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Reduced plasma angiotensin II levels are reversed by hydroxyurea treatment in mice with sickle cell disease

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ABSTRACT

Aims: Sickle cell disease (SCD) pathogenesis leads to recurrent vaso-occlusive and hemolytic processes, causing numerous clinical complications including renal damage. As vasoconstrictive mechanisms may be enhanced in SCD, due to endothelial dysfunction and vasoactive protein production, we aimed to determine whether the expression of proteins of the renin–angiotensin system (RAS) may be altered in an animal model of SCD.
Main methods: Plasma angiotensin II (Ang II) was measured in C57BL/6 (WT) mice and mice with SCD by ELISA, while quantitative PCR was used to compare the expressions of the genes encoding the angiotensin-II-receptors 1 and 2 (*AT1R* and *AT2R*) and the angiotensin-converting enzymes (*ACE1* and *ACE2*) in the kidneys, hearts, livers and brains of mice. The effects of hydroxyurea (HU; 50–75 mg/kg/day, 4 weeks) treatment on these parameters were also determined.
Key findings: Plasma Ang II was significantly diminished in SCD mice, compared with WT mice, in association with decreased *AT1R* and *ACE1* expressions in SCD mice kidneys. Treatment of SCD mice with HU reduced leukocyte and platelet counts and increased plasma Ang II to levels similar to those of WT mice. HU also increased *AT1R* and *ACE2* gene expression in the kidney and heart.
Significance: Results indicate an imbalanced RAS in an SCD mouse model; HU therapy may be able to restore some RAS parameters in these mice. Further investigations regarding Ang II production and the RAS in human SCD may be warranted, as such changes may reflect or contribute to renal damage and alterations in blood pressure.

Introduction

The pathogenesis of the genetic disorder, sickle cell disease (SCD), derives from the polymerization of deoxygenated hemoglobin S (HbS) in the red cells. Polymerized hemoglobin distorts the red blood cell (RBC), which becomes sickled in shape and less deformable (Stuart and Nagel, 2004). Vaso-occlusion is a characteristic manifestation of SCD and results from interactions between red cells, activated leukocytes, and other cells in the blood vessel, in a mechanism that is initiated and perpetuated by endothelial activation, reduced nitric oxide (NO) bioavailability, augmented oxidative stress and vascular inflammation (Conran and Costa, 2009). Together with hemolytic anemia, vaso-occlusive (VO) processes cause the clinical complications that are associated with SCD, including painful VO episodes, autoinfarction of the spleen, acute chest syndrome, stroke, pulmonary hypertension, organ damage, renal damage and a shortened lifespan (Steinberg et al., 2009).

Hydroxyurea (HU), or hydroxycarbamide, has been used with some success as a therapy in SCD, reducing crisis incidence and mortality, and is currently the only drug approved by the FDA for the treatment of this disease (Charache et al., 1995). This drug is generally administered to patients fulfilling the criteria for HU therapy under a chronic regime of between 15 and 30 mg/kg/day (McGann and Ware, 2011). HU therapy in SCD patients usually increases fetal hemoglobin (HbF) production (Charache et al., 1992) and there is evidence to suggest that HU can generate NO production *in vivo* (King, 2004). HU therapy has also been shown to decrease red blood cell (RBC) rigidity, modulate adhesion protein expression by RBC and endothelial cells (VEC), as well as reduce leukocyte counts (Conran and Costa, 2009).

While most adult SCD patients display lower systemic blood pressures (de Jong et al., 1982; Pegelow et al., 1997; Steinberg et al., 2009), vaso-constrictive mechanisms may be enhanced in SCD at sites of inflammation, with a probable contribution to vaso-occlusive processes (Ergul et al., 2004). Reduced levels of NO in the blood vessels, due to hemolytic processes and endothelial dysfunction (Reiter et al., 2002), may decrease NO-dependent vasodilation, while increased endothelin-1 (ET-1) production may augment vasoconstriction at sites of inflammation and endothelial activation (Ergul et al., 2004).

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Angiotensin II (Ang II) is a vasoactive peptide and constitutes one of the vasoactive products of the renin–angiotensin system (RAS). Ang II is produced from the cleavage of angiotensinogen by renin to form angiotensin I (Ang I), followed by conversion of Ang I to Ang II by the angiotensin-converting enzyme (ACE) (Grace et al., 2012). Ang II mediates its biological effects *via* two receptors, the Ang II type 1 receptor (AT_1R) and the Ang II type 2 receptor (AT_2R). AT_1R mediates much of the pathological effects of Ang II, such as vasoconstriction, inflammation, and sodium reabsorption. In contrast, AT_2R may mediate vasodilation, and inhibit growth and its expression can increase in pathological conditions such as hypertension, renal failure and diabetes (Savoia et al., 2011). While ACE is the major Ang-II-generating enzyme, it can also inactivate kinins, leading to a reduction in bradykinin-mediated vasodilation. In addition, the more recently identified ACE2 protein catalyzes Ang I and Ang II conversion into the small Ang peptides Ang-(1–9) and Ang-(1–7), which both have opposing effects to those of Ang II (Sampaio et al., 2007).

Renal disease is frequently experienced by SCD patients, especially in older individuals (Ataga et al., 2014; Sasongko et al., 2013; Sharpe and Thein, 2014). Repeated cycles of ischemic injury in the kidney may cause chronic microvascular disease, causing hyperfiltration and proteinuria and potentially contributing to the onset of chronic kidney disease in some individuals (Sharpe and Thein, 2014). Given that renal damage is often associated with SCD and the fact that the kidney is the major site of renin production, this study aimed to determine whether production of the vasoconstrictive peptide, Ang II, and proteins involved in Ang II production and signaling may be altered in SCD. We used transgenic Berkeley mice as an animal model of SCD for this study and investigated the effects of HU treatment on these parameters.

Methods

Animals

C57BL/6 mice were obtained from the animal housing facility (CEMIB) of the University of Campinas, Brazil. Berkeley mice (Tg[HuminiLCRα1GγAγδβS] $Hba^{-/-}Hbb^{-/-}$) were originally purchased from the Jackson laboratories (Bar Harbor, ME) and maintained and bred at CEMIB. The Berkeley mice used were age-matched (5–6 months) to C57BL/6 (WT) mice and are, hereafter, referred to as “Berk mice”. For some experiments, chimeric male SCD mice were generated by transplantation of nucleated bone marrow cells harvested from Berkeley SCD mice into lethally-irradiated C57BL/6 mice (2 months old), as previously described (Turhan et al., 2002). Only chimeric SCD mice expressing >97% human globin were utilized in experiments at 3 months after transplantation. Transplanted chimeric SCD mice are hereafter denominated “SCD mice”. All animal experimental procedures were carried out in accordance with the ‘Principles of Laboratory Animal Care’ (http://grants.nih.gov/grants/guide/notice-files/not96-208.html), as well as in accordance with current Brazilian laws for the protection of animals; this study was approved by the Animal Research Ethics Committee of the University of Campinas (CEUA/UNICAMP, protocol 3024-1, 2013). All animals were fed on a 22% protein diet (NUVILAB — CR1 irradiated) for at least 3 months before the experiments.

Treatment of SCD mice with hydroxyurea

For some experiments, chimeric SCD mice (5 months old) were treated with hydroxyurea (50 or 75 mg/kg/day i.p.) or saline vehicle (i.p.) 5-times-a-week for 4 weeks before sacrificing and storing plasma and organs at −80 °C. Hematological indices were obtained for blood samples using a Beckman Coulter hemolytic analyzer (Fullerton, CA).

Measurement of plasma angiotensin II

Blood samples were collected in EDTA by cardiac puncture from mice and Angiotensin II Inhibitor Cocktail (SPI-Bio Bertin Pharma, Montigny-le-Bretonneux, France) was immediately added to samples (30 μL/mL blood) to inhibit angiotensin II (Ang II) degradation and production. Samples were centrifuged (15 min, 3000 *g*, 4 °C) and the plasma was stored at −80 °C until the day of assay. On the day of assay, Ang II was extracted from plasma samples using phenyl Hypersep PH columns (Thermo Scientific, Waltham, MA) before determining Ang II concentrations using the Angiotensin II Enzyme Immunoassay (SPI-Bio Bertin Pharma), according to the manufacturer's instructions. Final plasma Ang II concentrations were calculated from a standard curve and adjusted according to the initial plasma volume from which Ang II was extracted.

Quantitative real time PCR (qPCR)

The expressions of the genes encoding angiotensin II type 1 receptor (*AT1R*), angiotensin II type 2 receptor (*AT2R*), ACE (*ACE1*) and ACE2 (*ACE2*) were determined in the tissues of the kidney, heart, brain and liver, which were isolated from mice at the time of sacrifice. mRNA was extracted from the organs of mice (entire organs) using Trizol (Invitrogen, Carlsbad, USA) and cDNA was synthesized using a reverse-transcription kit (RevertAid H Minus First Strand cDNA Synthesis, Thermo Scientific, Waltham, MA). Synthetic oligonucleotide primers were designed to amplify cDNA for conserved regions of the genes described, by Primer-Express (Applied Biosystems, Foster City, CA, USA; for primer sequences, see Table 1). Primers were synthesized by Invitrogen (São Paulo, Brazil) and *ACTB* was used as an internal control gene. All samples were assayed in a 12 μL volume containing 5 ng cDNA, 6 μL SYBR Green Master Mix PCR (Applied Biosystems, Foster City, California, USA) and gene primers (7500 Fast Real-Time PCR System — Applied Biosystems). To confirm the accuracy and reproducibility of the real-time PCR, the intra-assay precision was calculated according to the equation: E(−1 / slope). The dissociation protocol was performed at the end of each run to check for non-specific amplification. Two replicas were run on the plate for each sample. Results are expressed as arbitrary units (A.U.) of gene expression, when compared with the control gene.

Statistical analysis

Data are depicted in graphs as medians and interquartile ranges and in tables as means ± SEM. Values were compared by unpaired nonparametric analysis of variance (ANOVA; Kruskal–Wallis) and Dunn's post test, or the unpaired nonparametric Mann–Whitney test for comparisons between two groups. $P < 0.05$ was considered to be significant.

Table 1
Primer sequences employed for quantitative PCR.

Gene	Primers	Optimal primer concentration
AT1R-F	5′-GTCAGTTTCAACCTCTACGCCAG-3′	150 nM
AT1R-R	5′-ACAATGGCCAGGTAGCGATC-3′	
AT2R-F	5′-GAATCCCTGGCAAGCATCTTAT-3′	150 nM
AT2R-R	5′-ATGTTGGCAATGAGGATAGACAAG-3′	
ACE1-F	5′-GGGCATTGACCTAGAGACTGATG-3′	70 nM
ACE1-R	5′-CTTGGGCTGTCCGGTCATAC-3′	
ACE2-F	5′-ACCAAAGCATTAAAGTGAGGATAAG-3′	150 nM
ACE2-R	5′-GTTGTTGGTCCATTCATATGCATT-3′	
ACTB-F	5′-ACTGCCGCATCCTCTTCCT-3′	70 nM
ACTB-R	5′-GAACCGCTCGTTGCCAATA-3′	

F, forward; R, reverse; *AT1R*, gene encoding Ang II type 1 receptor; *AT2R*, gene encoding Ang II type 2 receptor; *ACE1*, gene encoding ACE; *ACE2*, gene encoding ACE2; *ACTB*, gene encoding beta actin.

Results

Plasma levels of angiotensin II are significantly lower in transgenic sickle cell mice

Levels of Ang II were found to be approximately 90% lower in the transgenic sickle Berkeley (Berk) mice compared with aged-matched C57BL/6 control (WT) mice (Fig. 1A).

Expression levels of angiotensin receptors and angiotensin converting enzymes in transgenic Berk sickle cell mice

The relative expressions of the genes encoding AT_1R (*AT1R*), AT_2R (*AT2R*), angiotensin converting enzyme (*ACE1*) and angiotensin converting enzyme 2 (*ACE2*) in the kidney, heart, brain and liver of C57BL/6 control (WT) mice and transgenic sickle Berkeley (Berk) mice are presented in Table 2. In the kidney, the expressions of the *AT1R* and *ACE1* genes were significantly lower in Berk mice than in WT. In contrast, *ACE1* expression was significantly higher in the liver of Berk mice, compared with WT mice. All other genes studied were expressed similarly in the organs analyzed.

Effects of hydroxyurea therapy on hematology and angiotensin parameters in sickle cell mice

Under basal conditions, sickle cell disease (chimeric; SCD) mice demonstrated significantly augmented leukocyte counts and mean corpuscular volume (MCV), as well as significantly reduced red blood cell counts, hemoglobin levels, mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), compared with age and sex-matched WT C57BL/6 control mice (Table 3). Consistent with previous studies demonstrating the action of HU on sickle cell mice (Lebensburger et al., 2010, 2012), chronic treatment of animals with 50 mg/kg/day hydroxyurea (HU; i.p.) for 4 weeks resulted in a reduction in leukocytosis, and increased MCH, while 75 mg/kg/day HU reduced RBC counts, hemoglobin levels and platelet counts after 4 weeks in sickle cell mice, possibly indicating a slight toxicity at this higher concentration (Table 3). As these mice do not produce the human gamma globin chain, these effects were independent of fetal hemoglobin production.

Basal plasma Ang II levels were also decreased in transplanted sickle chimeric mice compared with the WT C57BL/6 control mice (Fig. 1B). Four weeks of chronic HU treatment resulted in increased mean plasma Ang II levels (75 mg/kg/day) that reached levels that were similar to those of WT mice (Fig. 1B). With regard to the tissue expression of angiotensin related proteins, HU treatment (75 mg/kg/mL) was associated with a reversal in the (non-significantly) decreased expressions of the genes encoding the AT_1R and the ACE2 protein in the kidneys of SCD mice (increases of 40.6% and 35.7%, respectively, $P < 0.05$; Table 4). The decreased expression ($P > 0.05$) of *AT1R* in the heart of sickle cell mice was also augmented by 75 mg/kg/day HU (55.8%, $P < 0.05$; Table 4), while no significant effects of HU on the expressions of the *AT1R*, *AT2R*, *ACE1* and *ACE2* genes were observed in the brain or liver of SCD mice (Table 5).

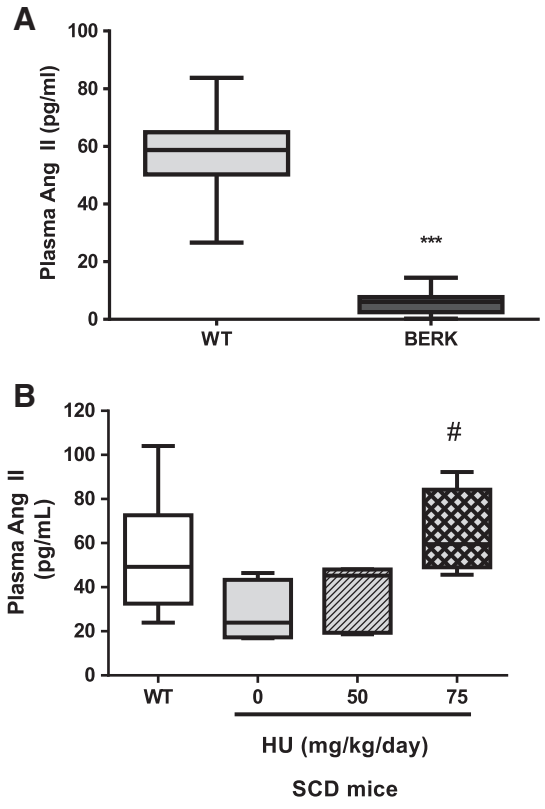

Fig. 1. Plasma angiotensin II in WT control mice and sickle cell mice. (A) Plasma levels of angiotensin II (Ang II) are significantly lower in transgenic Berkeley sickle mice (Berk, N = 7) than in wild type (WT; C57BL/6, N = 9) control mice. Mice were age-matched (5–6 months old); Ang II was determined by ELISA. ***$P < 0.001$, Mann Whitney test. (B) Effects of hydroxyurea treatment on plasma Ang II levels in sickle cell mice. Sickle cell mice (transplanted male chimeric mice; SCD; 5 months old) were treated (i.p.) with 0 (N = 5), 50 (N = 5) or 75 (N = 4) mg/kg/day hydroxyurea (HU) five times a week for four weeks. Levels of angiotensin II (Ang II) were then measured in the plasma of sickle cell mice and compared with those of age-matched male WT (C57BL/6, N = 10) control mice. #$P < 0.05$, compared with basal (0 mg/kg/day HU) (Kruskal–Wallis/Dunn's).

Discussion

Given the high levels of vascular inflammation, oxidative stress and production of vasoactive substances reported in SCD, we were surprised to find that Ang II levels were significantly diminished in the plasma of transgenic Berk mice and were also lower in chimeric SCD mice. Low Ang II was associated with decreased expressions of the genes encoding AT_1R and ACE in the kidneys of mice with SCD. It has been suggested that SCD individuals display relatively lower blood pressures, compared with age- and race-matched controls (Pegelow et al., 1997; Steinberg et al., 2009). ACE inhibitors and Ang II receptor blockers, such as enalapril and losartan, are commonly employed as antihypertensives (Burnier et al., 2014); furthermore, the absence of intrarenal ACE production has been shown to protect against hypertension in mice (Gonzalez-Villalobos et al., 2013). At present, there are no data available to indicate whether the parameters of the RAS system, including Ang II and RAS proteins, are altered in human SCD. It is difficult to extrapolate results from SCD animal models to the human form of the disease (Manci et al., 2006); however, it may be postulated that should down-regulation of renal RAS also occur in human SCD this may contribute to lower blood pressure in these individuals, although further data and evidence collected from humans with SCD are required to support such a hypothesis. The implications of an increased ACE expression in the liver of SCD mice are not clear; however liver injury is known to increase the hepatic expression of ACE (Grace et al., 2012).

Treatment of SCD mice with HU for four weeks led to reduced leukocyte and platelet counts in mice, without any improvement in red cell indices, which was consistent with previous studies examining the effects of chronic HU treatment on mice with SCD and with the fact that these mice do not express fetal hemoglobin (Lebensburger et al., 2010, 2012). Importantly, HU treatment (75 mg/kg/day) was found to increase Ang II to levels that were similar to those of age- and sex-matched WT mice and increased the expression of the genes encoding the AT_1R and ACE2 proteins in the kidney and heart. As such, in SCD mice, HU therapy appears to restore alterations in unbalanced parameters of the RAS. To date there is no evidence regarding basal RAS parameters or the effects of HU on the RAS in human SCD, and while HU

Table 2
Expression of genes encoding the angiotensin receptors and angiotensin converting enzymes in organs of wild type (WT) and Berkeley (Berk) sickle mice.

	Kidney		Heart		Brain		Liver	
	WT N = 8	Berk N = 9	WT N = 9	Berk N = 9	WT N = 5	Berk N = 5	WT N = 5	Berk N = 6
AT1R	0.76 ± 0.06	0.44 ± 0.06**	0.68 ± 0.06	0.52 ± 0.09	0.65 ± 0.07	0.82 ± 0.06	0.49 ± 0.11	0.66 ± 0.11
AT2R	0.25 ± 0.07	0.18 ± 0.10	0.52 ± 0.08	0.46 ± 0.15 (N = 5)	0.43 ± 0.09	0.63 ± 0.14	0.57 ± 0.20 (N = 3)	0.50 ± 0.14 (N = 5)
ACE1	0.85 ± 0.03	0.43 ± 0.07***	0.57 ± 0.04	0.49 ± 0.10	0.62 ± 0.08	0.63 ± 0.10	0.14 ± 0.05	0.52 ± 0.15*
ACE2	0.66 ± 0.06	0.59 ± 0.08	0.48 ± 0.07	0.50 ± 0.06	0.40 ± 0.05	0.57 ± 0.15	0.59 ± 0.17	0.21 ± 0.03

Expressions of the genes, *AT1R* (gene encoding Ang II type 1 receptor), *AT2R* (encoding Ang II type 2 receptor), *ACE1* (encoding angiotensin converting enzyme, ACE), and *ACE2* (encoding ACE2), in the kidney, heart, brain and liver of wild type (WT C57BL/6) control mice and transgenic sickle Berkeley (Berk) mice. Gene expressions are depicted as arbitrary units (A.U.) of expression compared with the expression of *ACTB*. The number of mice analyzed is reported for each tissue, unless specified for specific experiments.
* P < 0.05, compared with equivalent WT expression; Mann Whitney test.
** P < 0.01, compared with equivalent WT expression; Mann Whitney test.
*** P < 0.001, compared with equivalent WT expression; Mann Whitney test.

therapy has been suggested to have renoprotective effects in SCD, being associated with lower albuminuria and improved glomerular filtration, no significant effects of HU on blood pressure have been reported in these patients (Desai et al., 2012; Laurin et al.,2014; Silva Junior et al., 2014). As such, while observations of an HU-induced augmentation in Ang II in mice could indicate a potentially deleterious effect of this drug in SCD, further studies in humans are required especially given that concurrent increases in HbF in humans on HU may modulate these effects. The observation of HU-induced increased ACE2 activity in SCD mice could have potential benefits, as a consequent production of Ang-(1–7) could have vasodilatory and anti-inflammatory effects; however, Ang-(1–7) levels were not measured herein and further studies are required to determine whether HU may affect the levels of this Ang II-derived peptide in SCD.

ACE inhibitors (ACEi) and Ang II receptor blockers (ARB) are routinely used for the control of nephropathy in diseases such as diabetes and chronic kidney disease (CKD) (Lambers Heerspink and de Zeeuw, 2013; Lewis and Maxwell, 2014) and a number of reports relate the use of these classes of drugs in SCD patients presenting albuminuria and proteinuria with a view to preventing evolution to CKD (Ataga et al., 2014; Fitzhugh et al., 2005; Foucan et al., 1998; Lima et al., 2008; Sasongko et al., 2013; Sharpe and Thein, 2011). Higher relative systemic blood pressure in SCD has been associated with an increased risk for pulmonary hypertension and renal dysfunction (Gordeuk et al., 2008). As HU augmented the production of Ang II in SCD mice, investigations to look at the effects of HU on the human RAS are necessary with a view to evaluating whether the administration of ACEi or ARB together with HU may be indicated in patients with higher systemic blood pressures.

Conclusions

A mouse model of sickle cell disease appears to present an unbalanced RAS system, as demonstrated by decreased levels of plasma Ang II and the altered expression of major RAS proteins in the kidneys of these mice; HU treatment was able to restore some RAS parameters in these mice. Further studies are required to determine whether such alterations may occur in human SCD and the consequences of these alterations.

Conflicts of interest statement

The authors declare that they have no conflicts of interest relevant to this study.

Table 3
Hematological parameters of WT mice and of sickle cell mice following treatment, or not, with hydroxyurea (HU) for four weeks.

Hematological parameter	WT (N = 5)	Sickle cell mice		
		0 mg/kg/day HU (N = 5)	50 mg/kg/day HU (N = 5)	75 mg/kg/day HU (N = 4)
Leukocyte count ($\times 10^3/\mu L$)	7.24 (7.3/5.7/10.1)	30.20** (27.4/25.9/37.9)	21.47# (23.3/15.9/26.9)	22.91 (23.8/17.7/26.4)
RBC count ($\times 10^6/\mu L$)	8.86 (8.89/8.35/9.35)	3.71** (3.9/3.04/3.99)	3.52 (3.48/3.26/4.00)	2.80## (2.82/2.45/3.13)
Platelet count ($\times 10^3/\mu L$)	1001 (1000/921/1121)	991 (982/790/1225)	1012 (981/891/1198)	730# (738/548/898)
Hemoglobin (Hb, g/dL)	14.62 (14.8/13.8/15.5)	4.78** (4.9/4.1/5.2)	4.86 (4.7/4.6/5.4)	3.97# (4.00/3.4/4.5)
MCV (fL)	43.59 (43.91/42.68/43.97)	54.56* (54.7/52/56.7)	54.86 (54.8/50.9/58.8)	57.83 (57.8/57.4/58.3)
MCH (pg)	16.46 (16.5/16.1/16.7)	12.9** (12.8/12.5/13.5)	13.76# (13.9/13.3/14.1)	14.15## (14.2/13.7/14.5)
MCHC (g/dL)	37.8 (37.8/37.6/38.2)	23.68** (23.4/22.9/25.5)	25.1 (25.7/22.5/26.7)	24.52 (24.6/23.6/25.3)

Data expressed in mean (median/min/max); WT, WT (C57BL/6) control mice; sickle cell mice, transplanted chimeric sickle mice. Mice were treated, or not, with hydroxyurea (HU) five times a week for four weeks (*via* i.p.). RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.
* P < 0.05, compared to WT (Mann–Whitney test).
** P < 0.01, compared to WT (Mann–Whitney test).
# P < 0.05, compared with 0 mg/kg/day (Kruskal–Wallis, Dunn's test).
P < 0.01, compared with 0 mg/kg/day (Kruskal–Wallis, Dunn's test).

Table 4
Effect of hydroxyurea treatment on the expression of genes encoding the angiotensin receptors and angiotensin converting enzymes in the kidney and heart of sickle cell mice.

	Kidney				Heart			
	WT	Sickle cell			WT	Sickle cell		
		Basal	50 HU mg/kg/day	75 HU mg/kg/day		Basal	50 HU mg/kg/day	75 HU mg/kg/day
	N = 12	N = 5	N = 5	N = 4	N = 12	N = 5	N = 5	N = 4
AT1R	0.73 ± 0.05	0.64 ± 0.05	0.71 ± 0.06	0.90 ± 0.05#	0.71 ± 0.05	0.52 ± 0.07	0.41 ± 0.06	0.81 ± 0.03#
AT2R	0.20 ± 0.05	0.44 ± 0.19 (N = 4)	0.12 ± 0.01	0.26 ± 0.09	0.55 ± 0.07 (N = 11)	0.45 ± 0.11	0.47 ± 0.18	0.72 ± 0.13 (N = 3)
ACE1	0.80 ± 0.05	0.36 ± 0.02** (N = 4)	0.43 ± 0.02	0.45 ± 0.05 (N = 3)	0.65 ± 0.05	0.54 ± 0.06	0.44 ± 0.06	0.60 ± 0.07
ACE2	0.70 ± 0.05	0.56 ± 0.03	0.64 ± 0.06	0.76 ± 0.07#	0.58 ± 0.07	0.19 ± 0.03**	0.20 ± 0.03	0.32 ± 0.02

Expressions of the genes, *AT1R* (encoding Ang II type 1 receptor), *AT2R* (encoding Ang II type 2 receptor), *ACE1* (encoding ACE), and *ACE2* (encoding ACE2), in the kidney and heart of wild type (C57BL/6) control mice and sickle cell mice (transplanted chimeras). Gene expressions are depicted as arbitrary units (A.U.) of expression compared to the expression of *ACTB*. Numbers of mice analyzed are reported for each tissue, unless specified.
** $P < 0.01$, compared with equivalent WT expression; Mann Whitney test.
# $P < 0.05$, compared with basal expression (Kruskal–Wallis/Dunn's).

Table 5
Effect of hydroxyurea treatment on the expression of genes encoding the angiotensin receptors and angiotensin converting enzymes in the brain and liver of sickle cell mice.

	Brain				Liver			
	WT	Sickle cell			WT	Sickle cell		
		Basal	50 HU mg/kg/day	75 HU mg/kg/day		Basal	50 HU mg/kg/day	75 HU mg/kg/day
	N = 9	N = 5	N = 5	N = 4	N = 9	N = 5	N = 5	N = 4
AT1R	0.53 ± 0.07	0.51 ± 0.03	0.44 ± 0.05	0.56 ± 0.16	0.33 ± 0.09	0.45 ± 0.04	0.55 ± 0.07	0.73 ± 0.16
AT2R	0.51 ± 0.10	0.59 ± 0.10	0.59 ± 0.06	0.60 ± 0.18	0.36 ± 0.10 (N = 8)	0.32 ± 0.11	0.47 ± 0.18	0.33 ± 0.23
ACE1	0.58 ± 0.09	0.78 ± 0.02	0.81 ± 0.06	0.75 ± 0.08	0.14 ± 0.04	0.59 ± 0.13**	0.54 ± 0.13	0.38 ± 0.74
ACE2	0.48 ± 0.06	0.76 ± 0.03*	0.78 ± 0.06	0.78 ± 0.09	0.41 ± 0.14 (N = 8)	0.49 ± 0.10	0.53 ± 0.07	0.81 ± 0.19 (N = 3)

Expressions of the genes, *AT1R* (encoding Ang II type 1 receptor), *AT2R* (encoding Ang II type 2 receptor), *ACE1* (encoding ACE), *ACE2* (encoding ACE2), in the brain and liver of wild type (C57BL/6) control mice and sickle mice (transplanted chimeric mice). Gene expressions are depicted as arbitrary units (A.U.) of expression compared to the expression of *ACTB*. Numbers of mice analyzed are reported for each tissue, unless specified.
* $P < 0.05$, compared with equivalent WT expression; Mann Whitney test.
** $P < 0.01$, compared with equivalent WT expression; Mann Whitney test.

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