Evidence of protective effects of recombinant ADAMTS13 in a humanized model of sickle cell disease

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Abstract

Sickle cell disease (SCD) is an inherited red blood cell disorder that occurs worldwide. Acute vaso-occlusive crisis is the main cause of hospitalization in patients with SCD. There is growing evidence that inflammatory vasculopathy plays a key role in both acute and chronic SCD-related clinical manifestations. In a humanized mouse model of SCD, we found an increase of von Willebrand factor activity and a reduction in the ratio of a disintegrin and metalloproteinase with thrombospondin type 1 motif, number 13 (ADAMTS13) to von Willebrand factor activity similar to that observed in the human counterpart. Recombinant ADAMTS13 was administered to humanized SCD mice before they were subjected to hypoxia/reoxygenation (H/R) stress as a model of vaso-occlusive crisis. In SCD mice, recombinant ADAMTS13 reduced H/R-induced hemolysis and systemic and local inflammation in lungs and kidneys. It also diminished H/R-induced worsening of inflammatory vasculopathy, reducing local nitric oxidase synthase expression. Collectively, our data provide for the first-time evidence that pharmacological treatment with recombinant ADAMTS13 (TAK-755) diminished H/R-induced sickle cell-related organ damage. Thus, recombinant ADAMTS13 might be considered as a potential effective disease-modifying treatment option for sickle cell-related acute events.

Introduction

Sickle cell disease (SCD) is a hereditary red blood cell disorder caused by a single amino acid substitution in the β chain of hemoglobin and results in the production of pathological sickle hemoglobin (HbS). SCD is characterized by chronic hemolysis and inflammatory vasculopathy, which concur with acute vaso-occlusive crises. These main causes of hospitalization of SCD patients contribute to the disease's high mortality and morbidity.[1-3]

In the last decade, progress in the knowledge of the pathophysiology of SCD has highlighted the role of pro-adhesive cell-cell interactions involving dense red blood cells, reticulocytes, neutrophils, inflammatory activated endothelial cells, and plasma factors.[4-9] Collectively these factors contribute to the pro-thrombotic profile of SCD as supported by thrombin generation, depleted anticoagulant proteins, an activated fibrinolytic system, and increased tissue factor expression, even in steady state.[5,9-14] von Willebrand factor (VWF) and its regulatory protease ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motif, number 13) represent a critical axis in both hemostasis and inflammatory responses.[15,16] VWF activity has been suggested as a driving mechanism in various diseases such as atherosclerosis, diabetes, coronary artery disease, stroke, myocardial infarction, thrombotic microangiopathy, and sepsis. All these are characterized by inflammatory vasculopathy, amplified inflammatory response and vascular dysfunction.[4,5,8,11,17] Ultra-large multimers of VWF have been reported in these disorders, most likely associated with relative reduction of ADAMTS13 activity due to either inhibition of cleavage activity of ADAMTS13 or degradation of ADAMTS13 related to severe inflammation.[18]

Recombinant human ADAMTS13 (rADAMTS13; TAK-755) has been developed for the treatment of congenital and im-

mune-mediated thrombotic thrombocytopenic purpura (TTP). ADAMTS13 deficiency in TTP causes unusually large VWF multimers, thrombosis in the microcirculation and ischemic damage to multiple organs.[19] Administration of TAK-755 to patients with congenital TTP reduced the concentration of ultra-large VWF multimers and improved the clinical course of congenital TTP.[20] Accumulation of ultra-large VWF has been reported in patients with SCD, possibly related to the detrimental effect of plasma free hemoglobin that, binding to VWF, prevents its cleavage by ADAMTS13.[21-25] This is further supported by the observation of increased VWF antigen and decreased ADAMTS13/VWF:antigen ratio in SCD patients at both steady state and in acute pain crises.[23,24,26-28]

To address the question of whether the normalization of ADAMTS13 might mitigate sickle cell-related acute events, we administered recombinant human ADAMTS13 (TAK-755) to humanized sickle cell mice which were then exposed to hypoxia/reoxygenation (H/R) stress, a consolidated model mimicking sickle cell-related acute vaso-occlusive events.[4,17,29] Here, we showed that treatment with rADAMTS13 limits H/R-induced inflammatory vasculopathy in target organs for SCD such as lung and kidney, reducing vascular vulnerability with beneficial effects on disease progression. Taken together, our data provide a rationale to explore the use of rADAMTS13 in the treatment of sickle cell-related acute events.

Methods

Mice and study design

Mice humanized for human sickle hemoglobin ($Hba^{tm1(HBA)Tow}$ $Hbb^{tm2(HBG1,HBB*)Tow}$, HbS, SS mice) or human normal hemoglobin ($Hba^{tm1(HBA)Tow}$ $Hbb^{tm3(HBG1,HBB*)Tow}$, HbA, AA mice), were either directly supplied by The Jackson Laboratory (Jackson Laboratories, USA/ Charles River Laboratories, Sulzfeld, Germany) or bred at Verona University (CIRSAL), Italy. All animal studies complied with national laws governing animal experimentation. The experimental protocol was approved by the animal care and use committees of the respective institutions. Male and female AA and SS mice, aged 3 to 4 months old, were studied under ambient conditions (normoxia) or exposed to H/R (7 or 8% oxygen for 5 or 10 h, followed by 1 or 3 h at ambient atmosphere) to mimic sickle cell-related acute vaso-occlusive crisis.[4,29]

rADAMTS13 (TAK-755, SHP655, Baxalta Innovations GmbH, Orth an der Donau, Austria) was provided in sterile water for injection. In preliminary experiments, we verified the ability of human rADAMTS13 to cleave mouse VWF with an efficiency similar to that observed for human VWF.[30] Sequence analysis of human and mouse ADAMTS13 revealed a high interspecies identity and similarities in protein areas involved in either protease activity or VWF binding (*Online Supplementary Figure S1A, B*). rADAMTS13 was administered intravenously (via the tail vein) at a dose volume of 10 mL/kg 1 h before hypoxia. In preliminary pharmacokinetic experiments, we identified 2,940 U/kg as the optimal dose to reduce VWF activity/antigen ratio in SS mice.[31] Vehicle buffer was a solution in sterile water for injection of calcium chloride (2 mM), L-histidine (20 mM), mannitol (3% w/w), sucrose (1% w/w), and polysorbate 80 (0.05% w/w) at pH 6.9-7.1.

Hematologic parameters and red cell indices were determined as previously reported.[32-35] H/R-induced clinical signs were evaluated as previously described.[4,36,37] Details are reported in the *Online Supplementary Methods*.

Plasma assays and bioactivity of human recombinant ADAMTS13

Human ADAMTS13 antigen level was determined by an enzyme-linked immunosorbent assay (ELISA) using affinity purified polyclonal anti-human ADAMTS13 antibody from guinea pig and detection with horseradish peroxidase-conjugated polyclonal rabbit anti-human ADAMTS13 antibody. Human ADAMTS13 activity was determined by a fluorescence resonance energy transfer (FRET) assay[38] using FRETS-VWF73 quenching substrate (Peptanova). Mouse VWF antigen level was determined by ELISA (Asserachrom, VWF:Ag), mouse VWF activity by a VWF collagen binding ELISA method (Zymutest, VWF:CBA), and mouse VWF multimer analysis by low resolution agarose gel electrophoresis in combination with immunostaining with an anti-human VWF antibody (Hydragel).

Histological analysis of lungs and kidneys

Paraffin-embedded tissue blocks were cut into 2-3 µm sections and mounted on adhesion microscope glass slides for hematoxylin and eosin (H&E) and Perls' staining for iron content. The analysis was performed on four different fields at a 200X magnification. Tissue pathology, inflammatory cell infiltrate, the presence of thrombi and iron deposition were assessed by blinded pathologists as previously described.[4,29,32]

Molecular analysis

Real-time polymerase chain reaction analysis was carried out as previously described.[29] Details are reported in the *Online Supplementary Methods*.

Immunoblot analyses

Frozen lung, kidney and aorta samples were homogenized and lysed as previously reported.[4,29,32] Proteins were quantified and analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis. Gels were transferred to nitrocellulose membranes for immunoblot analysis with specific antibodies. Details are described in the O*nline*

Supplementary Methods.

In vitro blood cell adhesion study

The degree of calcein-labeled platelet adhesion (expressed as percent coverage over the total surface) was studied in a perfusion chamber (BioFlux 1000z System, Fluxion BioSciences, San Francisco, CA, USA), using SS mouse blood in a 48-well plate coated with equine tendon fibrillar collagen type I (Horm collagen reagent, Takeda, Linz, Austria) at a wall shear rate of 1500 s^{-1} (60 dyne/cm^2) with and without addition of rADAMTS13 (200 U/mL).

Statistical analysis

Statistical analysis was performed with GraphPad Prism 8.0, and *P* values were calculated using an unpaired one-tailed *t*-test with the Welch correction. Data were analyzed using either a *t*-test or one-way analysis of variance for repeated measures between the mice of various genotypes. A difference with a $P<0.05$ was considered statistically significant.

Results

Sickle cell disease mice showed raised von Willebrand factor activity and relative deficiency of ADAMTS13 activity

In humanized SCD mice, we found a 2-fold increase in VWF activity/antigen ratio compared to that in healthy AA mice. In agreement, a higher order and higher number of VWF multimers were detected in plasma from SS mice compared to AA animals, similarly to the situation in patients with SCD (Figure 1A, *Online Supplementary Figure S2A*).[24] We then evaluated VWF and ADAMTS13 protein activities and antigens under ambient air conditions or hypoxia (*Online Supplementary Figure S2B*). ADAMTS13 activity was similar in both mouse strains under either normoxia or hypoxia (Figure 1B). Increases in VWF activity, as determined by collagen binding, and in total VWF antigen levels were observed in SS mice under normoxia compared to those in AA animals (Figure 1B, *Online Supplementary Figure S2C*). Hypoxia further increased VWF activity in plasma from SS mice (Figure 1B). The ADAMTS13/VWF activity ratio was lower in SS mice, under either normoxia or hypoxia, than in healthy animals (Figure 1B). Our results agree with observations in patients with SCD,[25,26] characterized by increased VWF activity and relative deficiency of ADAMTS13 activity. This supported the translational relevance of the tested pharmacology. Thus, we evaluated the impact of rADAMTS13 on the humanized mouse model of SCD exposed to H/R stress to mimic acute vaso-occlusive crisis[4,36] (*Online Supplementary Figure S2D*).

In sickle cell mice, recombinant ADAMTS13 improved hypoxia/reoxygenation-induced hematologic changes and reduced systemic inflammation

Recombinant ADAMTS13 diminished the H/R-induced reduction in hematocrit and hemoglobin (Figure 1C, *Online Supplementary Figure S2E*). This was associated with a significant reduction in hemoglobin distribution width, used as a marker of a dense cell subpopulation, compared with that in vehicle-treated SS animals (Figure 1D, left panel). Hemoglobin distribution width corresponds to the standard deviation of the red cell hemoglobin histogram shown in the right panel of Figure 1D (see also *Online Supplementary Figure S2F* for the histogram for healthy, AA, mice). We also observed a reduction in plasma lactate dehydrogenase in rADAMTS13-treated SS mice exposed to H/R compared to the value in SS vehicle-treated animals (*Online Supplementary Figure S2G*). Previously, Nwankwo *et al*. reported lower platelet counts in SS mice under normoxia than in healthy (AA) controls.[40] Here, we found that, compared to treatment with vehicle, rADAMTS13 treatment ameliorated the H/R-induced thrombocytopenia in SS mice (Figure 1E). Since a functional connection between platelets and ADAMTS13 activity has been reported in other models of inflammatory vasculopathy associated with H/R stress, we conducted preliminary experiments of platelet adhesion to immobilized collagen in a perfusion chamber with or without rADAMTS13. We found increased adhesion of platelets from SS mice when compared to platelets from heathy controls. This was significantly reduced by rADAMTS13 (*Online Supplementary Figure S3A, B*). A significant decrease in neutrophil count was also found in rADAMTS13-treated SS mice exposed to H/R, compared to the count in vehicle-treated SS mice (Figure 1F). This was associated with a reduction in C-reactive protein, a marker of systemic inflammation (*Online Supplementary Figure S3C*). Taken together, our data indicate that rADAMTS13 reduces hemolysis and platelet adhesion and ameliorates systemic inflammation in SS mice exposed to H/R.

In sickle cell mice, recombinant ADAMTS13 diminished hypoxia/reoxygenation-induced lung injury and local inflammatory related vascular dysfunction

Lung is a target organ of SCD.[4,37] Thus, we evaluated the effects of rADAMTS13 on lungs of SS mice exposed to H/R stress. rADAMTS13 reduced inflammatory cell infiltrates and thrombi formation compared with those in vehicle-treated animals (Figure 2A, Table 1). This was associated with significant reductions in protein and leukocyte counts in bronchoalveolar lavage fluid, indicating a reduction of H/R-associated vascular leakage in rADAMTS13-treated SS mice compared with that in vehicle-treated animals (Figure 2B).

Previous studies have shown the crucial role of nuclear factor kappa B (NF-κB)-dependent pathways in the severity

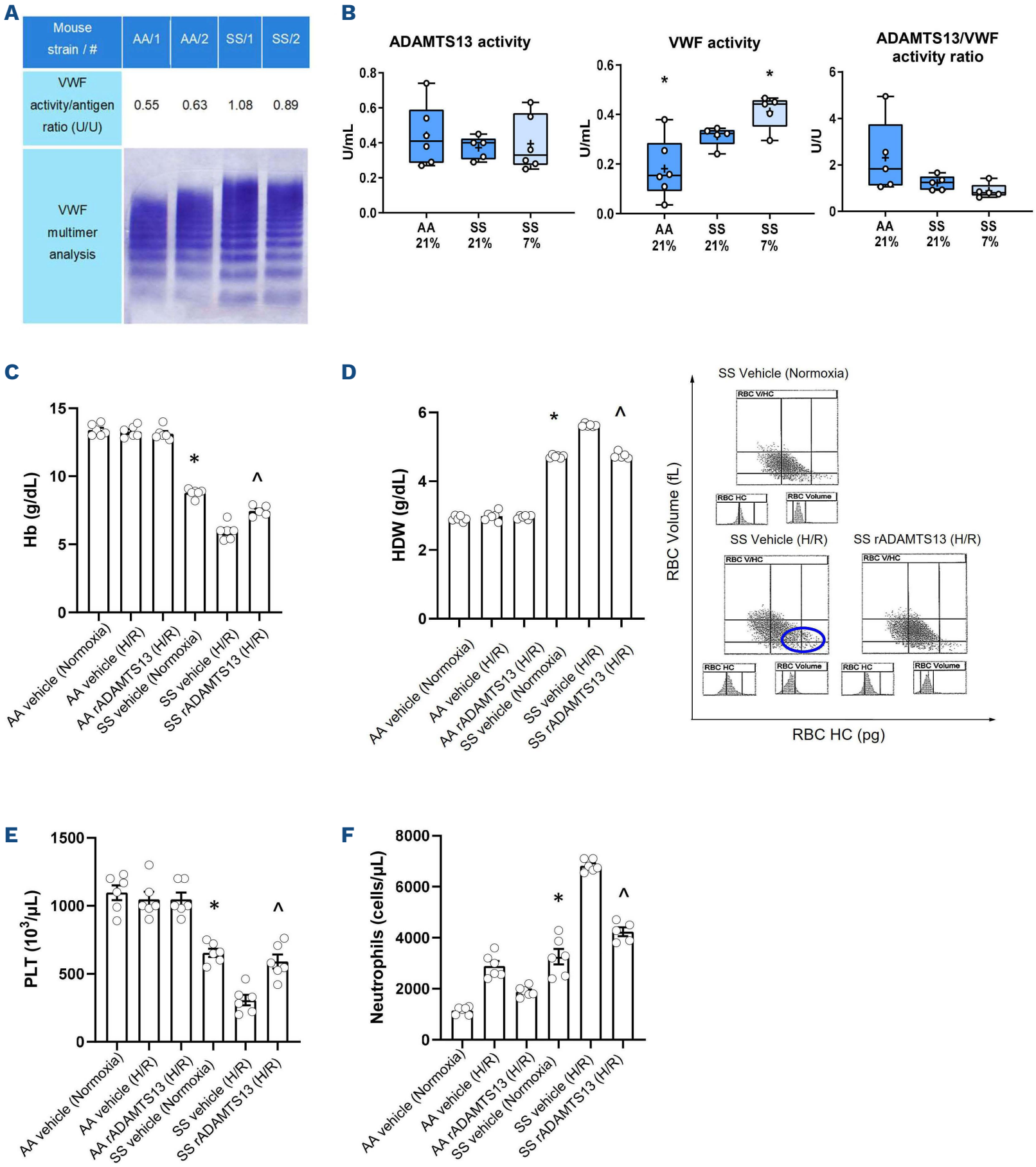

Figure 1. Recombinant ADAMTS13 reduced hypoxia/reoxygenation-induced hemolysis and modulated the systemic inflammatory response. (A) von Willebrand factor (VWF) activity/antigen concentration ratio in plasma of humanized healthy (AA) and sickle cell (SS) mice. VWF multimer gel analysis of SS and AA mouse plasma (see *Online Supplementary Figure S2A* for additional gels). (B) Plasma ADAMTS13 activity, VWF activity, ADAMTS13/VWF activity ratio in AA and SS mice (n=5-6 age-matched male mice; *P<0.05 *vs*. SS 21%). (C) Hemoglobin (Hb) in AA and SS mice under normoxia and exposed to hypoxia/reoxygenation (H/R) treated with either vehicle or recombinant ADAMTS13 (rADAMTS13); *P<0.05 compared to AA; ^P<0.05 compared to vehicle. (D) Left panel. Hemoglobin distribution width (HDW) in AA and SS mice under normoxia and exposed to H/R treated with either vehicle or rADAMTS13; *P<0.05 compared to AA; ^P<0.05 compared to vehicle. Right panel. Distribution histograms generated for red blood cell volume (RBC Volume) and cell hemoglobin concentration (RBC-HC) of red blood cells from humanized SS mice under normoxia and treated with either vehicle or rADAMTS13 and exposed to H/R. One experiment representative of six others with similar results is shown. The blue circle indicates the presence of a subpopulation of dense red cells. (E) Platelet (PLT) counts in humanized AA and SS mice under normoxia and treated with either vehicle or rADAMTS13 and exposed to H/R (n=6; age-matched female and male mice; ^P<0.05). (F) Peripheral neutrophils in AA and SS mice under normoxia or exposed to H/R (8% oxygen for 10 h followed by 3 h of reoxygenation) treated with either vehicle or rADAMTS13. Data are presented as box-and-whisker plots. *P<0.001 compared to vehicle-treated animals under normoxia. ^P<0.001 compared to vehicle-treated animals under hypoxia. P values were calculated using an unpaired one-tailed t-test with the Welch correction.

of SCD-related lung injury.[4,37] Here, we found that rADAMTS13 reduced H/R-induced activation of NF-κB p65 (pNF-κB/NF-κB ratio) compared with that in vehicle-treated SS mice (Figure 2C, *Online Supplementary Figure S4A*). Indeed, a downregulation of *Il1* gene expression was observed in lungs from rADAMTS13-treated SS mice compared with that in samples from vehicle-treated animals (*Online Supplementary Figure S4B*).

Markers of both vascular endothelial dysfunction and local inflammatory responses, such as vascular endothelial cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), endothelin-1 (ET-1) and E-selectin, were significantly lower in lungs of rADAMTS13-treated SS mice than in vehicle-treated SS animals (Figure 2D, *Online Supplementary Figures S4C* and *S5*). It is noteworthy that the expression of thromboxane synthase (TXAS), controlled by NF-κB, was significantly reduced in rADAMTS13-treated SS mice compared with that in vehicle-treated animals. This finding is in line with the downregulation of heme-oxygenase-1 (HO-1) expression, a known anti-oxidant and lung cytoprotective system[4,41] (Figure 2D, *Online Supplementary Figure S4C*). Indeed, we found a reduction in oxidation of lung proteins from rADAMTS13-treated SS mice exposed to H/R stress compared with that in vehicle-treated animals (*Online Supplementary Figure S6A*). Previous studies showed that a local reduction in nitric

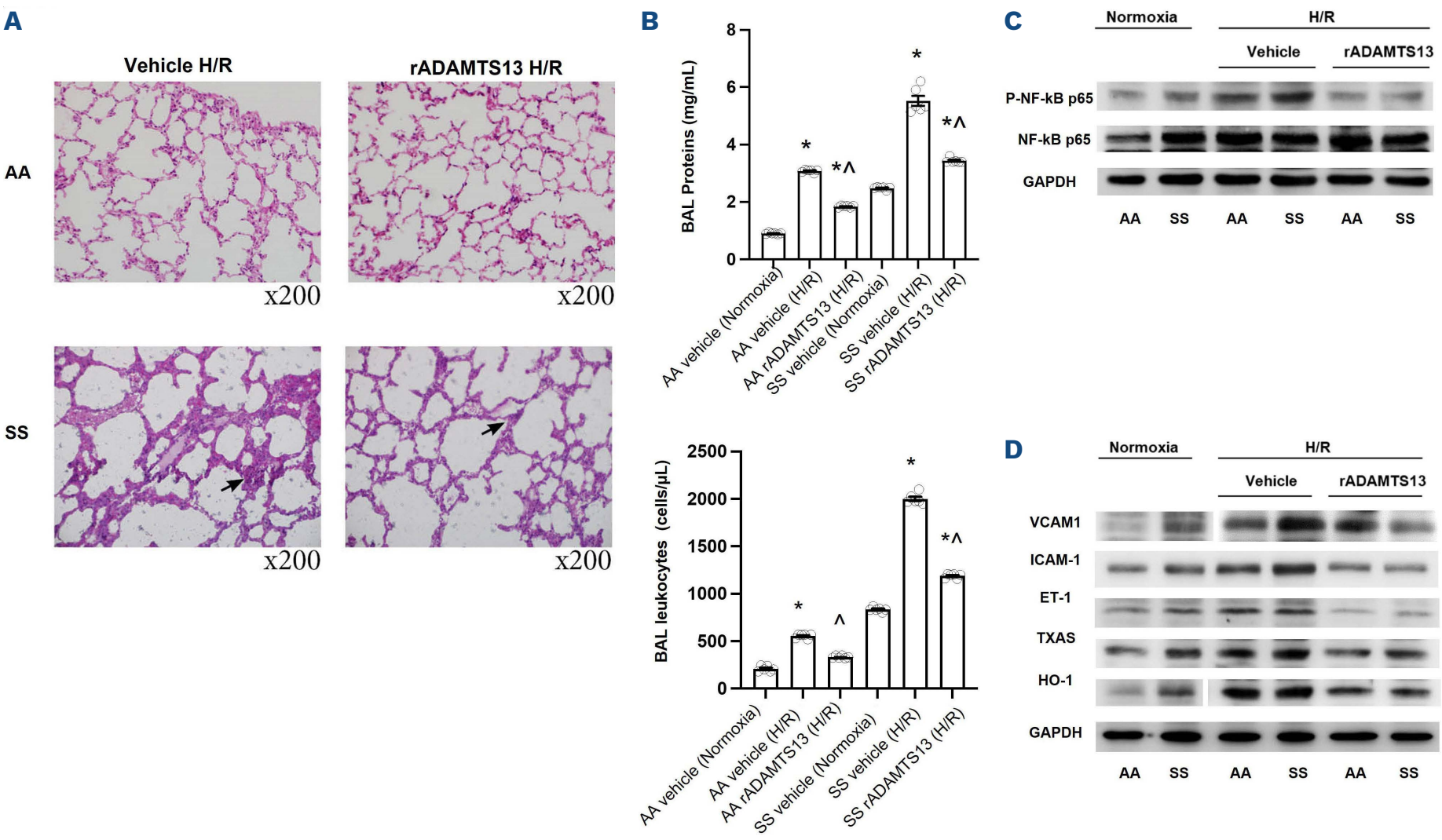

Figure 2. In sickle cell disease mice, recombinant ADAMTS13 reduced hypoxia/reoxygenation-induced lung damage and inflammatory vasculopathy. (A) Representative hematoxylin and eosin-stained sections of lung tissue from healthy (AA) and sickle cell (SS) mice exposed to hypoxia (8% oxygen; 10 h), followed by reoxygenation (21% oxygen; 3 h) treated with vehicle or recombinant ADAMTS13 (rADAMTS13) (2940 U/kg), see also Table 1; black arrows indicate inflammatory cell infiltrate. (B) Leukocyte content (lower panel) and protein content (upper panel) in bronchoalveolar lavage (BAL) from AA and SS mice under normoxia or exposed to hypoxia (8% oxygen; 10 h), followed by reoxygenation (21% oxygen; 3 h) treated with either vehicle or rADAMTS13. Data are presented as mean ± standard error of mean (n=6, age-matched male and female animals). *P<0.001 compared to vehicle-treated animals under normoxia. ^P<0.001 compared to vehicle-treated animals under hypoxia. P values were calculated using an unpaired one-tailed t-test with the Welch correction. (C) Immunoblot analysis, using specific antibodies against phosphorylated (P-)NF-κB p65 and NF-κB p65 of lung from AA and SS mice under normoxia or exposed to hypoxia (8% oxygen; 10 h), followed by reoxygenation (21% oxygen; 3 h) treated with either vehicle or rADAMTS13. GAPDH was used as a protein loading control. One representative gel from three with similar results is shown. Densitometric analysis of immunoblots is shown in *Online Supplementary Figure 4SA*. (D) Immunoblot analysis, using specific antibodies against VCAM-1, ICAM-1, ET-1, TXAS and HO-1 of lung from AA and SS mice treated as in (C). GAPDH was used as a protein loading control. One representative gel from three with similar results is shown (n=3 age-matched male and female mice in each group). Densitometric analysis of immunoblots is shown in *Online Supplementary Figure S4C*. H/R: hypoxia/reoxygenation; VCAM-1: vascular endothelial cell adhesion molecule-1; ICAM-1: intracellular adhesion molecule-1; ET-1: endothelin-1; TXAS: thromboxane synthase; HO-1: heme oxygenase-1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Table 1. Effects of recombinant ADAMTS13 on lung and kidney pathology of healthy and sickle cell mice exposed to hypoxia/reoxygenation stress.

Pathology endpoints			AA mice		SS mice	
Tissue	**Marker**	**Measure**	**vehicle - H/R (N=5)**	**rADAMTS13 - H/R (N=5)**	**vehicle - H/R (N=5)**	**rADAMTS13 - H/R (N=4)**
Lung	Inflammatory cell infiltrate	grade	(+)	(+)	(+) - (++)	(+)
		incidence	3/5	1/5	2/5 - 3/5	1/4
	Thrombi	grade	(0)	(0)	(+)	(+)
		incidence	0/5	0/5	5/5	1/4
		mean per field	0	0	3	3
Kidney	Inflammatory cell infiltrate	grade	(+)	(+)	(+) - (++)	(0)
		incidence	1/5	1/5	2/5 - 1/5	0/4
	Thrombi	grade	(0)	(0)	(+)	(+)
		incidence	0/5	0/5	5/5	4/4
		mean per field	0	0	5	2.5

AA. healthy; SS: sickle cell; rADAMTS13: recombinant ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motif, number 13); H/R: hypoxia/reoxygenation.

oxide is critical to the pathogenesis of acute sickle cell-related organ damage. Two main nitric oxide synthases (NOS) have been described to be important in SS. eNOS is constitutively expressed in the endothelium, whereas iNOS is inducible by cytokines and the inflammatory response.[37,42-44] It is worth noting that prolonged or severe oxidation might transform eNOS from a coupled to uncoupled state, resulting in superoxide production.[45] In our model, we found increased expression of eNOS in SS mice under normoxia when compared with that in healthy mice (*Online Supplementary Figure S6B*). H/R stress further upregulates eNOS expression in SS mice. rADAMTS13 reduced the H/R-induced upregulation of eNOS in SS mice. No major change was observed in rADAMTS13-treated AA mice compared with vehicle-treated animals (*Online Supplementary Figure S6B)*. No difference in iNOS expression was observed in both mouse strains exposed to H/R treated with either vehicle or rADAMTS13 (*Online Supplementary Figure S6B*). Collectively, our data indicate that rADAMTS13 reduces local inflammatory and vascular dysfunction in the lung by modulation of vascular adhesion markers and eNOS expression.

In sickle cell mice, recombinant ADAMTS13 reduced hypoxia/reoxygenation-induced kidney injury and modulated local inflammatory response

Sickle cell-related nephropathy is one of the most common complications in both children and adults with SCD.[36,46,47] In SCD, renal vasculopathy has been linked to both ischemic/reperfusion damage, resulting in vascular dysfunction and pro-fibrotic stimuli.[1,33] Here, we found that treatment with rADAMTS13 reduced atrophic tubules, glomerular inflammatory cell infiltration and decreased thrombi formation in SS mice compared with vehicle-treated SS animals (Figure 3A, Table 1). This was associated with significant reductions in both creatinine and blood urea nitrogen in rADAMTS13-treated SS mice exposed to H/R compared with their levels in vehicle-treated animals (Figure 3B). This agrees with the lower activation of NF-κB p65 observed in kidneys from rADAMTS13-treated SS mice than in vehicle-treated animals (Figure 3C, *Online Supplementary Figure S7A*), suggesting an amelioration of the local inflammatory response to H/R stress. Indeed, we found downregulation of VCAM-1, ET-1, TXAS and E-selectin expression in kidney from rADAMTS13 SS mice compared with the expression in vehicle-treated SS animals (Figure 3D, *Online Supplementary Figure S7B*). Taken together our data indicate that rADAMTS13 reduced H/R-induced kidney damage and renal inflammatory vasculopathy in humanized SCD mice. Indeed, we found reduction in oxidation of proteins in kidney from rADAMTS13-treated SS mice exposed to H/R stress compared with that in vehicle-treated animals (Figure 4A). This was associated with upregulation of both eNOS and iNOS in kidney from SCD mice exposed to H/R compared with the levels in either SS mice under normoxia or AA mice exposed to H/R stress. rADAMTS13 reduced the H/R-induced increased expression of both eNOS end iNOS in kidney from SS mice compared with that in vehicle-treated SS animals (Figure 4B).

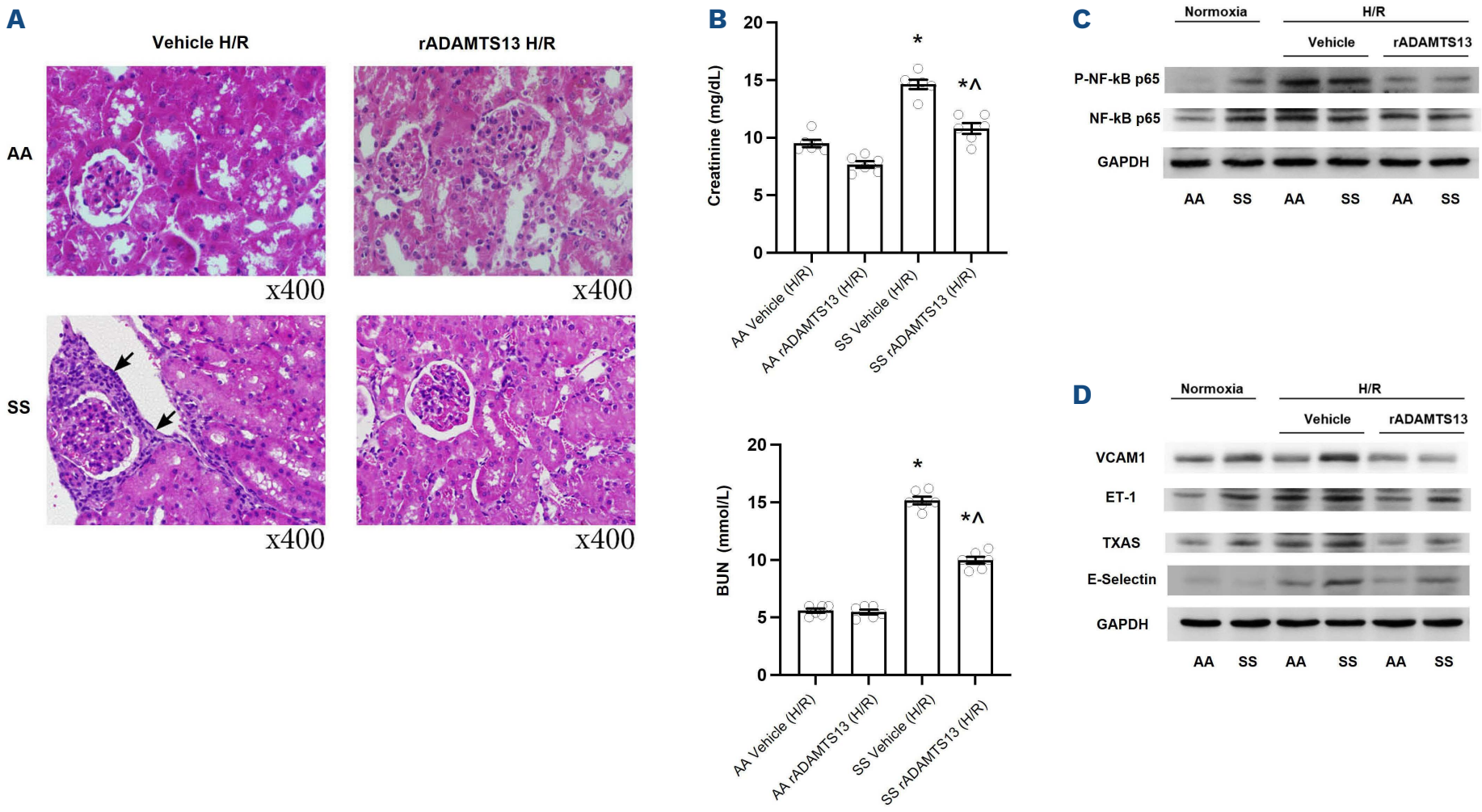

Figure 3. In sickle cell disease mice, recombinant ADAMTS13 diminished hypoxia/reoxygenation-induced kidney damage and vascular inflammatory activation. (A) Representative hematoxylin and eosin-stained sections of kidney tissue from healthy (AA) and sickle cell (SS) mice exposed to hypoxia (8% oxygen; 10 h), followed by reoxygenation (21% oxygen; 3 h) treated with either vehicle or recombinant ADAMTS13 (rADAMTS13) (2940 U/kg); see also Table 1; black arrows indicate inflammatory cell infiltrate. (B) Plasma creatinine (upper panel) and blood urea nitrogen (BUN) (lower panel) in AA and SS mice treated as in (A). Data are mean ± standard error of mean (n=6 age-matched male and female animals). *P<0.05 compared to AA, ^P<0.05 compared to vehicle-treated animals. (C) Immunoblot analysis, using specific antibodies against phosphorylated (P-)NF-κB p65 and NF-κB p65 of kidney from AA and SS mice under normoxia or exposed to hypoxia (8% oxygen; 10 h), followed by reoxygenation (21% oxygen; 3 h) treated with vehicle or rADAMTS13. GAPDH served as the protein loading control. One representative gel from three with similar results is shown (n=3 age-matched male and female in each group). Densitometric analysis of immunoblots is shown in *Online Supplementary Figure 7SA*. (D) Immunoblot analysis, using specific antibodies against VCAM-1, ET-1, TXAS and E-Selectin of kidney from AA and SS mice treated as in (A). GAPDH served as a protein loading control. One representative gel from three with similar results is shown (n=3 age-matched male and female in each group). Densitometric analysis of immunoblots is shown in *Online Supplementary Figure 7SB*. H/R: hypoxia/reoxygenation; VCAM-1: vascular endothelial cell adhesion molecule-1; ET-1: endothelin-1; TXAS: thromboxane synthase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Collectively, our data show that rADAMTS13 reduces H/R-induced kidney injury, and improves the local inflammatory response and vascular dysfunction.

In sickle cell mice, recombinant ADAMTS13 diminished hypoxia/reoxygenation-induced inflammatory vasculopathy

Since inflammatory vasculopathy plays a key role in the pathogenesis of both acute and chronic sickle cell-related organ damage,[4,7,9,29] we studied isolated aorta from both mouse strains exposed to H/R stress. As shown in Figure 4C, we confirmed the H/R-induced upregulation of both ET-1 and E-selectin in aorta from vehicle-treated SS mice compared with that of SS animals under normoxia.[4,29,36] This effect was reduced by rADAMTS13 treatment. No major change in ET-1 was observed in AA mice exposed to H/R stress compared with that in normoxic AA animals (Figure 4C). The expression of E-Selectin was increased in aorta from both mouse strains exposed to H/R compared with the expression in normoxic animals. This effect was reduced by rADAMTS13 treatment in both mouse strains (Figure 4C).

Discussion

Here, we show for the first time that exogenous ADAMTS13 reduces acute SCD-related vascular activation and H/R-induced organ damage in humanized sickle cell mice. Previous observations in human subjects with SCD suggested a relative ADAMTS13 deficiency that contributes to the accumulation of VWF and participates in the inflammatory vasculopathy that characterizes the disease.[24]

Administration of rADAMTS13 to SCD mice was shown to reduce H/R-induced hemolysis and H/R-induced throm-

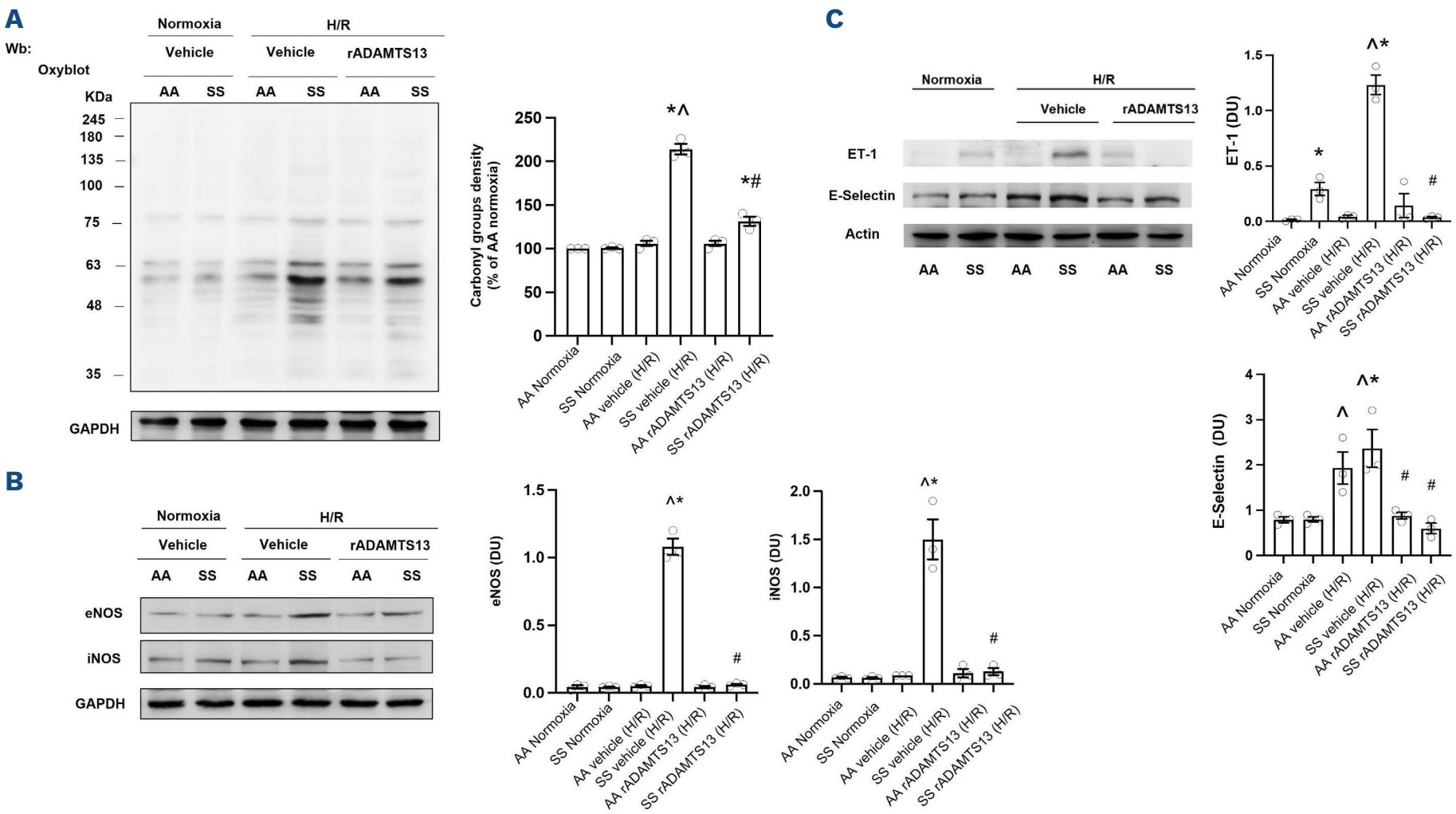

Figure 4. Recombinant ADAMTS13 reduces hypoxia/reoxygenation-induced kidney over-activation of endothelial nitric oxide synthase and protects against hypoxia/reoxygenation-induced worsening of inflammatory vasculopathy. (A) Left panel. Kidney soluble fraction from healthy (AA) and sickle cell (SS) mice treated under normoxia or exposed to hypoxia (8% oxygen; 10 h), followed by reoxygenation (21% oxygen; 3 h) treated with either vehicle or recombinant ADAMTS13 (rADAMTS13) (2940 U/kg). Samples were analyzed by 11% sodium dodecylsulfate polyacrylamide gel electrophoresis and subjected to OxyBlot. The carbonylated proteins (1 mg) were detected by treating with 2,4-dinitrophenylhydrazine (DNP) and blotted with anti-DNP antibody. Right panel. Band area was quantified by densitometry and expressed as percentage of that in AA mice in normoxia. The data are presented as means ± standard error of mean (SEM) (n=3 age-matched male and female mice in each group); ^P<0.05 compared to normoxia, *P<0.05 compared to AA mice; #P<0.05 compared to vehicle-treated animals by a one-tailed t-test with Welch correction. (B) Left panel. Immunoblot analysis, using specific antibodies against eNOS and iNOS in kidney from AA and SS mice under normoxia or exposed to hypoxia (8% oxygen; 10 h), followed by reoxygenation (21% oxygen; 3 h) treated with either vehicle or rADAMTS13 (2940 U/kg). GAPDH served as a protein loading control. One representative gel from three with similar results is shown. Right panel. Densitometric analysis of the immunoblot. Data are presented as means ± SEM (n=3 age-matched male and female mice in each group); ^P<0.05 compared to normoxia, *P<0.05 compared to AA mice; $^{\#}P$<0.05 compared to vehicle-treated animals by a one-tailed t-test with Welch correction. (C) Left panel. Immunoblot analysis, using specific antibodies against ET-1 and E-Selectin of isolated aorta from AA and SS mice under normoxia or exposed to hypoxia (8% oxygen; 10 h), followed by reoxygenation (21% oxygen; 3 h) treated with either vehicle or rADAMTS13 (2940 U/kg). Actin was used as a protein loading control. One representative gel from three with similar results is shown. Right panels. Densitometric analysis of immunoblots. Data are presented as means ± SEM (n=3 age-matched male and female in each group); ^P<0.05 compared to normoxia, *P<0.05 compared to AA; $^{\#}P$<0.05 compared to vehicle-treated animals, one-tailed t-test with Welch correction. H/R: hypoxia/reoxygenation; ET-1: endothelin-1; eNOS: endothelial nitric oxide synthase; iNOS: inducible nitric oxide synthase; DU: densitometric units.

bocytopenia, suggesting that rADAMTS13 can beneficially affect microangiopathy related to acute vaso-occlusive crises. Indeed, rADAMTS13 decreased the adhesion of platelets from SS mice, further corroborating the working hypothesis of rADAMTS13 as a new therapeutic option for management of sickle cell-related acute events.

The beneficial effects of rADAMTS13 on clinical manifestation related to H/R stress is also supported by: (i) the decrease of systemic inflammation and of local inflammatory cell infiltrates in target organs for SCD, such as the lungs and kidneys; (ii) the downregulation of VCAM-1 and ICAM-1 in both lungs and kidneys; and (iii) the reduction in thrombi formation observed in both organs from SS mice exposed to H/R stress and treated with rADAMTS13. This was paralleled by modulation of both markers of vascular activation and pro-adhesion molecules in isolated aorta from rADAMTS13-treated SS mice, further supporting the role of the relative deficiency of ADAMTS13 activity in sickle cell-related inflammatory vasculopathy.[11,48] The amplified and sustained inflammatory response participates in ADAMTS13 dysfunction in SCD (Figure 5), as supported by modulation of eNOS/iNOS expression in both lung and kidney by rADAMTS13 treatment.[11,49]

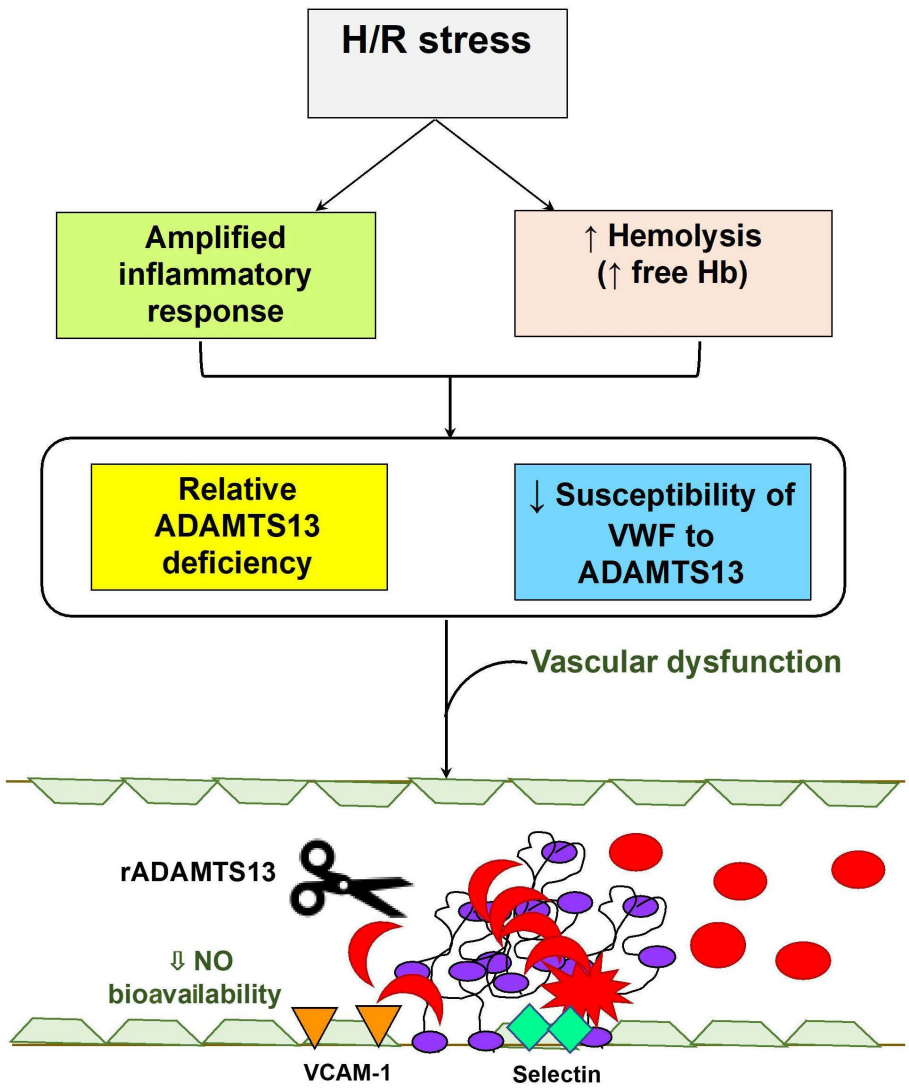

Figure 5. Schematic diagram of mechanisms involved in sickle cell-related microangiopathy and the role of recombinant ADAMTS13 as a novel therapeutic option for acute vaso-occlusive crises. Vaso-occlusive crises in sickle cell disease are characterized by hypoxia/reoxygenation stress, promoting an amplified inflammatory response and severe hemolysis. Both factors contribute to a relative deficiency of ADAMTS13 associated with decreased susceptibility of von Willebrand factor (VWF) to ADAMTS13. This potentiates vascular endothelial activation and damage, characterized by increased expression of vascular pro-adhesion markers and abnormal local bioavailability of nitric oxide as part of severe sickle cell-related inflammatory vasculopathy and vascular dysfunction involved in acute organ damage. H/R: hypoxia/reoxygenation; Hb: hemoglobin; ADAMTS13: a disintegrin and metalloproteinase with thrombospondin type 1 motif, number 13; VWF: von Willebrand factor; rADAMTS13: recombinant ADAMTS13; NO: nitric oxide; VCAM-1: vascular cell adhesion molecule-1.

Although hydroxyurea is the standard therapy for both children and adults with SCD, the biocomplexity of SCD requires a multimodal therapeutic approach to prevent more severe acute and chronic organ complications. Novel therapeutic strategies such as anti-P-selectin antibody (crizanlizumab), which interferes with pro-adhesive events, and voxelotor, a small anti-sickling agent, have recently been approved by the Food and Drug Administration.[5,8,50] Both agents act in the long-term, as chronic treatments. In contrast, rADAMTS13 might be used in the early phase of severe vaso-occlusive events to reduce vascular dysfunction and to limit sickle cell-related acute organ damage. rADAMTS13 (TAK-755) has been tested in a series of safety and toxicity studies in rats and cynomolgus monkeys (in rats at doses up to 1,980 U/kg, C_{max} up to 108.5 U/mL and treatment duration up to 26 weeks). Adverse events (including bleeding episodes) directly related to rADAMTS13 were not observed, even at the highest doses tested, in any of the studies.[51]

In conclusion, our data indicate that treatment with rADAMTS13, via regulation of ultra-large VWF multimer cleavage, might provide a pharmacological benefit and disease-modifying activity. Collectively our findings provide the rationale to enter rADAMTS13 into a clinical trial of SCD (ClinicalTrials.gov Identifier: NCT03997760) testing the applicability of this agent in the clinical management of acute events in patients with SCD.

Disclosures

SC, GS, and HGr are employees of Baxalta Innovations GmbH, a member of the Takeda group of companies and are Takeda stock owners. PR, HGl, FC, MS, DV, MD, BP, HR, FS and WH were employees of Baxalta Innovations GmbH, a member of the Takeda group of companies at the time of the study. LDF has received grant/research support from: Baxalta Innovations GmbH, a Takeda company; and Roche; Agios. EF and AM have no conflicts of interest to declare.

Contributions

LDF, PR, MD, FS and WH designed the experiments. EF, AM, IA, AI, FC, MS, SC, GS, DV, HGr. and BP performed and interpreted the experiments. EF, AM, HR, WH and LDF reviewed the experimental data and the manuscript. PR, HG, WH and LDF contributed to writing the manuscript.

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Data-sharing statement

The data that support the findings of this study are available from the corresponding author, [Gerald Schrenk], upon reasonable request.

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