

## RESEARCH PAPER

# Endothelin-1 contributes to the progression of renal injury in sickle cell disease via reactive oxygen species

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## BACKGROUND AND PURPOSE

Endothelin-1 (ET-1) is increased in patients with sickle cell disease and may contribute to the development of sickle cell nephropathy. The current study was designed to determine whether ET-1 acting via the ET<sub>A</sub> receptor contributes to renal injury in a mouse model of sickle cell disease.

## EXPERIMENTAL APPROACH

Adult, humanized HbSS (homozygous for sickle Hb) mice had increased ET-1 mRNA expression in both the cortex and the glomeruli compared with mice heterozygous for sickle and Hb A (HbAS controls). In the renal cortex, ET<sub>A</sub> receptor mRNA expression was also elevated in HbSS (sickle) mice although ET<sub>B</sub> receptor mRNA expression was unchanged. Ligand binding assays confirmed that sickle mice had increased ET<sub>A</sub> receptors in the renal vascular tissue when compared with control mice.

## KEY RESULTS

In response to PKC stimulation, reactive oxygen species production by isolated glomeruli from HbSS sickle mice was increased compared with that from HbSA controls, an effect that was prevented by 1 week *in vivo* treatment with the selective ET<sub>A</sub> antagonist, ABT-627. Protein and nephrin excretion were both elevated in sickle mice, effects that were also significantly attenuated by ABT-627. Finally, ET<sub>A</sub> receptor antagonism caused a significant reduction in mRNA expression of NADPH oxidase subunits, which may contribute to nephropathy in sickle cell disease.

## CONCLUSIONS AND IMPLICATIONS

These data support a novel role for ET-1 in the progression of sickle nephropathy, specifically via the ET<sub>A</sub> receptor, and suggest a potential role for ET<sub>A</sub> receptor antagonism in a treatment strategy.

## Abbreviations

ET-1, endothelin-1; HbAS, heterozygous for sickle and Hb A; HbSS, homozygous for sickle Hb; ROS, reactive oxygen species; SAD, HbS Antillies and D-Punjab; SCD, sickle cell disease; SCN, sickle cell nephropathy

## Tables of Links

TARGETS
ET <sub>A</sub> receptor
ET <sub>B</sub> receptor

LIGANDS	
[ <sup>125</sup> I]-ET-1	Endothelin-1 (ET-1)
ABT-627	Endothelin-3 (ET-3)
Angiotensin II	PMA

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

## Introduction

Despite significant gains in life expectancy over the past three decades, adult patients with sickle cell disease (SCD) continue to suffer from progressive organ damage. Although hydroxyurea typically confers significant long-term treatment benefits, most patients still suffer chronic effects of the disease. Sickle cell nephropathy (SCN) has emerged as one of the major causes of morbidity and mortality in SCD (Abbott *et al.*, 2002; Yanni *et al.*, 2009). Nearly 70% of adults with SCD will develop either micro or macroalbuminuria with about 30% developing overt proteinuria, an early indicator for chronic kidney disease (Guasch *et al.*, 2006). Currently, it is estimated that nearly one in five of all SCD patients will progress to end-stage renal disease requiring long-term dialysis (Powars *et al.*, 1991).

Renal pathology in SCD is thought to begin in childhood and progress through adulthood. Early stages are characterized by ischaemic medullary damage, urine concentrating deficiency and increased GFR (Stadius van Eps *et al.*, 1970). This is followed by progressive proteinuria, glomerulosclerosis and eventual decline in GFR (Falk *et al.*, 1992; Falk and Jennette, 1994). Despite the growing prevalence of this chronic manifestation, the mechanisms behind SCN are largely unknown and commonly believed to be due to recurring vaso-occlusive crises and intermittent hypoxia. Currently, there are no effective treatment modalities to adequately address the long-term effects of this progressive renal disease.

The endothelium-derived peptide, endothelin-1 (ET-1), has strong vasoconstrictor, proliferative and pro-inflammatory effects that are mediated primarily through endothelin type A (ET<sub>A</sub>) receptors (Kohan *et al.*, 2011). ET-1 acts in either a paracrine or autocrine fashion and is released in response to shear stress, hypoxia, hemin, angiotensin II, thrombin activation and inflammatory cytokines – all are prevalent in SCD. Indeed, ET-1 is elevated in the plasma and urine of patients with SCD (Rybicki and Benjamin, 1998; Tharaux *et al.*, 2005). Furthermore, ET-1 was recently shown to have potent systemic adverse effects in one of the first mouse models of SCD, potentially exacerbating hypoxia/reoxygenation injury and stimulating the progression of SCN (Sabaa *et al.*, 2008).

ET-1 also increases the production of reactive oxygen species (ROS). ROS are chemically active molecules containing oxygen and persist physiologically as a product of metabolism and as a defence mechanism utilized by the immune system to neutralize bacterial threats. However, when ROS are produced in amounts that overwhelm endogenous antioxidants, significant cellular damage can result due

to oxidative stress. ET-1 stimulates the production of ROS in arterial smooth muscle cells, endothelial cells and isolated arteries, demonstrating a putative mechanism for the pathological actions of ET-1 (Dong *et al.*, 2005; Laplante *et al.*, 2005; Loomis *et al.*, 2005).

ROS are implicated in a wide range of disease processes such as hypertension and chronic kidney disease and also SCD (Wolin, 2000; Wilcox, 2012). SCD creates a constant state of increased oxidative stress, owing to increased ROS and chemically active nitrogen metabolites, the effects of which are thought to contribute significantly to the long-term pathology of SCD (Kupesiz *et al.*, 2012). Previous studies demonstrated that neutrophils, erythrocytes and platelets from patients with SCD produce significantly higher levels of ROS compared with controls (Fibach and Rachmilewitz, 2008; Marcal *et al.*, 2008). Furthermore, SCD patients have decreased antioxidant capacity, disabling protective endogenous mechanisms to prevent oxidative stress-induced injury (Almeida *et al.*, 2010).

While there are multiple enzymatic sources of ROS, one of the most important and perhaps ubiquitous is NADPH oxidase. NADPH oxidase is a multi-subunit enzyme that produces superoxide when activated. Plasma from SCD patients stimulates the production of ROS by leukocytes from unaffected controls via NADPH oxidase activation, and another recent report observed mRNA expression of NADPH oxidase subunits was elevated in neutrophils from SCD patients (Marcal *et al.*, 2008; Almeida *et al.*, 2010). NADPH oxidase participates in the later phase of hypoxia/reoxygenation injury through the overproduction of superoxide, further implicating NADPH oxidase and subsequent ROS over production in SCD (Wood *et al.*, 2005).

All of these studies suggest that ET-1 is involved in the initiation of SCN and contributes to the progression of this form of chronic kidney disease. However, pre-clinical support for the use of selective ET<sub>A</sub> blockade as a viable treatment for SCN is lacking. Therefore, we sought to test the hypothesis that ET-1 via the ET<sub>A</sub> receptor is responsible for elevated glomerular ROS production and other markers of renal injury seen in a mouse model of SCD.

## Methods

### Animals

All mice were maintained and studied in accordance with the US National Research Council's *Guide for the Care and Use of Laboratory Animals* and the experiments were approved and

monitored by the Georgia Regents University or the University of Alabama at Birmingham Institutional Animal Care and Use Committees. All animal care and experimental procedures complied with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). Studies utilized humanized homozygous for sickle Hb (HbSS)-only transgenic knock-in mice with notation: B6;129-*Hba*<sup>tm1(HbA)Tow</sup> *Hbb*<sup>tm2(HBG1,HBB\*)Tow</sup>/*Hbb*<sup>tm3(HBG1,HBB)Tow</sup>/J, originally developed by Townes *et al.* (Wu *et al.*, 2006; Belcher *et al.*, 2014; Rivers *et al.*, 2015). These animals will be referred to as 'humanized sickle mice' or simply 'sickle mice'. These animals are bred on a mixed C57BL/6J and 129 backgrounds. Control animals used in the current experiments were heterozygotes [heterozygous for sickle and Hb A (HbAS)] from the same colony. Animals in all groups ranged in age from 12 to 16 weeks-old at the time of experimentation and were age and sex matched with the exception of the binding studies, which were carried out with age-matched mice of mixed sexes. Group numbers varied depending on availability of mice. Mice were housed under conditions of constant temperature and humidity and exposed to a 12 h light/dark cycle. All mice were given free access to mouse chow (Harlan Teklad) and tap water. Separate groups of mice were placed in standard metabolic cages and allowed to adapt for 2 days prior to obtaining a 24 h urine sample.

### Glomerular isolation

Glomeruli were isolated as previously described (Savin *et al.*, 1992; Saleh *et al.*, 2010). Renal cortices were isolated and passed through a series of cellulose filters to separate the tubular and vascular elements from the glomeruli. Final cellulose filter pore size was 70  $\mu\text{m}$  to ensure uniform collection of glomeruli. The resulting suspension was purified via centrifugation twice at 1000 $\times$  *g*, and the pellet was resuspended in HBSS (5.33 mM potassium chloride, 0.44 mM potassium phosphate monobasic, 138 mM sodium chloride, 4 mM sodium bicarbonate, 0.3 mM sodium phosphate dibasic and 5.6 mM glucose) to produce a suspension of >95% glomeruli. Isolations were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for mRNA analysis or used immediately for ROS measurements.

### Renal pre-glomerular vessel isolation

Arterioles, interlobular and arcuate arteries were isolated as previously reported (Schneider *et al.*, 2010). Mice were anaesthetized by isoflurane induction and then given an injection of sodium pentobarbital (65  $\text{mg}\cdot\text{kg}^{-1}$  i.p.). Kidneys were removed, decapsulated and placed in ice-cold HBSS. One kidney at a time was gently pressed against a 70  $\mu\text{m}$  microcellulose filter while submerged in HBSS to separate vascular from tubular and microvascular renal tissue using a flat, rounded spatula. Care was taken to minimize cellular damage; rather, tissue was gently massaged until the tubular structures were separated from the vasculature. Once separated, kidney vessels were placed in a 1.5 mL tube, re-suspended in HBSS and snap-frozen with liquid nitrogen for later use in the binding assay.

### Drug treatments

Where indicated, the  $\text{ET}_A$  antagonist, ABT-627 (also known as atrasentan; AbbVie, Inc., Abbott Park, IL, USA), was administered

via drinking water and the concentration adjusted daily according to water intake to deliver a consistent  $5\text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$  for 7 days as previously reported (Honing *et al.*, 2000; Elmarakby *et al.*, 2004; Sasser *et al.*, 2007; Boesen *et al.*, 2011; Saleh *et al.*, 2011b). ABT-627 is an orally bioavailable  $\text{ET}_A$  receptor antagonist considered highly specific for the  $\text{ET}_A$  receptor ( $K_i$  of 69 pM) (Oppenorth *et al.*, 1996; Winn *et al.*, 1996; Wessale *et al.*, 2002).

### Glomerular ROS assay

Glomerular ROS production was quantified using a luminescence assay (Heimlich *et al.*, 2015). Isolated glomeruli were plated on a 96-well clear-bottom plate and incubated with 1 mM L012, a luminol derivative. Baseline luminescence was obtained for 5 min prior to exposure to 1 mM PMA. ROS production was calculated by the change in luminescence in response to PMA stimulation. Data were collected at 1 min intervals for 25 min. AUC based on an average of the baseline period was calculated with Graph Pad Prism Software (San Diego, CA, USA).

### Assays

Urinary protein concentration was measured by Bradford Assay (BioRad, Hercules, CA, USA) and multiplied by 24 h urine volume to attain excretion rate. Absorbance was measured at 595 nm and compared with a known quantity of protein. The glomerular barrier protein, nephrin, was measured using an indirect competitive ELISA according to the manufacturer's instructions (Exocell, Philadelphia, PA, USA).

To determine the quantity of  $\text{ET}_A$  and  $\text{ET}_B$  receptors present in the renal vasculature, tissue was harvested and frozen at  $-80^{\circ}\text{C}$ . Tissues were thawed and homogenized in homogenization buffer (250 mM sucrose, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 15  $\mu\text{M}$  PMSF). The homogenate was centrifuged at 1000 $\times$  *g* for 30 min at  $4^{\circ}\text{C}$ . The supernatant was collected and centrifuged at 30 000 $\times$  *g* for 45 min at  $4^{\circ}\text{C}$  and the pellet resuspended in homogenization buffer to obtain the membrane fraction. Protein concentrations of membrane fractions were determined by Comassie blue Bradford assay. Binding assays were conducted as previously reported (Pollock *et al.*, 2000; Taylor *et al.*, 2002; Kittikulsuth *et al.*, 2011; Kittikulsuth *et al.*, 2012). In brief, [ $^{125}\text{I}$ ]-ET-3 binding was used to determine the maximum binding ( $B_{\text{max}}$ ) values for  $\text{ET}_B$  receptor expression. To determine  $\text{ET}_A$  receptor number, the value for [ $^{125}\text{I}$ ]-ET-3 binding was subtracted from the  $B_{\text{max}}$  value for [ $^{125}\text{I}$ ]-ET-1 binding.

### mRNA isolation and measurement

mRNA was isolated using the Pure Link Mini RNA extraction kit (Ambion, Austin, TX, USA) as previously described (Boesen *et al.*, 2008). Real-time PCR was carried out using iTaq Universal Probes Mastermix (BioRad) and TaqMan primer gene expression assays (Applied Biosystems, Foster City, CA, USA;  $\text{ET}_1$ : Mm00438656\_m1,  $\text{ET}_A$ : Mm01243722\_m1,  $\text{ET}_B$ : Mm00432989\_m1, NADP oxidase 2 (NOX2): Mm01287743\_m1, NADP oxidase 4 (NOX4): Mm00479246\_m1, p47PHOX: Mm00447921\_m1). Results were quantified using  $2^{-\Delta\Delta\text{CT}}$  method and calculated as a fold change from the appropriate control values (Livak and Schmittgen, 2001).

## Statistical analysis

All groups of data were assessed for normality using the Shapiro–Wilk test. Where appropriate, Student's *t* test was used to compare normally distributed means of two groups, whereas the non-parametric Kruskal–Wallis test was used to compare non-normally distributed groups. For two-factor analysis, a two-way ANOVA was used with Bonferroni's *post hoc* correction to compare individual means. Results are expressed as means  $\pm$  SEM, with  $P < 0.05$  being considered statistically significant.

## Results

### Physical data

Mice were assessed for physical characteristics and results displayed in Table 1. Sickle mice and heterozygous controls

showed no difference in overall body mass or kidney-to-body mass ratio. The spleens of sickle mice were significantly larger when compared with body weight than the spleens of controls ( $P < 0.05$ ). Sickle mice also had lower haematocrit than their heterozygous counterparts ( $P < 0.05$ ).

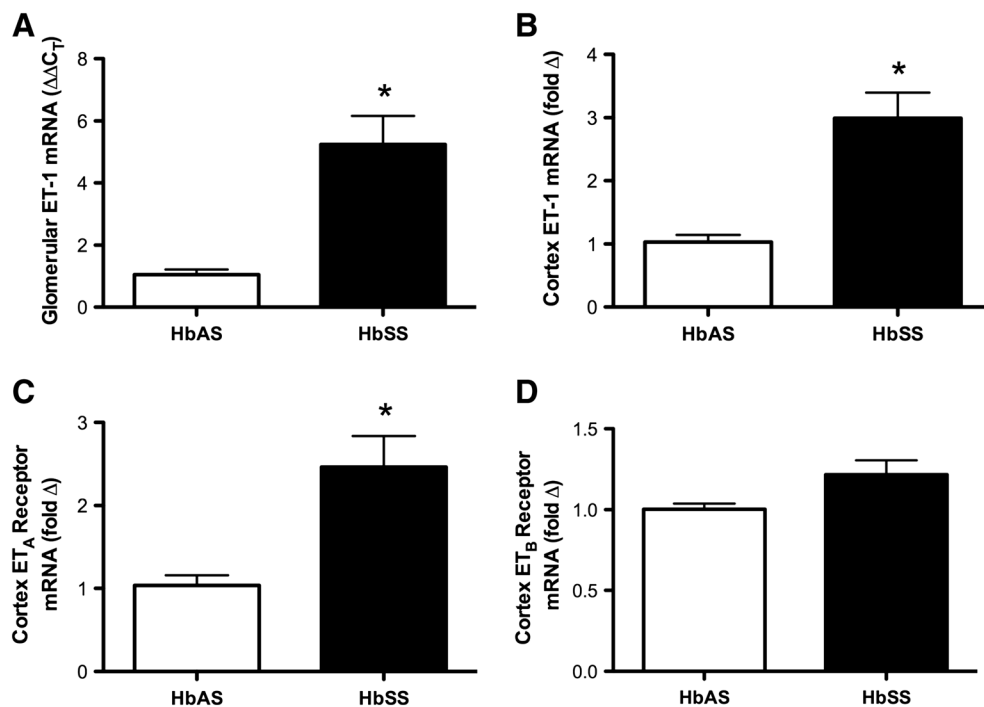
### ET-1, ET<sub>A</sub> and ET<sub>B</sub> receptor mRNA expression

Renal cortical tissue and isolated glomeruli from HbAS and HbSS mice were collected for determination of ET-1 mRNA expression. HbSS mice had significantly higher levels of ET-1 mRNA at baseline in both cortex (Figure 1) and glomeruli (Figure 1), confirming the presence of elevated tissue ET-1 mRNA in the humanized mouse similar to previous models of SCD. ET<sub>A</sub> and ET<sub>B</sub> receptor expression was also determined in the cortex of HbAS and HbSS mice. HbSS mice had

**Table 1**

Physical data for sickle and homozygous control mice

Parameter	HbAS	HbSS	Units	<i>P</i> value
Haematocrit	39.5 $\pm$ 0.6	31.2 $\pm$ 1.3	%	<0.05
Body mass	26.4 $\pm$ 0.7	28.8 $\pm$ 1.2	g	0.14
Spleen/body mass	9.6E <sup>-3</sup> $\pm$ 8.2E <sup>-4</sup>	6.7E <sup>-2</sup> $\pm$ 6.1E <sup>-3</sup>	g	<0.05
Kidney/body mass	1.5E <sup>-2</sup> $\pm$ 8.5E <sup>-4</sup>	1.6E <sup>-2</sup> $\pm$ 5.6E <sup>-4</sup>	g	0.23
Urine output	1.1 $\pm$ 0.3	2.2 $\pm$ 0.2	mL	<0.05
Water consumption	4.5 $\pm$ 0.4	5.1 $\pm$ 0.7	mL	0.46



**Figure 1**

ET-1 mRNA expression in heterozygous control and SCD mice in isolated glomeruli (A) and renal cortex (B). ET<sub>A</sub> (C) and ET<sub>B</sub> (D) receptor mRNA expression was measured in the renal cortex. Data are mean  $\pm$  SEM. \* $P < 0.05$ ;  $n = 5, 6$  or 7.

increased expression of ET<sub>A</sub> receptor but not ET<sub>B</sub> receptor compared with controls (Figure 1).

### ET receptor binding in renal vasculature

To further characterize the renal endothelin system in the Townes humanized mouse model, we isolated renal vascular tissue of HbAS and HbSS mice to determine ET receptor binding activity. Specific binding of [<sup>125</sup>I]-ET-1 in the renal vessel preparation was higher in HbSS animals than in the HbAS controls (Figure 2; B<sub>max</sub> = 181 ± 19 vs. 117 ± 11 fmol·mg<sup>-1</sup> respectively); however, [<sup>125</sup>I]-ET-3-specific binding was similar between the groups (Figure 2; B<sub>max</sub> = 116 ± 14 vs. 94 ± 18 fmol mg<sup>-1</sup> for HbAS vs. HbSS respectively). ET-1 has equal affinity for both ET<sub>A</sub> and ET<sub>B</sub> receptors, while ET-3 only binds to ET<sub>B</sub> receptors at low concentrations. Therefore, B<sub>max</sub> values for ET-1 represent the total binding to both receptors, while B<sub>max</sub> values for ET-3 binding at the concentrations used represent ET<sub>B</sub> receptor-specific binding. ET<sub>A</sub> receptor-specific binding was then determined by subtracting ET-1 B<sub>max</sub> from ET-3 B<sub>max</sub>. HbSS cell mice had significantly increased ET<sub>A</sub> receptor-specific binding in the renal vasculature but had comparable ET<sub>B</sub>-specific binding (Figure 2). The K<sub>D</sub> for ET-1 and ET-3 was determined from the Scatchard analysis. ET-1 K<sub>D</sub> in HbSS mice was significantly increased compared with that in HbAS controls (0.11 ± 0.02 nM vs. 0.05 ± 0.01 nM, n = 9–11, P < 0.05), while there were no significant differences

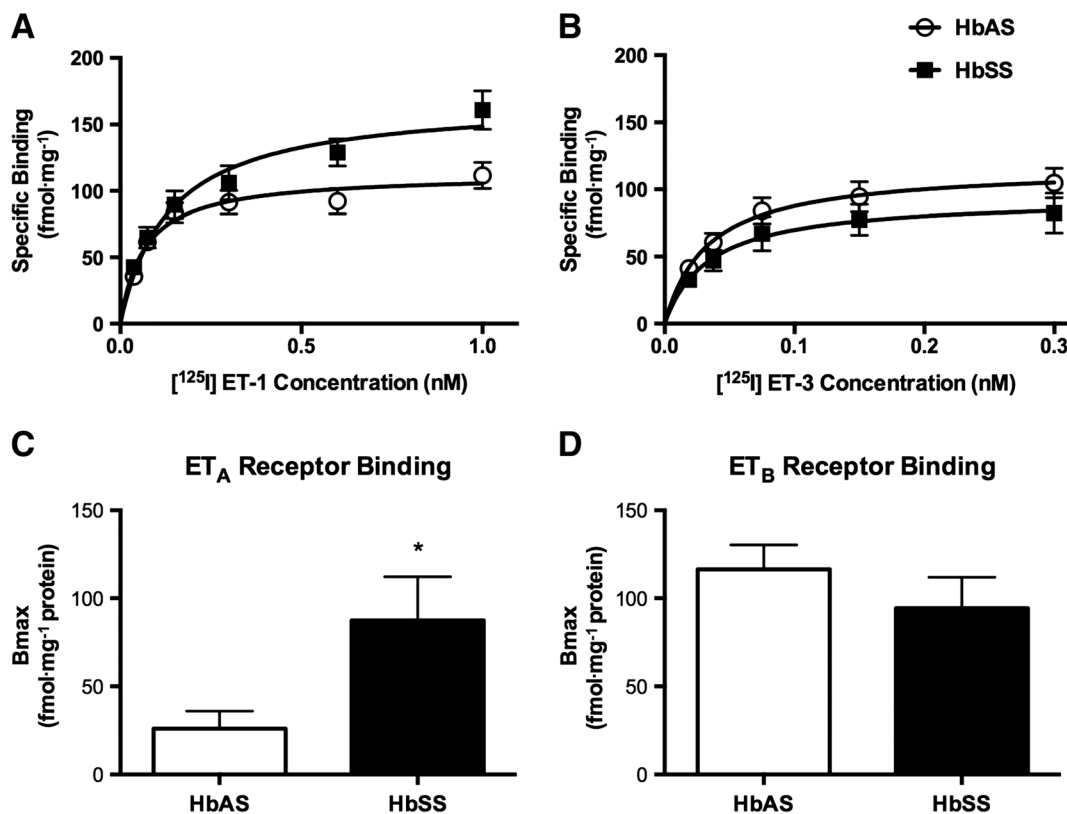
in the K<sub>D</sub> for ET-3 binding between genotypes (0.032 ± 0.004 nM and 0.034 ± 0.005 nM).

### Stimulation of glomerular ROS production via ET<sub>A</sub> receptor activation

To determine the level of ET<sub>A</sub> receptor contribution to glomerular ROS production, HbSS mice were treated with vehicle or ABT-627 for 1 week. ROS production was measured by PMA stimulation of isolated mouse glomeruli, and representative data from each treatment group are displayed in Figure 3A. First, ROS production in glomeruli from untreated HbSS mice was significantly greater than that from HbAS control mice (Figure 3). After *in vivo* ET<sub>A</sub> receptor blockade with ABT-627, the average response to PMA for glomeruli from HbSS animals was significantly reduced compared with that from untreated HbSS mice (Figure 3). To test the etiology of the ROS in glomeruli, glomeruli from HbSS mice were treated *in vitro* with the ROS inhibitor apocynin (100 μM) before stimulation with PMA. Apocynin completely abolished the PMA-stimulated ROS production, suggesting that NADPH oxidase could be a potential source of glomerular ROS (0.82 ± 0.02 × 10<sup>3</sup> ALU·mg<sup>-1</sup> protein vs. 26.3 ± 4.8 10<sup>3</sup> ALU·mg<sup>-1</sup> protein, n = 6–9, P < 0.05).

### Effects of ET<sub>A</sub> receptors on renal injury markers

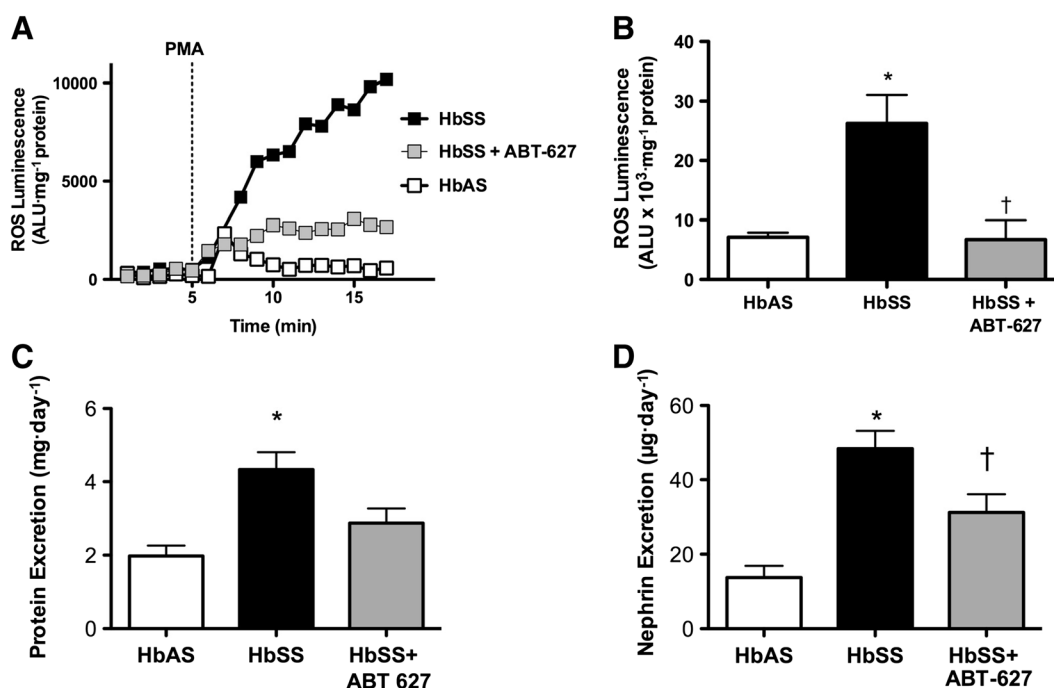
To test the hypothesis that ET<sub>A</sub> receptors contribute to proteinuria in SCD, HbSS mice were treated with or without



**Figure 2**

Specific ET-1 (A) and ET-3 (B) binding in membrane preparations from dissected renal vasculature. B<sub>max</sub> for the ET<sub>A</sub> receptor was calculated as the difference between B<sub>max</sub> for ET-1 and ET-3 binding (C), while ET<sub>B</sub> receptor binding was equated to maximum ET-3-specific binding (D). Data are mean ± SEM. \*P < 0.05; n = 9, 10 or 11.





**Figure 3**

Representative luminescence reading in glomeruli isolated from control, SCD and SCD mice treated with the ET<sub>A</sub> receptor antagonist, ABT-627. (A) Total ROS luminescence was calculated from the area under the luminescence curve and normalized to mg protein (B). Panels C and D depict the average protein and nephrin excretion, respectively, for the three groups of mice. Data are mean ± SEM. \**P* < 0.05 versus control; †*P* < 0.05 versus untreated sickle mice; *n* = 6 or 8.

ABT-627. Urine was collected over a 24 h period following 1 week of treatment. In untreated mice, HbSS animals had significantly higher levels of proteinuria compared with heterozygous HbAS mice (Figure 3). As a result of treatment with ABT-627, HbSS mice exhibited significantly decreased 24 h protein excretion when compared with untreated HbSS mice (Figure 4). HbSS mice also had significantly reduced nephrin excretion when treated with the ET<sub>A</sub> antagonist. The untreated HbSS animals had significantly elevated nephrinuria compared with heterozygous HbAS controls, an effect that was significantly attenuated by ABT-627 (Figure 3).

ET<sub>A</sub> receptor antagonism with ABT-627 significantly decreased mRNA expression of oxidative stress markers in glomeruli of HbSS mice. Both NOX4 and the subunit p47<sup>phox</sup> were significantly decreased compared with glomeruli from untreated HbSS (Figure 4). In contrast, there was no discernible difference in either NOX4 or p47<sup>phox</sup> between HbAS and HbSS within whole cortex homogenates (Figure 4). NOX2 was comparable between treatment groups in both the cortex and the glomeruli (Figure 4).

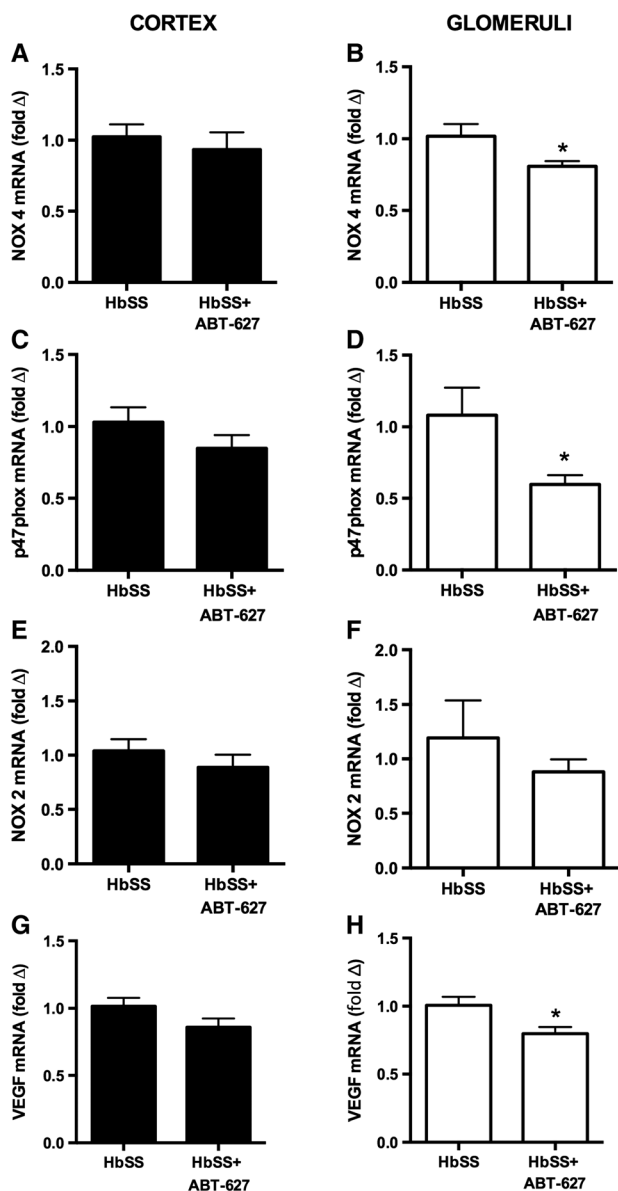
## Discussion

These data support a novel role for ET-1 via the ET<sub>A</sub> receptor in the progression of SCN in a humanized mouse model of SCD. ET-1 as well as ET<sub>A</sub> receptor mRNA was elevated in renal tissue from sickle mice. Additionally, sickle mice exhibited enhanced ET-1 binding and, specifically, increased ET<sub>A</sub> receptor

binding in the renal vasculature. Because oxidative stress is well-established in SCD, our findings also support the hypothesis that ET-1 contributes to oxidative stress and glomerular injury in this model. *In vivo* and *in vitro* ET<sub>A</sub> receptor antagonism decreased glomerular ROS production, proteinuria and nephrinuria in addition to down-regulating mRNA expression for NADPH oxidase subunits.

The sickle mice utilized in these studies expressed similar overall body mass and kidney-to-body mass ratio between genotypes. Spleen-to-body mass ratio was expectedly elevated in sickle mice. This aspect was recorded in the first publication of this mouse model, by noting marked expansion of red pulp and intrasplenic pooling of erythrocytes (Levasseur *et al.*, 2003). Haematocrit is a rough estimate of the percentage erythrocyte volume in whole blood and can indicate anaemia. Haematocrit was also significantly decreased but not to the extent that is typically seen in humans, usually around 0.25 (Kaul *et al.*, 1983). Sickle cell mice as well as humans are anaemic by definition, but the degree of anaemia is variable. Given the current data, our sickle mice might experience less anaemia than a typical human SCD patient. However, haematocrit does not necessarily indicate disease severity except in the more extreme values such as those in the teens and lower, which might indicate aplastic crisis (Mallouh and Qudah, 1993). Therefore, we believe this model to represent the typical anaemia status found in SCD.

Multiple studies have reported the presence of increased plasma and urine ET-1 in humans with SCD (Rybicki and Benjamin, 1998; Tharaux *et al.*, 2005; Hatzipantelis *et al.*,



**Figure 4**

Expression of NADPH oxidase subunits, NOX4 and p47<sup>phox</sup>, in renal cortex and glomeruli of SCD mice treated with or without the ET<sub>A</sub> receptor antagonist, ABT-627. Data are mean ± SEM. \**P* < 0.05; *n* = 6 or 7.

2013). The SAD mouse was one of the first models developed that used a combination of human Hb mutations (HbS, Antillies and D-Punjab, SAD) in the mouse genome to increase the propensity for RBCs to sickle and mirror human pathophysiology (Trudel *et al.*, 1991). However, the SAD differs in certain aspects of the human disease course such as the lack of chronic anaemia and a more severe response to hypoxia/reoxygenation. Our current studies were motivated, in part, by observations that SAD mice have increased ET-1 mRNA expression as determined in whole kidney homogenates, which is exacerbated by hypoxia–reoxygenation (Sabaa *et al.*, 2008). To our knowledge, tissue levels of ET-1 have not been previously evaluated for the humanized

knock-out, knock-in mouse model of SCD developed by the Townes lab where mouse genes for  $\alpha$ ,  $\beta$  and  $\gamma$  Hb have been replaced by corresponding human genes that include the mutation for SCD. Using this model, we observed increased ET-1 mRNA both in the cortex and to a greater extent in the glomeruli of the humanized sickle mouse. Based on previous experiments in our laboratory, the endothelium is the most likely source of elevated ET-1 following hypoxia (Heimlich *et al.*, 2015). Vascular endothelial ET-1 knock-out animals exhibited no change in ET-1 mRNA expression after acute hypoxia, whereas animals with intact endothelial ET-1 showed significant elevations in ET-1 mRNA after hypoxia (Heimlich *et al.*, 2015). Further supporting this theory is the finding that cultured endothelial cells incubated with sickled RBCs produce increases in ET-1 (Phelan *et al.*, 1995; Shiu *et al.*, 2002; Ergul *et al.*, 2004). Nonetheless, it remains possible that cell types other than the endothelium, such as macrophages, may be important sources of ET-1 production in SCD.

ET-1 at physiological levels is necessary for proper renal function, especially in the collecting duct system to stimulate natriuresis via the ET<sub>B</sub> receptor (Kohan *et al.*, 2011). However, transgenically-derived mice constitutively overexpressing ET-1 mRNA have a glomerulosclerosis phenotype without increased BP lending support to the notion that ET-1 has powerful trophic effects, independent of BP (Hochoer *et al.*, 1997; Amiri *et al.*, 2004). In the Townes humanized sickle mice, increased ET-1 mRNA expression in the glomeruli combined with enhanced ET<sub>A</sub> receptor expression within the renal cortex provides the circumstances requisite for extensive renal injury induced by overactivation of ET<sub>A</sub> receptors.

Because SCD creates a chronic state of oxidative stress, we aimed to evaluate ROS production in the glomeruli with the understanding that increased levels of ROS can cause cellular damage and appear to contribute to glomerular injury in other disorders such as diabetic nephropathy (Sharma *et al.*, 2005). ROS production was elevated upon stimulation with PMA in the HbSS mouse, a phenomenon that was preventable by *in vivo* treatment with ABT-627 and *in vitro* incubation with apocynin. PMA stimulation gives little insight as to the cellular origin of the ROS production; however, we would suspect the source to be endothelial. Given our findings that ET<sub>A</sub> receptor blockade reduces the expression of NADPH oxidase subunits, these data indicate that glomerular ROS are most likely enzymatically derived from NADPH oxidase and that the ET<sub>A</sub> receptor perhaps promotes the activity of this ROS-producing enzyme, especially in the setting of chronic exposure to elevated ET-1. Previous studies also discovered that ET-1 increases oxidative stress via up-regulation of NADPH oxidase through ET<sub>A</sub> receptor activation, but the mechanism for this effect is not completely clear (Touyz *et al.*, 2004; Loomis *et al.*, 2005). These findings do not exclude the possibility of additional enzymatic sources of ROS. Nonetheless, our findings provide mechanistic insight into the pathophysiology of SCN with particular emphasis on the role of ET<sub>A</sub> receptor activation.

NOX4, a gp91<sup>phox</sup> homologue, and p47<sup>phox</sup> were decreased in response to ET<sub>A</sub> receptor blockade only in the glomeruli, while NOX2 showed no change in response to ET<sub>A</sub> antagonism in either tissue. NOX4 is the predominant isoform found in the kidney but is also expressed throughout the vasculature (Geiszt *et al.*, 2000; Ellmark *et al.*, 2005; Hu *et al.*, 2005). Previous studies have shown

that increased superoxide production was associated with increased mRNA expression of gp91<sup>phox</sup> and p47<sup>phox</sup> (Kityyakara *et al.*, 2003). Our data indicate that the ET<sub>A</sub> receptor has a glomerular-specific modulatory effect upon important NADPH oxidase subunits that contribute to oxidative stress in SCD.

The humanized SCD mice exhibited proteinuria at baseline that was reduced by ET<sub>A</sub> receptor blockade. Albuminuria and proteinuria represent an early sign of glomerular injury, and their presence predicts not only an elevated risk for nephropathy but also cardiovascular disease (Raptis and Viberti, 2001). ET<sub>A</sub> receptor activation has been reported in an array of proteinuric renal diseases including both diabetic and non-diabetic forms of nephropathy. ET-1 and ET<sub>A</sub> receptor involvement in chronic kidney disease is well-established in a large variety of animal models as well (Benigni *et al.*, 1993; Kohan and Pollock, 2013). Previous studies attempting to address proteinuria in SCD have revealed ACE inhibitors can reduce proteinuria and microalbuminuria in humans (Falk *et al.*, 1992). ET-1 production is stimulated by angiotensin II, thus providing a potential explanation for how both ACE inhibition and ET<sub>A</sub> antagonism would be beneficial in SCD-associated proteinuria (Resink *et al.*, 1990; Emori *et al.*, 1991).

Chronic administration of sub-pressor doses of ET-1 is also known to directly cause nephrin shedding *in vivo* and can be reversed by ET<sub>A</sub> receptor blockade (Saleh *et al.*, 2010). Similarly, ET<sub>A</sub> antagonism reduces urinary nephrin excretion in a model of diabetic nephropathy (Saleh *et al.*, 2011b; Saleh *et al.*, 2011a). Our current data showing increased urinary nephrin excretion in the humanized SCD mouse provide a novel biomarker for glomerular injury in SCN. Similar to other models of kidney disease, nephrinuria in sickle mice is corrected through ET<sub>A</sub> receptor antagonism, further validating the paradigm of renal injury via ET<sub>A</sub> receptor activation. Further studies examining the structural and histological changes associated with ET<sub>A</sub> receptor activation are needed to confirm the role of ET-1 in the progression of renal disease in SCD.

## Conclusions

Overall, our results point to a mechanism describing the initiation and progression of nephropathy in a humanized mouse model of SCD. Our experiments offer a potential pathophysiological explanation for elevated ROS production in the setting of SCD within the kidney. There are many aspects of SCD that trigger elevated activity of the ET-1 system such as hypoxia, inflammation, reduced NO bioavailability, increased angiotensin II and a potentially vicious cycle of ROS production. In any event, our findings offer convincing evidence to support ET<sub>A</sub> receptor blockade as a potential treatment strategy for approaching the increasingly prevalent and deadly manifestation of kidney injury in human SCD.

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## Author contributions

J. B. H. and J. S. S. performed the research. J. B. H., S. E. M., J. S. P., A. K. and D. M. P. designed the research study. T. M. T., S. E. M. and J. S. P. contributed essential reagents or tools. J. B. H., P. M. O. and D. M. P. analysed the data. J. B. H. and D. M. P. wrote the paper.

## Conflict of interest

The authors declare no conflict of interest.

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