels were evaluated. Assessment of percent cortical surface area with acute tubular necrosis, as defined by tubular dilation, nuclear karyolysis, and cytoplasmic hypereosinophilia was performed in a blinded fashion. Renal function in this protocol and Protocol III was evaluated by measuring blood urea nitrogen (BUN) with the use of BUN analyzer 2 (Beckman Instruments, Fullerton, CA).

Protocol III: Effect of SnPP on Renal Ischemia-Reperfusion Injury in Wild-Type and Sickie Mice

In these studies, we used a model of renal ischemic injury as previously used and described by our laboratory.³¹

Wild-type and sickle mice were pretreated with a single i.p. injection of either SnPP (50 μ mol/kg of body weight) or vehicle (0.9% saline), administered 14 hours before the ischemic insult. Mice were anesthetized with pentobarbital (60 mg/kg of body weight), the kidneys were exposed via a midline abdominal incision, and the renal pedicles carefully dissected. Fifteen minutes of ischemia were imposed by clamping the renal pedicles bilaterally with nontraumatic sterile clamps (RS5426, Micro Aneurysm clip, straight, 10 mm, 125 g pressure; Roboz Surgical Instruments, Rockville, MD). A sham procedure in wild-type and sickle mice included anesthesia and laparotomy but omitted dissection and clamping of the renal pedicle. Twenty-four hours after the ischemic insult, blood samples were drawn for the measurement of BUN, mice were euthanized, and tissues were harvested for histological analysis.

Statistical Analysis

Data are expressed as mean $\pm$ SE. The effect of SnPP as compared to vehicle in wild-type and sickle mice was analyzed by Student's *t*-test for parametric data and the Mann-Whitney test for nonparametric data. Data were considered significant for *P* < 0.05.

Results

Effect of SnPP on Renal Hemodynamics in Wild-Type and Sickie Mice

The administration of SnPP did not affect the body weights of either the wild-type or sickle mice, and the mean body weights of all wild-type and sickle mice that underwent studies of renal hemodynamics were not significantly different (35 ± 1 vs. 36 ± 4 g, respectively, *P* = NS, *n* = 15 in each group). The kidney weights were significantly greater in sickle mice as compared with wild-type mice expressed either as absolute weight of both kidneys (0.54 ± 0.03 vs. 0.39 ± 0.02 g, *P* < 0.05, *n* = 15 in each group) or as the weight of both kidneys factored for body weight

Figure 1. Renal hemodynamics in wild-type and sickle mice treated with vehicle or SnPP. **A:** RBF in wild-type mice (WT) treated with vehicle (open bar, $n = 7$) or SnPP (solid bar, $n = 5$) and in sickle mice (Sickle) treated with vehicle (open bar, $n = 7$) or SnPP (solid bar, $n = 7$). SnPP ($50 \mu\text{mol/kg}$ of body weight, i.p.) or vehicle was administered 24 hours before study. * $P < 0.05$ versus vehicle-treated group in wild-type mice; # $P < 0.05$ versus vehicle-treated group in sickle mice. **B:** GFR in wild-type mice (WT) treated with vehicle (open bar, $n = 7$) or SnPP (solid bar, $n = 7$) and sickle mice (Sickle) treated with vehicle (open bar, $n = 7$) or SnPP (solid bar, $n = 6$). **C:** Calculated renal vascular resistances (RVRs) in wild-type mice (WT) treated with vehicle (open bar, $n = 7$) or SnPP (solid bar, $n = 5$) and sickle mice (Sickle) treated with vehicle (open bar, $n = 7$) or SnPP (solid bar, $n = 7$). * $P < 0.05$ versus vehicle-treated group in wild-type mice; # $P < 0.05$ versus vehicle-treated group in sickle mice.

(0.015 ± 0.001 vs. 0.011 ± 0.001 , $P < 0.05$, $n = 15$ in each group).

In these studies, mean arterial pressure determined during the renal hemodynamic studies was not significantly altered by SnPP in wild-type mice (86 ± 3 and 81 ± 3 mmHg, vehicle-treated vs. SnPP-treated in these and all subsequent data) or in sickle mice (63 ± 3 and 64 ± 3 mmHg). SnPP significantly reduced RBF in wild-type and sickle mice, leading to 40% and 44% reductions in RBF as compared with values determined in vehicle-treated wild-type and vehicle-treated sickle mice, respectively (Figure 1); in neither wild-type nor sickle mice was GFR significantly altered by SnPP (Figure 1). Calculated renal vascular resistances significantly increased in both wild-type and sickle mice following treatment with SnPP; the percent increments in renal vascular resistance in SnPP-treated wild-type and SnPP-treated sickle mice above respective vehicle-treated groups were not significantly different (41 ± 4 vs. $71 \pm 25\%$, $P = 0.34$) (Figure 1).

To assess the efficacy of SnPP as an inhibitor of HO, HO activity was measured in the kidney following these hemodynamic studies. In several of the kidneys of wild-type and sickle mice treated with SnPP, no HO activity was measurable, and the mean values of HO activity in SnPP-treated wild-type mice and SnPP-treated sickle mice (11.8 ± 4.0 and 7.1 ± 3.0 pmol/mg of protein/hour, respectively) were reduced to ~6% and 3% of HO activity measured in the kidneys in vehicle-treated wild-type mice and vehicle-treated sickle mice (192.2 ± 15.0 and 276.4 ± 16.0 pmol/mg of protein/hour, respectively), the latter being significantly different from each other. From these studies, we conclude that SnPP imposed highly effective and comparable inhibition of HO activity in both groups and that the basal HO activity in the kidney is higher in vehicle-treated sickle mice than in vehicle-treated wild-type mice.

Renal Effects of Chronic Administration of SnPP in Wild-Type and Sickle Mice

Chronic administration of SnPP exerted significant and divergent effects on renal histological injury in wild-type and sickle mice. In wild-type mice, chronic administration of SnPP, as compared with similarly administered vehicle, induced foci of tubulointerstitial inflammation and fibrosis (Figure 2). In sickle mice, SnPP exerted renal histological effects that differed markedly from that observed in wild-type mice (Figure 3). Vehicle-treated sickle mice exhibited medullary congestion and, to a lesser extent, congestion in glomerular and cortical capillaries. Sickle mice treated with SnPP exhibited less medullary, cortical, and glomerular congestion; notably, the effect of SnPP observed in the wild-type mice, namely, the induction of tubulointerstitial disease, was not observed in sickle mice.

In an attempt to determine the mechanism underlying the divergent effects of SnPP, we assessed the renal expression of genes relevant to tubulointerstitial disease. Remarkably, the expression of such genes closely tracked with the renal histological changes observed. SnPP increased mRNA expression for an interstitial collagen [collagen III(α 1)] in wild-type mice but decreased such expression in sickle mice (Figure 4); SnPP tended to exert directionally similar effects on a glomerular basement collagen [collagen IV(α 1)] in wild-type and sickle mice (Figure 4). SnPP significantly increased mRNA expression for a fibrogenic cytokine (transforming growth factor- β 1) in wild-type mice and tended to decrease such expression in sickle mice (Figure 4). Finally, SnPP significantly increased mRNA expression for a pro-inflammatory cytokine (interleukin-6 [IL-6]) in wild-type mice but significantly decreased such expression in sickle mice (Figure 4). SnPP thus exhibits divergent effects on the expression of renal injury and injury-related genes in wild-type and sickle mice: SnPP promotes the expression of tubulointerstitial inflammation and injury-related genes in wild-type mice, whereas in sickle mice, SnPP reduces congestion in the renal microcirculation in the absence of any inflammatory effect in the kidney, indeed, suppressing renal expression of injury-related genes. These effects of SnPP did not eventuate in changes in GFR as reflected by measurements of the glomerular filtration marker, BUN: SnPP did not significantly affect BUN in wild-type mice (17 ± 2 vs. 19 ± 1 mg/dl, $P = \text{NS}$) or in sickle mice (13 ± 1 vs. 14 ± 2 mg/dl, $P = \text{NS}$).

Effects of SnPP on Renal Ischemia-Reperfusion Injury in Wild-Type and Sickle Mice

In our prior studies, we demonstrated that sickle mice exhibit increased sensitivity to ischemic injury, exhibiting a greater rise in BUN and worse histological injury as compared to that observed in ischemic wild-type mice.³¹ In the present studies, we examined the effect of SnPP on the sensitivity of sickle mice to such injury. In wild-type mice, the BUN in vehicle-treated and

Figure 2. Histological examination of the kidney cortex in vehicle-treated wild-type mice (**A** and **C**) and SnPP-treated wild-type mice (**B** and **D**). Original magnification, $\times 200$ (**A** and **B**), $\times 400$ (**C** and **D**). All sections are stained with hematoxylin and eosin.

SnPP-treated wild-type mice subjected to ischemia were comparably elevated (62 ± 13 and 60 ± 17 mg/dl) and were markedly higher than the BUN in vehicle-treated and SnPP-treated wild-type mice subjected to sham ischemia (9 ± 2 and 9 ± 2 mg/dl). Treatment with SnPP, as compared with vehicle, significantly reduced the BUN in sickle mice subjected to ischemia (115 ± 17 and 60 ± 17 mg/dl, $P < 0.05$); both values were still markedly higher as compared with vehicle-treated and SnPP-treated sickle mice subjected to sham ischemia (10 ± 1 and 13 ± 1 mg/dl). These data are summarized in Figure 5. These findings thus demonstrate that, following renal ischemia, renal function is more severely worsened in sickle mice as compared with wild-type mice. Whereas SnPP does not exert any appreciable effect on renal function in wild-type mice subjected to ischemia, SnPP affords significant protection against the exacerbation of renal dysfunction observed in sickle mice following renal ischemia.

These changes in renal function, as assessed by BUN, were corroborated by alterations in renal histo-

logical injury, as assessed by qualitative and semi-quantitative analyses. In vehicle-treated wild-type mice, ischemia induced acute tubular necrosis, which was quite patchy and involved only a minority of the tubules; these changes were not significantly altered after administration of SnPP, as shown by semiquantitative analyses depicted in Figure 6. In contrast, in vehicle-treated sickle mice, acute tubular necrosis involved the majority of the cortex and renal tubules and was much more severe as compared with the histological changes observed in wild-type mice subjected to ischemia. SnPP significantly reduced the severity of acute tubular necrosis incurred by ischemia in sickle mice, restricting such changes mainly to the deep cortical and corticomedullary zones (Figure 6). Representative sections depicting the effect of SnPP in sickle mice are displayed in Figure 7. Ischemia induced vascular congestion in sickle mice but not in wild-type mice, and SnPP reduced such ischemia-induced vascular congestion in sickle mice (semiquantitative scores: 2.7 ± 0.1 vs. 1.4 ± 0.3 , $P < 0.05$).

Figure 3. Histological examination of the kidney in vehicle-treated sickle mice (**A** and **C**) and SnPP-treated sickle mice (**B** and **D**). **A** and **B** represent the renal medulla and are at an original magnification of $\times 200$, whereas the **C** and **D** are sections of the kidney cortex and are at an original magnification of $\times 400$. All sections are stained with hematoxylin and eosin.

Discussion

The renal phenotype and hemodynamic profile in this transgenic sickle strain are consistent with findings in human SCD. In this murine model, mean arterial pressure was lower than, whereas GFR and RBF were comparable to, these parameters in age-matched and sex-matched wild-type mice. Reduced blood pressure and a lower incidence of systemic hypertension occur in human SCD,⁴⁰ and while GFR is commonly elevated in relatively younger patients with SCD,^{1–4} GFR decreases to a normal range with age, and indeed may steadily decline when progressive glomerulopathies and tubulointerstitial disease involve the kidney.^{1–4} Clinically relevant features of this transgenic model also include renal hypertrophy and medullary congestion.^{1–4}

Assessment of the involvement of HO activity in maintaining renal hemodynamics in sickle mice failed to reveal a contribution from HO activity that significantly differed from that observed in the wild-type strain. In wild-type mice, SnPP did not affect GFR or mean arterial pressure

but did reduce renal blood flow, a finding similarly observed in other studies of metalloporphyrin inhibitors in renal hemodynamics in rats.⁴¹ In sickle mice, the renal hemodynamic response to SnPP was comparable to that observed in wild-type mice, thereby indicating that the maintenance of renal hemodynamics in SCD is not inordinately dependent on the HO system, as has been described for other vasodilator systems such as vasorelaxant prostaglandins.⁴²

In contrast, chronic administration of SnPP exerted divergent effects in wild-type and sickle mice. In wild-type mice, chronic administration of SnPP induced tubulointerstitial inflammation and fibrosis, which were accompanied by up-regulation of genes for interstitial and basement membrane collagens, a fibrogenic cytokine (transforming growth factor- $\beta 1$), and a pro-inflammatory cytokine (IL-6). Studies of the expression of these proteins in response to SnPP would complement the effects of SnPP on histological injury and gene expression and would be of interest. It is notable that the unstressed,

Figure 4. Gene expression in the kidney in wild-type mice (WT) and sickle mice (Sickle) treated with SnPP or vehicle. WT and sickle mice were treated with SnPP (30 μ mol/kg of body weight, solid bar, $n = 7$) or vehicle (open bar, $n = 5$) every other day over 25 days. Quantitative real-time RT-PCR was used to determine mRNA expression for collagen III ($\alpha 1$) (A), collagen IV ($\alpha 1$) (B), transforming growth factor- $\beta 1$ (C), and IL-6 (D). * $P < 0.05$ versus vehicle-treated group in wild-type mice; # $P < 0.05$ versus vehicle-treated group in sickle mice.

intact kidney in wild-type rodents exhibits basal HO activity that reflects, mainly, activity originating from the HO-2 isoform. These data for wild-type mice thus imply that HO-2 exerts an anti-inflammatory effect in the kidney,

Figure 5. BUN determined 24 hours after renal ischemia in wild-type mice (WT) treated with vehicle (open bar, $n = 15$) or SnPP (solid bar, $n = 14$) and sickle mice (Sickle) treated with vehicle (open bar, $n = 9$) or SnPP (solid bar, $n = 9$). SnPP (50 μ mol/kg of body weight, i.p.) or vehicle was administered 14 hours before the induction of bilateral renal ischemia imposed for 15 minutes. * $P < 0.05$ versus vehicle-treated sickle mice.

Figure 6. Semiquantitative scores for acute tubular necrosis determined 24 hours after renal ischemia in wild-type mice (WT) treated with vehicle (open bar, $n = 15$) or SnPP (solid bar, $n = 14$) and sickle mice (Sickle) treated with vehicle (open bar, $n = 9$) or SnPP (solid bar, $n = 9$). SnPP (50 μ mol/kg of body weight, i.p.) or vehicle was administered 14 hours before the induction of bilateral renal ischemia imposed for 15 minutes. * $P < 0.05$ versus vehicle-treated sickle mice.

because chronic inhibition of such activity, *in the absence of any other insult*, leads to tubulointerstitial inflammation and fibrosis, a finding that, to the best of our knowledge, has not been described in the literature. For at least two reasons, it seems unlikely that this effect of SnPP, chronically administered to wild-type mice, reflects the reduction in RBF that was observed in wild-type mice 24 hours following the administration of a single dose of SnPP. First, in the short-term protocol, a comparable reduction in RBF was observed in sickle mice treated with a single dose of SnPP, yet sickle mice failed to evince any tubulointerstitial inflammation in the chronic study. Second, the effect of SnPP on RBF as observed in the short-term protocol may not necessarily predict the changes that occur following more chronic administration of SnPP, because, as is well recognized, chronic inhibition of a given vasoactive system is commonly accompanied and offset by secondary changes in other vasoactive systems.

In sickle mice, the observed effect of chronic administration of SnPP was quite contrary to the exacerbatory effect that we initially hypothesized would occur. Instead, SnPP decreased vascular congestion and, unlike what was observed in the wild-type mice, SnPP failed to induce either tubulointerstitial disease or injury-related genes. Remarkably, chronically administered SnPP significantly reduced the elevated IL-6 mRNA expression observed in the kidney in vehicle-treated sickle mice to the levels observed in the vehicle-treated wild-type mice. It is thus possible that the reduced vascular congestion and the relative absence of inflammation observed in sickle mice may be related to the normalization of IL-6 by SnPP. In this regard, it is notable that in rat macrophages, hypoxia-reoxygenation induces HO-1 and IL-6, and the

Figure 7. Histological examination of the kidney 24 hours after ischemia in vehicle-treated sickle mice (**A** and **C**) and SnPP-treated sickle mice (**B** and **D**). **A** and **B** represent the kidney cortex and are at an original magnification of $\times 100$, whereas the **C** and **D** represent the outer medulla and are at an original magnification of $\times 200$. All sections are stained with hematoxylin and eosin.

latter is suppressed when SnPP is present at the time of hypoxia-reoxygenation.⁴³ In human SCD, serum levels of IL-6 are increased during steady state^{44–46} and are incriminated in the evolution of the pro-inflammatory phenotype of SCD.⁴⁷ Although IL-6 is pleiotropic in nature, pro-inflammatory and procoagulant effects of IL-6 are well described and are incriminated in the pathogenesis of certain nephritides and acute ischemic injury.^{48,49} We speculate that the ameliorative effect of SnPP in the kidney in sickle mice may reflect, at least in part, the normalization of the expression of IL-6.

A protective effect of SnPP was also seen in studies in which sickle mice were stressed by an episode of renal ischemia. As previously reported,³¹ and again observed in the present studies, sickle mice exhibit, as compared with wild-type mice, increased sensitivity to acute renal ischemia. In the protocol of renal ischemia used in the current study, SnPP did not affect renal function in wild-type mice or significantly worsen renal histological injury. However, in sickle mice subjected to ischemia, the effect of SnPP was again contrary to the hypothesized exacer-

batory effect: specifically, prior treatment with SnPP protected against renal ischemic injury in sickle mice, preventing the deterioration of renal function in sickle mice above that observed in the ischemic wild-type mice. Accompanying this protection against renal dysfunction by SnPP was a reduction in tubular necrosis and vascular congestion as assessed by semiquantitative analyses.

In the vast majority of states of tissue injury in which SnPP is used as an inhibitor of HO, exacerbation of tissue injury is clearly observed^{10–13}; however, in a minority of settings, a protective effect of SnPP and other metalloporphyrins is also described, including in models of renal injury. For example, in studies of heme protein-induced injury to proximal tubules *in vitro*, prior treatment with SnPP protects against such injury,⁵⁰ whereas the administration of SnPP in relatively high doses reduces renal ischemic injury.⁵¹ The mechanisms underlying the protection conferred by SnPP in SCD remain to be explored and, to the extent that such mechanisms are derived from inhibiting HO activity, may include the following possibilities.^{52,53} First, HO activity releases iron from the heme

prosthetic group, and if induction of HO activity (which occurs in this murine model of SCD) is inadequately or ineffectively coupled to iron-storage and iron-transport processes that restrain elevations in cellular levels of "free" iron, iron-catalyzed oxidative stress may occur. In this regard, in fibroblasts with varying levels of HO overexpression, relatively lower levels of overexpression protect against oxidant injury, whereas higher levels of HO-1 expression exacerbate such injury, the latter associated with elevation in cellular iron content.^{54,55} It is conceivable that the induction of HO-1, which occurs in sickle mice, may represent a level of overexpression of HO-1 that is maladaptive and injurious rather than adaptive and protective. Second, other products of HO, such as CO and bilirubin, although clearly cytoprotective in lower amounts, can be toxic at higher concentrations, and if these products were excessively generated in SCD, SnPP would confer protection by interrupting the heightened generation of these products and attendant tissue injury. Third, the inhibition of HO activity by SnPP and other metalloporphyrins is accompanied by a reciprocal increase in HO-1 mRNA and protein.^{51,56} This remarkable effect of SnPP (inhibiting HO activity and inducing HO-1 protein) was first discovered by Sardana and Kappas⁵⁶ some 20 years ago in studies undertaken in the liver, and more recent studies have confirmed that SnPP evinces a similar reciprocal effect in the kidney.⁵¹ In this regard, it is possible that the HO system may confer protection not simply and only from HO activity but also through signaling and other effects of the HO-1 protein per se.⁵¹ Relevant to this consideration is the fact that when HO-1 is induced in injured tissue, the extent of elevation in HO-1 protein commonly outstrips the degree of elevation in HO activity, and when HO activity is inhibited by SnPP, there is further augmentation in the expression of HO-1 protein. It is possible that through protein-protein interactions or other effects of the HO-1 protein, beneficial effects may be conferred. Fourth, by inhibiting HO, SnPP may reduce systemic and tissue levels of bilirubin (an antioxidant), thus potentially fostering a pro-oxidant state. Such oxidative stress by SnPP may oxidatively denature sickle red blood cells, the latter effect promoting their removal from the circulation by the reticuloendothelial system, and thereby leading, as was observed in the present study, to reduced vascular congestion in the kidneys from sickle mice chronically treated with SnPP.

The observed protective effects of SnPP in the current study in murine SCD, a disease in which tissues are exposed to increased amounts of an abnormal hemoglobin and heme, diverge from findings in prior studies that have examined the effect of modulating HO activity in tissues exposed to increased amounts of heme proteins and/or heme. For example, SnPP exacerbates renal injury in the glycerol model of heme protein-mediated, acute renal injury;¹⁷ zinc protoporphyrin worsens heme-induced apoptosis of renal tubular epithelial cells;⁵⁷ overexpression of HO-1 protects against heme protein-induced endothelial cell injury;¹⁸ and an HO-1 deficiency state markedly exacerbates acute heme protein-mediated nephrotoxicity and chronic heme protein-mediated

renal inflammation.^{19,20} Moreover, in quite recent studies in murine SCD using the dorsal skin fold chamber to assess venular blood flow, SnPP exacerbated stasis in venules that was induced by exposing sickle mice to hypoxia-reoxygenation.⁵⁸ To the extent that tissue injury in SCD reflects heme protein/heme-induced injury, and the biological effects of SnPP result from inhibition of HO activity, it is notable, and quite unexpected, that in the present studies SnPP reduces vascular congestion and tissue injury in unstressed and stressed sickle mice.

It is also possible that the protective effects of SnPP may be entirely independent of the HO system, and in this regard there is evidence that SnPP inhibits nitric-oxide synthase activity, guanylyl cyclase activity, and the generation of atrial natriuretic peptide^{59,60}; metalloporphyrins may also inhibit caspase activity.⁶¹ It should also be emphasized that, because it is quite possible that the effects of SnPP may be entirely independent of the HO system, then clearly additional strategies are needed to precisely determine the functional significance of HO activity and HO-1 in SCD.

The doses and dosing of SnPP used in the present study are based on regimens used in published studies. The acute studies (Protocols I and III) used a single dose of SnPP, 50 $\mu\text{mol/kg}$ of body weight i.p., as previously used, for example, in studies that examined the anti-thrombotic effect of HO-1 in microvascular beds.³⁵ The chronic study (Protocol II) used the dose and dosing regimen (30 $\mu\text{mol/kg}$ of body weight i.p. every other day) previously used in studies of a murine cardiac allograft model³⁸; this dose of SnPP has also been administered daily for more than 50 days in a model of mouse-to-rat cardiac transplants.⁶² Similarly, a comparable dose and dosing regimen for tin mesoporphyrin (5 mg/100 g body weight i.p., equivalent to 66 $\mu\text{mol/kg}$ of body weight i.p.), was administered three times per week for 3 weeks in a two-kidney, one-clip model in the rat.⁶³ These regimens for the administration of SnPP may lead to an accumulation of the compound in the kidney, and as discussed above there may be other effects of SnPP besides the inhibitory effect on HO activity. Additionally, it is possible that SnPP may be differentially accumulated in the kidney in wild-type and sickle mice. Another consideration is that tin, per se, is an inducer of HO-1 and exerts biologically active effects.⁶⁴ Nonetheless, we wish to underscore that our measurements of HO activity confirmed that the dose and dosing regimen for the administration of SnPP exerted quite comparable and highly effective inhibition of HO activity in wild-type and sickle mice.

In summary, we demonstrate that SnPP, a widely used inhibitor of HO activity, exerts the following renal effects: 1) comparable effects on renal hemodynamics in wild-type and sickle mice following short-term administration, 2) divergent effects when chronically administered, inducing renal injury in wild-type mice, while attenuating vascular congestion and expression of injury-related genes in sickle mice, and 3) a protective effect against renal ischemic injury in sickle mice. Whatever the mechanisms underlying the beneficial effects of SnPP, and the extent to which these effects of SnPP are affected by inhibiting HO activity, these unexpected findings uncover

an experimental maneuver that protects against renal injury in murine SCD.

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