Long-Term Endothelin-A Receptor Antagonism Provides Robust Renal Protection in Humanized Sickle Cell Disease Mice

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ABSTRACT

Sickle cell disease (SCD)—associated nephropathy is a major source of morbidity and mortality in patients because of the lack of efficacious treatments targeting renal manifestations of the disease. Here, we describe a long-term treatment strategy with the selective endothelin-A receptor (ETA) antagonist, ambrisentan, designed to interfere with the development of nephropathy in a humanized mouse model of SCD. Ambrisentan preserved GFR at the level of nondisease controls and prevented the development of proteinuria, albuminuria, and nephrinuria. Microscopy studies demonstrated prevention of podocyte loss and structural alterations, the absence of vascular congestion, and attenuation of glomerulosclerosis in treated mice. Studies in isolated glomeruli showed that treatment reduced inflammation and oxidative stress. At the level of renal tubules, ambrisentan treatment prevented the increased excretion of urinary tubular injury biomarkers. Additionally, the treatment strategy prevented tubular brush border loss, diminished tubular iron deposition, blocked the development of interstitial fibrosis, and prevented immune cell infiltration. Furthermore, the prevention of albuminuria in treated mice was associated with preservation of cortical megalin expression. In a separate series of identical experiments, combined ETA and ETB receptor antagonism provided only some of the protection observed with ambrisentan, highlighting the importance of exclusively targeting the ETA receptor in SCD. Our results demonstrate that ambrisentan treatment provides robust protection from diverse renal pathologies in SCD mice, and suggest that long-term ETA receptor antagonism may provide a strategy for the prevention of renal complications of SCD.


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Sickle cell disease (SCD) is an autosomal recessive monogenic blood disorder that affects approximately 100,000 people in the United States and millions globally.1,2 The central pathology of the disease is the polymerization of mutant hemoglobin S (HbS). This process sets in motion a cascade of events, beginning with erythrocyte sickling, endothelial activation, and vaso-occlusion, that ultimately result in chronic tissue hypoxia, inflammation, and organ damage.3 Advances in the management of SCD throughout the past half-century have led to a progressive increase in life expectancy with a concomitant increase in the prevalence of chronic disease manifestations.4,5 Sickle cell nephropathy now represents one of the most serious organ-specific complications facing patients with SCD. Renal pathologies in SCD occur at
both the structural and functional level and include deteriora-
tion of the glomerular filtration barrier, progressive loss of
functional glomeruli, tubulointerstitial injury, defective
urine-concentrating ability, and dysfunction of the distal
nephron. Evidence of nephropathy, as manifested by albu-
minuria or proteinuria, occurs in nearly two-thirds of adults
with SCD, and presently, almost one in five patients with SCD
die of renal disease. This level of disease burden occurs due
to lack of availability of robustly renal-protective therapies
targeted to the mechanisms that underlie sickle nephropathy.

Endothelin-1 (ET-1) is a signaling peptide produced by di-
verse cell types that exerts its physiologic and pathophysiologic
actions by binding to two receptor subtypes, ET$_A$ and ET$_B$.
ET$_A$ receptor activation induces vasoconstriction, inflam-
maciation, mitogenesis, and nociception. These effects can be
counteracted by ET$_B$ activation in some tissues, highlighting
the importance of the balance between the two receptor
subtypes. ET-1 expression is induced by established hall-
marks of the SCD milieu, including hypoxia, oxidative stress,
and thrombosis. This theoretic connection between SCD and
urine-concentrating ability, and dysfunction of the distal
tubule.6 Evidence of nephropathy, as manifested by albu-
minuria or proteinuria, occurs in nearly two-thirds of adults
with SCD, and presently, almost one in five patients with SCD
die of renal disease.7,8 This level of disease burden occurs due
to lack of availability of robustly renal-protective therapies
targeted to the mechanisms that underlie sickle nephropathy.

Characteristics of Experimental Animals
Vehicle-treated 14-week-old HbSS mice had significantly
higher spleen-to-body weight ratio and daily urine volume,
and decreased urine osmolality, when compared with age-
matched vehicle-treated HbAA mice. Two-week treatment
with selective ET$_A$ receptor antagonist, ambrisentan, signifi-
cantly reduced spleen-to-body weight ratio of HbSS mice. No
parameters were changed with nonselective ET$_{A/B}$ receptor
antagonist, A-182086, treatment in HbSS mice (Tables 1 and
2). Neither antagonist had any effect on the characteristics of
tubule in control HbAA mice (Table 3). Ambrisentan signifi-
cantly reduced albuminuria, nephrinuria, and P$_{lab}$ in HbSS mice,
whereas A-182086 significantly attenuated only P$_{lab}$ and the
effect of dual antagonism did not reach statistical significance in the
reduction of albuminuria and nephrinuria (Table 3). These
results suggest that ET$_A$ activation contributes to the main-
tenance of dysfunction of the glomerular filtration barrier in
established sickle nephropathy, a finding that is consistent
with previous results from our laboratory.22

We also determined if ET signaling contributes to the main-
tenance of tubular injury in SCD, because final urinary albu-
nin excretion is the result of both glomerular filtration and
proximal tubular uptake. Thus, we measured urinary excre-
tion of kidney injury marker 1 (KIM-1) and N-acetyl-\(\beta\)-D-
-glucosaminidase (NAG), markers of proximal tubule injury.
Both markers were significantly increased in vehicle-treated
HbSS mice compared with controls. Ambrisentan signifi-
cantly attenuated KIM-1 and NAG, whereas A-182086 had
no significant effect on these markers in HbSS mice (Table 3).
Neither antagonist had any effect on markers of glomerular or
tubular injury in control HbAA mice (Table 3).

Long-Term Ambrisentan Treatment Prevents Onset of
Glomerular Injury
To investigate the pathophysiologic relevance of ET signaling in
the development of sickle nephropathy, we examined the effect of
10-week treatment (from weaning into adulthood) with ET

RESULTS

Short-Term ET Receptor Antagonism Attenuates
Glomerular and Tubular Injury
To investigate the contribution of ET signaling to the mainte-
nance and progression of established nephropathy in SCD, we
first examined the effect of 2-week treatment with ET receptor
antagonists on glomerular and tubular dysfunction in HbSS
and HbAA mice. Vehicle-treated HbSS mice had significantly
higher proteinuria, albuminuria, nephrinuria, and glomerular
permeability to albumin (P$_{lab}$) when compared with HbAA
controls (Table 3). Ambrisentan significantly reduced albu-
minuria, nephrinuria, and P$_{lab}$ in HbSS mice, whereas
A-182086 significantly attenuated only P$_{lab}$ and the effect of
dual antagonism did not reach statistical significance in the
reduction of albuminuria and nephrinuria (Table 3). These
results suggest that ET$_A$ activation contributes to the main-
tenance of dysfunction of the glomerular filtration barrier in
established sickle nephropathy, a finding that is consistent
with previous results from our laboratory.22
Table 1. Characteristics of experimental animals after 2 weeks of treatment with ET receptor antagonists

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HBAA</th>
<th>HBSS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Ambrisentan</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>M: 25.7±1.2</td>
<td>M: 28.8±0.8</td>
</tr>
<tr>
<td></td>
<td>F: 22.3±1.0</td>
<td>F: 21.7±0.5</td>
</tr>
<tr>
<td>Right kidney/body weight, mg/g</td>
<td>6.6±0.3</td>
<td>6.5±0.4</td>
</tr>
<tr>
<td>Left kidney/body weight, mg/g</td>
<td>6.2±0.3</td>
<td>5.8±0.3</td>
</tr>
<tr>
<td>Spleen/body weight ratio, %</td>
<td>0.9±0.1</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>Water intake, ml/24 h</td>
<td>3.2±0.3</td>
<td>4.7±0.5</td>
</tr>
<tr>
<td>Urine excretion, ml/24 h</td>
<td>1.0±0.2</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td>Urine osmolality, mOsm/kg</td>
<td>2482±147</td>
<td>2299±121</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>120±1</td>
<td>119±2</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.09±0.01</td>
<td>0.10±0.01</td>
</tr>
</tbody>
</table>

Data are means±SEM (n=12–14 in HBSS groups and n=6–9 in HBAA groups). M, male; F, female.

Table 2. Characteristics of experimental animals after 10 weeks of treatment with ET receptor antagonists

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HBAA</th>
<th>HBSS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Ambrisentan</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>M: 32.2±0.9</td>
<td>M: 30.5±0.9</td>
</tr>
<tr>
<td></td>
<td>F: 24.9±1.5</td>
<td>F: 24.3±0.7</td>
</tr>
<tr>
<td>Right kidney/body weight, mg/g</td>
<td>6.6±0.3</td>
<td>6.4±0.3</td>
</tr>
<tr>
<td>Left kidney/body weight, mg/g</td>
<td>5.6±0.5</td>
<td>5.8±0.2</td>
</tr>
<tr>
<td>Spleen/body weight ratio, %</td>
<td>0.8±0.1</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>Water intake, ml/24 h</td>
<td>3.8±0.6</td>
<td>4.0±0.3</td>
</tr>
<tr>
<td>Urine excretion, ml/24 h</td>
<td>1.0±0.3</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>Urine osmolality, mOsm/kg</td>
<td>2574±415</td>
<td>2257±185</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>121±2</td>
<td>115±2</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.10±0.01</td>
<td>0.11±0.01</td>
</tr>
</tbody>
</table>

Data are means±SEM (n=9–10 in HBSS groups and n=6–9 in HBAA groups). M, male; F, female.

Table 3. Effects of ambrisentan and A-182086 on proteinuria in HbSS mice

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HbAA Vehicle</th>
<th>Ambrisentan</th>
<th>A-182086</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinuria, mg/dl</td>
<td>0.1±0.01</td>
<td>0.1±0.01</td>
<td>0.1±0.01</td>
</tr>
</tbody>
</table>

Data are means±SEM (n=9–10 in HBSS groups and n=6–9 in HBAA groups). M, male; F, female.

Overall ambrisentan significantly prevented or attenuated all of these changes in glomerular morphology in HbSS mice (Figure 2). Neither antagonist had any effect on markers of glomerular structure or function in control HbAA mice (Supplemental Figures 1 and 2). These results suggest that ET receptor activation contributes to the development and progression of glomerulopathy in SCD.

Next, we examined the role of ET signaling in the development of sickle cell glomerulopathy by measuring structural and functional markers of glomerular injury in HbSS mice. Both ambrisentan and A-182086 prevented the increase in PAH in glomeruli isolated from HbSS mice (Figure 1C). Moreover, both antagonists prevented increases in nephrinuria (Figure 1E). However, only the selective ETA receptor antagonist prevented albuminuria in HbSS mice (Figure 1D). Importantly, ambrisentan treatment preserved GFR at the level of normal controls (Figure 1F), whereas A-182086 treatment had no effect on the decline in GFR observed in HbSS mice. (Figure 1F). Histologic analysis of glomerular structure showed glomerular hypertrophy, basement membrane of Bowman’s capsule thickening, glomerulosclerosis, and vascular congestion in vehicle-treated HbSS mice. Only ambrisentan significantly prevented or attenuated all of these changes in glomerular morphology in HbSS mice (Figure 2). Neither antagonist had any effect on markers of glomerular structure or function in control HbAA mice (Supplemental Figures 1 and 2). These results suggest that ETA activation contributes to the development and progression of glomerulopathy in SCD.

Long-Term Ambrisentan Treatment Prevents Onset of Podocyte Injury

Podocyte injury is a key feature of alteration in the integrity of the glomerular filtration barrier and, ultimately, proteinuric nephropathy. Thus, we examined the effect of the long-term ET receptor antagonism on podocyte phenotypic changes. Podocyte number per glomerulus, as determined by Wilms tumor 1 (WT-1) immunohistochemistry, was significantly decreased in vehicle-treated HbSS mice, and was concomitant with reduced mRNA levels of the podocyte markers nephrin, podocin, and synaptopodin. Both antagonists prevented podocyte loss, demonstrated by WT-1-positive glomerular cells and mRNA expression of podocyte markers (Figure 3, A–F). Transmission

electron microscopy studies in glomeruli of vehicle-treated HbSS mice showed evidence of more podocyte foot process fusion and effacement, and glomerular basement membrane thickening, whereas prevalence of these structural defects was similar to vehicle-treated nondisease controls in mice treated with both antagonists (Figure 3G). Neither antagonist had any effect on markers of podocyte injury in control HbAA mice (Supplemental Figure 3). These results suggest that ETA activation contributes to the development and progression of podocyte injury in SCD.

**Long-Term Ambrisentan Treatment Prevents Glomerular Dysfunction**

To elucidate the mechanism underlying the nephroprotective effect of ET receptor antagonism on glomeruli of HbSS mice, we examined endothelial activation, inflammation, and oxidative stress, hallmarks of glomerular dysfunction. Isolated glomeruli from HbSS mice treated with both antagonists demonstrated nondisease control–level sensitivity to phorbol 12-myristate 13-acetate–stimulated reactive oxygen species (ROS) production (Figure 4G). By contrast, only the selective ETA receptor antagonist prevented the induction of vascular adhesion molecules and proinflammatory marker expression in glomeruli from HbSS mice (Figure 4, D–F). Neither antagonist had any effect on glomerular dysfunction in control HbAA mice (Supplemental Figure 4). These results suggest that ETA receptor antagonism successfully protects from ET-mediated glomerular alterations in sickle nephropathy.

**Long-Term Ambrisentan Treatment Prevented Tubular Injury**

On the basis of data from the short-term treatment protocol, we analyzed markers of injury and histologic structure of the proximal tubules in HbSS mice. Increased urinary KIM-1 and NAG excretion were prevented by ambrisentan compared with vehicle-treated HbSS mice (Figure 5, A and B). Moreover, ambrisentan prevented renal inflammation and fibrosis, possibly through blocking T cell and macrophage infiltration (Figure 6, A–F) as well as the induction of proinflammatory mediators (Figure 6, G–I). In contrast, antagonism of both ET receptors had no significant effect on tubular injury in HbSS mice when compared with the selective ETA receptor antagonist (Figures 5 and 6). These results were confirmed by histologic analysis where...
ambrisentan preserved brush border thickness (Figure 5, D and G), prevented interstitial fibrosis (Figure 5, E and H), and reduced tubular iron deposition associated with nondisease levels of urinary neutrophil gelatinase-associated lipocalin (NGAL), iron binding protein, excretion in HbSS mice (Figure 5, C, F and I). Neither antagonist had any effect on tubular structure and function in control HbAA mice (Supplemental Figures 5 and 6).

**DISCUSSION**

Renal complications of SCD include a variety of glomerular and tubular abnormalities, however, the pathogenesis of these complications remains unclear. This study demonstrated that long-term ETA receptor antagonism prevented the development and progression of sickle nephropathy, thus demonstrating that ETA receptor signaling is a critical mechanistic mediator in renal complications associated with SCD. These studies provide the basis for the use of ETA receptor antagonism as a potential therapeutic approach for the prevention of renal involvement in SCD.

Abundant evidence implicates endothelial dysfunction in many proteinuric renal diseases, suggesting that activation of ET receptors may lead to glomerular endothelial and tubular dysfunction, thereby contributing to proteinuria. Our group has previously demonstrated that hypoxia increases glomerular ET-1 expression in C57BL/6J mice, but not in endothelial-specific ET-1 knockout mice. Also, ET-1 mRNA expression in glomeruli isolated from HbSS mice was shown to be significantly elevated. These data may suggest a mechanism for ET-1–induced nephropathy in SCD. In this study, we observed significantly elevated plasma levels of ET-1 and proteinuria in HbSS mice, consistent with prior reports of elevated ET-1 levels and proteinuria in patients with SCD. Albuminuria is linked with progression of nephropathy in SCD and has been associated with a decline in GFR and glomerular permeselectivity in SCD. This is consistent with other proteinuric renal diseases where proteinuria correlates with decline in GFR. In line with human disease, our results in humanized SCD mice provided evidence that prevention of proteinuria, with selective ETA receptor antagonism, was associated with preserved GFR.

Our observations suggest a pathophysiologic link between increased ET signaling and the impairment of glomerular function in SCD. Moreover, given the often opposing actions of ETA and ETB receptors, we asked the important question of whether selective ETA or nonselective ETA/ETB receptor antagonism provides superior or equivalent renal protection in SCD. This is particularly relevant given that both the combined ETA/ETB receptor antagonists, bosentan and macitentan, and the selective ETA receptor antagonist, ambrisentan, are currently Food and Drug Administration–approved for treatment of pulmonary arterial hypertension. The clinical experience that now exists with both selective and nonselective ET receptor antagonists minimizes barriers in the path to clinical translation of our findings.

The contribution of ETB receptor signaling to proteinuria and P_{ab} in SCD could not be directly assessed using a selective ETB receptor antagonist in vivo because of hypertension, reduced ET-1 clearance, and increased ETA receptor activation.
Figure 2. Histological analysis reveals that selective ET\(_A\) receptor antagonism preserves glomerular morphology and prevents vascular congestion in humanized sickle cell mice. Histologic examination of the renal cortex of genetic control (HbAA) and humanized sickle mice (HbSS) treated with vehicle, the selective ET\(_A\) antagonist, ambrisentan, or the combined ET\(_{A/B}\) antagonist, A-182086, for 10 weeks beginning at 4 weeks of age. (A) Depicts representative Masson trichrome–stained sections of glomeruli. Original magnification, \( \times 40 \) (scale bar=50 \( \mu \)m). (B) Depicts quantification of (A) represented as sclerosis index score. (C) Depicts glomerulomegaly represented as mean area of glomeruli (square micrometer). (D) Depicts number of glomeruli per square millimeter. (E) Depicts representative hematoxylin and eosin–stained sections of glomeruli. Original magnification, \( \times 40 \) (scale bar=50 \( \mu \)m). (F) Depicts glomerular congestion represented as percentage of glomeruli with congestion. (G) Depicts basement membrane of Bowman’s capsule thickening score. Data are mean±SEM; \( n=5 \) in HbAA and HbSS groups; *\( P<0.05 \) versus vehicle-treated HbAA; #\( P<0.05 \) versus vehicle-treated HbSS. All of the glomerular characteristics were counted in ten sections per slide (minimum 20 glomeruli).
Figure 3. ET<sub>A</sub> receptor antagonism prevents podocyte loss and preserves podocyte structure in humanized sickle cell mice. Immunohistochemical examination and mRNA expression of markers of podocyte injury of kidneys from genetic control (HbAA) and humanized sickle mice (HbSS) treated with vehicle, the selective ET<sub>A</sub> antagonist, ambrisentan, or the combined ET<sub>A/B</sub> antagonist, A-182086, for 10 weeks beginning at 4 weeks of age. (A) Depicts representative WT-1–positive stained sections of glomeruli. Original
that result from ET<sub>B</sub> receptor antagonism. Our results demonstrated that treatment with a selective ET<sub>A</sub> receptor antagonist significantly attenuated P<sub> Alb</sub> in glomeruli from HbSS mice. Treatment with the combined ET<sub>A/B</sub> receptor antagonist had a similar effect, indicating that the effect of ET-1 on P<sub> Alb</sub> is most likely exclusively mediated by ET<sub>A</sub> receptors. On the basis of the lower expression of ET<sub>B</sub> receptors compared with ET<sub>A</sub> receptors in glomeruli, our results support a specific role of ET<sub>A</sub> receptors in controlling P<sub> Alb</sub> and are consistent with previous studies that have shown that ET<sub>B</sub> receptor antagonism does not alter ET-1–induced elevation of P<sub> Alb</sub> in vitro. However, because P<sub> Alb</sub> measurements are performed in isolated decapsulated glomeruli independent of hemodynamic influences, a potential role of ET<sub>B</sub> receptors on glomerular filtration through hemodynamic mechanisms in SCD cannot be excluded. The mechanism contributing to glomerular injury in mice treated with the combined ET<sub>A/B</sub> receptor antagonist may be an inability to prevent congestion in glomerular capillaries, ultimately leading to impairment of renal hemodynamics evidenced by reduced GFR. This idea is in accordance with other studies showing a relationship between early alterations in renal hemodynamics and heightened histologic injury after renal ischemia in SCD. Moreover, rats lacking ET<sub>B</sub> receptors develop more severe renal injury and proteinuria in response to hyperglycemia or nephrectomy. In contrast, we observed significant decreases in urinary albumin and protein excretion only in HbSS mice treated with the selective ET<sub>A</sub> receptor antagonist, again suggesting a crucial role of ET<sub>A</sub> receptors in mediating glomerular injury in SCD.

Intact podocytes are essential for the integrity of the glomerular filtration barrier and podocyte injury leads to albuminuria. Importantly, podocytes express ET<sub>A</sub> and ET<sub>B</sub> receptors suggesting a target for elevated ET-1. In addition, several studies have shown that disruption of the podocyte actin cytoskeleton occurs after exposure to ET-1, and may contribute to the antiproteinuric effect of the ET<sub>A</sub> receptor antagonist. Our data highlight the importance of selectively targeting the ET<sub>A</sub> receptor to achieve maximal renal protection in SCD.

Recent studies have re-emphasized the role of proximal tubular uptake of albumin in protection against albuminuria. Renal tubules are known to be highly susceptible to hypoxic conditions and it has been reported that urinary KIM-1 and NAG levels, biomarkers of tubular injury, strongly correlate with albuminuria in patients with SCD. Therefore, we investigated the contribution of proximal tubular injury to albuminuria in SCD. We found that selective ET<sub>A</sub> receptor antagonism protects against tubular injury in HbSS mice, which may contribute to the antiproteinuric effect of the ET<sub>A</sub> receptor antagonist. These observations are also in agreement with those reported by Hocher et al. and may suggest an additional benefit of the use of ET<sub>A</sub> receptor antagonists in the treatment of sickle nephropathy. Our results suggest that renal tubular protein uptake may involve ET<sub>B</sub> receptor activation. Although proteinuria is often presumed to be a result of glomerular damage in SCD, the question of whether tubular injury contributes to impaired albuminuria or whether tubular injury is the consequence of a continual protein load remains unanswered. One explanation of the lack of reduction in albuminuria and proteinuria after administration of the dual ET<sub>A/B</sub> receptor antagonist in HbSS mice is that disruption of glomerular structure by ET-1, via ET<sub>A</sub> receptor activation, leads to alterations in podocyte morphology and function, resulting in increased P<sub> Alb</sub> and albuminuria/proteinuria in SCD mice. However, a recently published study demonstrated beneficial effects of podocyte-specific deletion of both ET<sub>A</sub> and ET<sub>B</sub> receptors, resulting in protection from podocyte loss and mesangial matrix expansion in diabetic nephropathy. Further studies with the use of podocyte-specific ET<sub>A</sub> or ET<sub>B</sub> receptor knockout mice are required to resolve this important question in SCD.

In this study, a protective effect on glomerular congestion and size was observed in HbSS mice treated with the selective ET<sub>A</sub> but not the dual ET<sub>A/B</sub> receptor antagonist. In contrast, Lenoir et al. reported significant benefit on glomerular structure in hypoxia-exposed SCD mice treated with the dual ET receptor antagonist, bosentan. However, bosentan is 30 times more selective for ET<sub>A</sub> versus ET<sub>B</sub> receptors, and when considered in the context of this study, this suggests that renal protection in previous studies may have been mediated primarily by blockade of ET<sub>A</sub> receptors in bosentan-treated SCD mice. Thus, our data highlight the importance of selectively targeting the ET<sub>A</sub> receptor to achieve maximal renal protection in SCD.
mice may involve increased albumin endocytosis in the proximal tubule followed by cell proliferation and injury. A second hypothesis is that blocking of ETₐ receptors in renal proximal tubules may cause a decrease in tubular albumin uptake, an increase in urinary albumin excretion, and ultimately tubular apoptosis and injury. This hypothesis is
supported by several studies implicating ET-1 as proapoptotic in the kidney.55–58 Our results demonstrate that, although the nonselective ET\textsubscript{A/B} receptor antagonist improved glomerular structure and function to some extent in HbSS mice, increased urinary albumin excretion was still present, thus excluding glomerular injury as the only cause of albinunuria. Also, the observed downregulation of megalin and FcRn may be related to the oxidative stress–mediated transcription factor, NFκB, activation and facilitated release of proinflammatory and profibrotic mediators, including MCP-1 or P-selectin.59 These results are also consistent with the protective role of ET\textsubscript{B} receptor in proximal tubules. Moreover, data from our laboratory has shown a protective role of ET\textsubscript{B} receptor activation in tunicamycin–induced renal tubular apoptosis.60 Thus, our results suggest that the albuminuria observed in HbSS mice treated with the dual ET\textsubscript{A/B} receptor antagonist is a combination of glomerular and tubular injury or dysfunction.

In conclusion, this study implicates the ET\textsubscript{A} receptor as an important mediator in the development and progression of renal

Figure 5. Long-term ET\textsubscript{A} receptor antagonism prevents renal tubular injury in humanized sickle cell mice. Histologic examination of the renal cortex and urinary excretion of markers of tubular injury of genetic control (HbAA) and humanized sickle mice (HbSS) treated with vehicle, the selective ET\textsubscript{A} antagonist, ambrisentan, or the combined ET\textsubscript{A/B} antagonist, A-182086, for 10 weeks beginning at 4 weeks of age. (A) Depicts the average urinary KIM-1 excretion after 10-week treatment protocol. (B) Depicts the average urinary NAG excretion after 10-week treatment protocol. (C) Depicts the average urinary NGAL excretion, iron-binding protein, after 10-week treatment protocol. Data are mean±SEM; *P<0.05 versus vehicle-treated HbAA; #P<0.05 versus vehicle-treated HbSS; n=9–10 in HbSS and n=9 in untreated HbAA group. (D) Depicts brush border thickness index score. (E) Depicts interstitial fibrosis index score. (F) Depicts quantification of iron deposition in the whole kidney sections (megapixel per micrometer). (G) Depicts representative periodic acid Schiffs–hematoxylin–stained sections of renal cortex. Original magnification, ×40 (scale bar=50 μm). (H) Representative Masson trichrome–stained sections of renal cortex and medulla. Original magnification, ×10 (scale bar=200 μm, respectively). (I) Depicts Prussian blue iron–stained sections of renal cortex. Original magnification, ×20 (scale bar=100 μm). Fibrosis and brush border thickness were assessed in ten sections per slide. Data are mean±SEM; *P<0.05 versus vehicle-treated HbAA; #P<0.05 versus vehicle-treated HbSS; n=5 in HbAA and HbSS groups.
Figure 6. Selective ETα receptor antagonism prevents renal cortical inflammation and immune cell infiltration in humanized sickle cell mice. Immunohistochemical examination and mRNA expression of proinflammatory and profibrotic markers in renal cortex of genetic control (HbAA) and humanized sickle mice (HbSS) treated with vehicle, the selective ETα antagonist, ambrisentan, or the combined ETαβ antagonist, A-182086, for 10 weeks beginning at 4 weeks of age. (A) Depicts representative CD3+–stained cortical sections. Original magnification, ×40 (scale bar=50 μm). (B) Depicts the quantification of glomerular CD3+ cells represented as the average
injury in SCD, and suggests that selective ET_{A} receptor antagonism is sufficient to prevent development and progression of renal injury in SCD. These findings provide rigorous proof-of-concept evidence to support the use of ET_{A} receptor antagonism in the treatment of sickle nephropathy, and by utilizing a well tolerated and clinically approved drug in our treatment strategy, the barriers to the next steps of clinical investigation are minimal.

CONCISE METHODS

Animals
Studies used humanized SCD knock-in mice, with notation: B6;129-HbaA^{tm1(HBA)/Tow}Hbbt^{tm2(HBG1,HBQ)/Tow}/Hbbt^{tm3(HBG1,HBQ)/Tow}, developed by Townes et al. Experimental animals (HbSS) were homozygous for mutant β globin (E to V at position 6) and expressed human HbS. Control animals (HbAA), derived from the same colony, were homozygous for wild-type β globin and expressed human HbA. All studies were performed in 14-week-old HbSS mice with similar numbers of males and females in each group and age-matched genetic controls. Group numbers varied depending on availability of mice from the breeding colony. All mice were housed under conditions of constant temperature, humidity, and 12-hour light/dark cycle, and were provided with food (Harlan Teklad) and water ad libitum. Separate groups of mice were placed in standard metabolic cages for 48 hours before the experiments. Mice were allowed to adapt to metabolic cages for 1 day before collection of 24-hour urine samples. All mice were maintained and studied in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals following a protocol reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committees.

Drug Treatment Protocols
The selective ET_{A} receptor antagonist ambrisentan (10mg/kg per day; AbbVie, Inc., Abbott Park, IL), nonselective ET_{A}/ET_{B} receptor antagonist A-182086 (10mg/kg per day; AbbVie, Inc., Abbott Park, IL), or vehicle were administrated in drinking water and the concentrations were adjusted daily according to the water intake, as previously described. Experimental animals (HbSS) were homoygous for mutant β globin and expressed human HbS. Control animals (HbAA), derived from the same colony, were homoygous for wild-type β globin and expressed human HbA. All studies were performed in 14-week-old HbSS mice with similar numbers of males and females in each group and age-matched genetic controls. Group numbers varied depending on availability of mice from the breeding colony. All mice were housed under conditions of constant temperature, humidity, and 12-hour light/dark cycle, and were provided with food (Harlan Teklad) and water ad libitum. Separate groups of mice were placed in standard metabolic cages for 3 days before collection of 24-hour urine samples. All mice were maintained and studied in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals following a protocol reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committees.

CONCISE METHODS

Animals
Studies used humanized SCD knock-in mice, with notation: B6;129-HbaA^{tm1(HBA)/Tow}Hbbt^{tm2(HBG1,HBQ)/Tow}/Hbbt^{tm3(HBG1,HBQ)/Tow}, developed by Townes et al. Experimental animals (HbSS) were homozygous for mutant β globin (E to V at position 6) and expressed human HbS. Control animals (HbAA), derived from the same colony, were homozygous for wild-type β globin and expressed human HbA. All studies were performed in 14-week-old HbSS mice with similar numbers of males and females in each group and age-matched genetic controls. Group numbers varied depending on availability of mice from the breeding colony. All mice were housed under conditions of constant temperature, humidity, and 12-hour light/dark cycle, and were provided with food (Harlan Teklad) and water ad libitum. Separate groups of mice were placed in standard metabolic cages for 48 hours before the experiments. Mice were allowed to adapt to metabolic cages for 1 day before collection of 24-hour urine samples. All mice were maintained and studied in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals following a protocol reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committees.

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BP Measurements
Standard tail-cuff BP measurements, using a pneumatic transducer, were obtained on five consecutive days from 13-week-old HbSS and HbAA mice that had undergone 5 days of prior training as previously described. Data are presented as the average of five measurements for each animal.

GFR Measurements
GFR was measured in 14-week-old HbSS and HbAA mice (1 day before metabolic cage studies) using transcutaneous measurement of fluorescein isothiocyanate–labeled sinestrin technique as previously described.

Measurements of P_{ab}
Glomeruli were isolated on ice by consecutive sieving technique as previously described with minor modifications. Briefly, renal cortical tissue was passed through a series of filters (with pore size 180, 100, and 50 μm) concluding in suspension of decapsulated, intact glomeruli in ice-cold Hanks Balanced Salt Solution, pH 7.4 (Corning, NY). Freshly isolated glomeruli were used for measurements of P_{ab} as previously described. In brief, change in glomerular volume was measured in response to an oncotic gradient induced by defined concentrations of albumin in the buffer solution.

Glomerular ROS Assay
Glomerular ROS production was measured using a luminescence assay as previously described.

Plasma and Urine Analyses
All urine analyses were performed on 24-hour urine samples collected in metabolic cages. Urinary protein concentration was measured using the Bradford assay (BioRad Laboratories, CA). Urinary albumin concentration was determined using an immunoperoxidase assay according to the manufacturer’s instructions (GenWay Biotech Inc., CA). Urinary nephrin concentration was measured using a mouse NPHN ELISA kit (Uscn Life Science Inc., TX). Urine levels of tubular injury markers were measured using ELISA for mouse KIM-1 (ab119596-KIM-1 [TIM-1] Mouse ELISA kit; Abcam, UK), NAG (Mouse NAG kit; Crystal Chem, IL), and NGAL (Lipocalin-2 [NGAL] Mouse ELISA kit; Abcam, UK). Plasma levels of ET-1 were determined by number of CD3^{+} cells per glomerulus (minimum 20 glomeruli counted). (C) Depicts the quantification of cortical nonglomerular CD3^{+} cells represented as the average number of nonglomerular CD3^{+} cells per field. (D) Depicts representative F4/80^{+}-stained cortical sections. Original magnification, ×10 (scale bar=50 μm). Arrows indicate F4/80^{+} cells. (E) Depicts the quantification of glomerular F4/80^{+} cells represented as the average number of F4/80^{+} cells per glomerulus (minimum 20 glomeruli counted). (F) Depicts the quantification of cortical nonglomerular F4/80^{+} cells represented as the average number of nonglomerular F4/80^{+} cells per field. CD3^{+} and F4/80^{+} cells were assessed in ten sections per slide and calculated. Data are mean±SEM; n=5 in HbAA and HbSS groups. (G) Depicts the relative MCP-1 mRNA expression in cortex after 10-week treatment protocol. (H) Depicts the relative P-selectin mRNA expression in cortex after 10-week treatment protocol. (I) Depicts the relative VCAM-1 mRNA expression in cortex after 10-week treatment protocol. Data are mean±SEM *P<0.05 versus vehicle-treated HbAA; #P<0.05 versus vehicle-treated HbSS; n=5–6 in HbSS and n=6 in vehicle-treated HbAA group.
Figure 7. Analysis of tubular albumin transporters reveals that ET₄₅ receptor antagonism preserves megalin expression in the renal cortex of humanized sickle cell mice. Expression of receptors that participate in albumin handling from renal cortex of genetic control (HbAA) and humanized sickle mice (HbSS) treated with vehicle, the selective ET₄₅ antagonist, ambrisentan, or the combined ET₄₅/B antagonist, A-182086, for 10 weeks beginning at 4 weeks of age. (A) Depicts the relative megalin mRNA expression in cortex after 10-week treatment protocol. (B) Depicts Western blot analysis of megalin expression in cortical extracts. (C) Depicts quantification of Western blot bands for megalin. (D) Depicts the relative cubilin mRNA expression in cortex after 10-week treatment protocol. (E) Depicts the relative amnionless (AMN) receptor mRNA expression in cortex after 10-week treatment protocol. (F) Depicts the relative FcRn mRNA expression in cortex after 10-week treatment protocol. Data are mean±SEM; *P<0.05 versus vehicle-treated HbAA; #P<0.05 versus vehicle-treated HbSS; n=5–6 in HbSS and n=6 in vehicle-treated HbAA group. a-18, A-182086; Amb., Ambrisentan; MW, molecular weight; RDU, relative densitometry units; Veh., vehicle.
Histologic Analysis

Kidneys isolated from 10-week treatment protocol mice were immersed in 10% formalin and embedded in paraffin. Kidney sections (4-μm thick) were processed for histopathology studies and immunohistochemistry assay. Sections were stained with hematoxylin and eosin, Masson trichrome, periodic acid Schiff–hematoxylin, or Prussian blue using standard protocols. Tissues were evaluated blindly according to the criteria used for quantification of the changes in renal structures, as previously described.69–72 Briefly, the extent of glomerular sclerosis was assessed on Masson trichrome–stained sections using a grading score from 0 to 4: 0 for no sclerosis; 1 for sclerosis in up to 25% of the glomerulus; 2 for sclerosis in up to 50% of the glomerulus; 3 for sclerosis in up to 75% of glomerulus; and 4 for sclerosis in up to 100% of the glomerulus. Glomerular basement membrane thickening score was assessed using the following 0–4 scale: 0 for no basement membrane thickening; 1 for mild thickening; 2 for moderate thickening; 3 for severe thickening; and 4 for massive thickening. Glomerular vascular congestion was assessed on hematoxylin and eosin–stained sections and calculated as the percentage of total glomeruli with congestion present in at least 25% of the glomerulus. A minimum of 20 glomeruli were evaluated under 400× magnification from ten different bright-field regions of renal cortex and results were averaged for each kidney. Tubular brush border thickness was assessed on periodic acid Schiff–hematoxylin–stained sections using a 0–4 grading scale: 0 for no changes; 1 for lesions involving <25% of the area; 2 for lesions involving 25%–50% of the area; 3 for lesions involving >50% of the area; and 4 for lesions involving nearly 100% of the area. Results were averaged for each animal. Tubulointerstitial fibrosis was assessed in ten fields per kidney section and graded as follows: 0 for no fibrosis; 1 for mild fibrosis; 2 for moderate fibrosis; 3 for severe fibrosis; and 4 for fibrosis involving nearly the entire area, and results averaged for each animal. Renal iron deposition was assessed on scanned images of whole kidney by MetaMorph software (MetaMorph; Molecular Devices LLC, CA). For immunohistochemistry, sections were stained for WT-1 using anti–Wilms tumor protein antibody (CAN-R9[1HC]; 56–2; ab89901, Abcam, UK), anti-CD3 (T cells) antibody (ab16669; 1:600; Abcam, UK), and anti-F-I4/80 (macrophages) antibody (MCA497GA; 1:200; BioRad). WT-1–positive cells were counted under 400× magnification from a minimum of 20 glomeruli from ten different bright-field regions of the cortex and results were averaged for each kidney. Renal cortical CD3+ and F4/80+ cells were blindly quantified in ten microscopic fields (200 × 200 μm, 400× magnification) and results were averaged for each kidney.

Electron Microscopy

Small cubes of renal cortex (1.5 × 1.5 × 1.5 mm) were drop-fixed in 2.5% glutaraldehyde/2.5% formalin, postfixed in 1% osmium tetroxide, and processed into resin blocks. Ultrathin sections were cut using a diamond knife, placed in a copper grid, stained with uranyl acetate and lead citrate, and examined using an FEI Tecnai T12 Spirit 20–120 kv transmission electron microscope.

Total RNA Extraction and Quantitative Real Time PCR

Total RNA extraction of renal cortex or glomeruli was performed using Ambion PureLink RNA Mini Kit or RNAqueous-Micro Total RNA Isolation Kit (Ambion, Austin, TX), and reverse transcription with the iScript cDNA synthesis kit (BioRad, Hercules, CA). Real-time amplification was performed with ABI 7300 Real-Time PCR System using iTaq Universal Probes Supermix (BioRad, Hercules, CA) and TaqMan primer gene expression assay (Applied Biosystems, Foster City, CA) of ET-1 (Mn00438656_m1), ETα (Mn01243722_m1), ETβ (Mn00452989_m1), WT-1 (Mn01357048), nephrin (Mn00497828), podocin (Mn01292252_m1), synaptopodin (Mn03433333_m1), MCP-1 (Mn00441242_m1), P-selectin (Mn01295931_m1), V-CAM1 (Mn01320970_m1), megalin (Mn01328171_m1), cubilin (Mn01325077_m1), amnionless (AMN; Mn00473870_m1), and FcRn (Mn01205451_m1) according to the manufacturer’s instructions. The comparative method of relative quantification (2-ΔΔCT) was used to calculate the expression level of each target gene, normalized to β-actin. Data are presented as the relative mRNA expression of the specific gene of interest.

Western Blotting

Thirty micrograms of protein was electrophoresed on 4%–15% gels (#4561086; Bio-Rad) as previously described.73 Blots were probed with a rabbit polyclonal antibody against the megalin c-terminus (ab76969; Abcam, UK). Equivalent protein loading was confirmed by Coomassie staining.

Statistical Analyses

Analyses were performed using GraphPad Prism 6.0 software (GraphPad Software Inc., CA). Data were compared using paired t test or two-way ANOVA with Bonferroni post hoc correction (variables: genotype, treatment). One-way ANOVA with Bonferroni’s post hoc correction for multiple comparisons was utilized for Western blot data. Results are expressed as mean ± SEM with α=0.05.

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DISCLOSURES
None.

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