Haemoglobin induces podocyte injury in mice and humans with pathologies associated with massive intravascular hemolysis.

Melania Guerrero-Hue[1], Alfonso Rubio-Navarro[1], María Dolores Sánchez-Niño[1,2], Eduardo Gutiérrez [2,3], Claudia Yuste[2,3], Ángel Sevillano[2,3], Manuel Praga[2,3], Javier Egea[4], Elena Román[5], Pablo Cannata[2,6], Rosa Ortega[7], Isabel Cortegano[8], Belén de Andrés[8], María Luisa Gaspar[8], Alberto Ortiz[1,2], Jesús Egido[1], Juan Antonio Moreno[1]

1 Renal, Vascular and Diabetes Research Laboratory, Instituto de Investigación Sanitaria-Fundación Jiménez Díaz. Autónoma University, Madrid, Spain. 2 Red de Investigación Renal (REDINREN), Madrid, Spain. 3 Department of Nephrology, Hospital 12 de Octubre, Madrid, Spain. 4 IIS-Hospital Universitario de la Princesa, Madrid, Spain; Instituto Teófilo Hernando, Departamento de Farmacología y Terapéutica, Facultad de Medicina, UAM, Madrid, Spain. 5 Pediatric Nephrology Department, La Fe Hospital, Valencia, Spain. 6 Pathology Department, Fundación Instituto de IIS-FJD, Autónoma University, Madrid, Spain. 7 Pathology Department, Hospital Universitario Reina Sofia, Córdoba, Spain. 8 Department of Immunology, Centro Nacional de Microbiologìa, Instituto de Salud Carlos III (ISCIII), Madrid, Spain.

Background

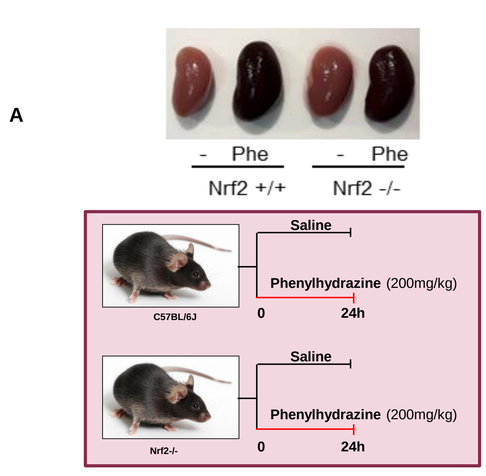
Recurrent and massive intravascular hemolysis is associated with progressive impairment of renal function, proteinuria and glomerulosclerosis, suggesting podocyte involvement. Podocytes are highly differentiated epithelial cells that play a key role in preserving glomerular filtration barrier. Structural alterations in podocyte foot processes occur in many glomerular diseases.

However, the cellular and molecular mechanisms involved in Hb-renal damage remains unclear. Control of ROS is important to restore the balance between oxidants and antioxidants in cells. In this regard, nuclear factor erythroid-2-related factor 2 (Nrf2) plays a central role in the defense against oxidative stress by activating the expression of a number antioxidants and phase 2 detoxifying enzymes.

Aims

Our objective was to determine the capacity for Hb uptake by murine podocyte *in vivo* and *ex vivo*, its injurious actions and the molecular pathways involved in this context. We also analyzed whether Hb-loaded podocytes are detected in kidney biopsies and urinary sediments from patients with severe intravascular hemolysis.

Intravascular hemolysis promoted Hb accumulation and heme catabolism activation in podocytes *in vivo*



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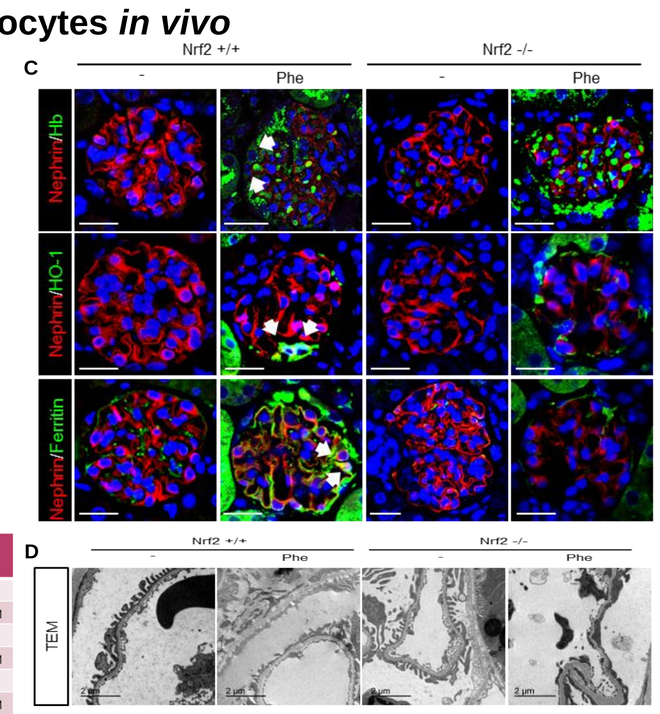
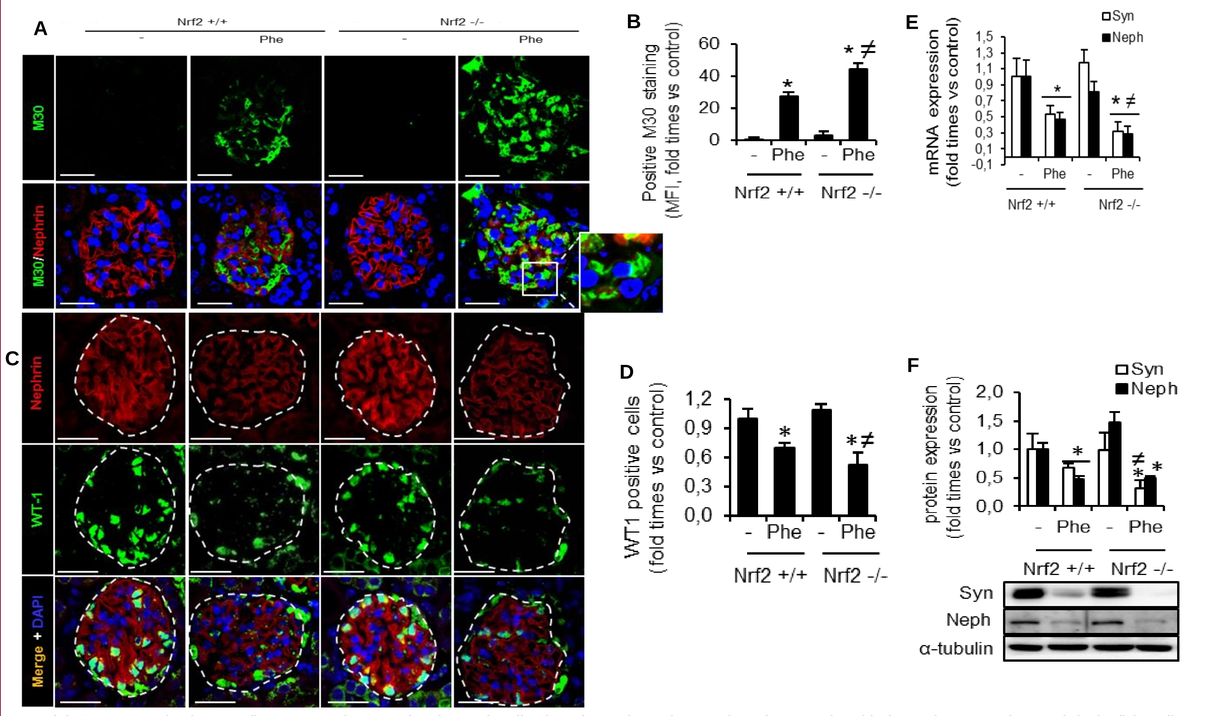
	Nrf2+/+		Nrf2-/-		P
	Control	Phe	Control	Phe	
Serum creatinine (mg/dL)	0.20±0.03	0.30±0.04 [a]	0.23±0.02	0.45±0.05 [a,b,c]	<0.05
BUN (mg/dL)	31.42±1.54	48.08±2.76 [a]	23.33±1.47	56.42±9.01 [a,c]	<0.001
Hematocrit (%)	49.99±2.76	33.96±3.51 [a]	46.60±1.68	31.25±2.77 [c]	<0.05
RBC (10^6 cells/mm^3)	9.78±0.37	7.21±0.31 [a]	10.12±0.26	7.01±0.50 [c]	<0.001
Serum hemoglobin (g/dL)	15.74±0.28	27.38±4.32 [a]	15.44±0.69	25.86±1.15 [c]	<0.05
Urinary albumin/creatinine (µg/µmol)	8.53±1.44	41.72±11.20[a]	5.98±1.24	109.21±33.43 [a,b,c]	<0.001
Urinary hemoglobin (µg/ml)	0.35±0.13	3.40±0.74 [a]	0.29±0.07	3.79±1.40 [a]	<0.001



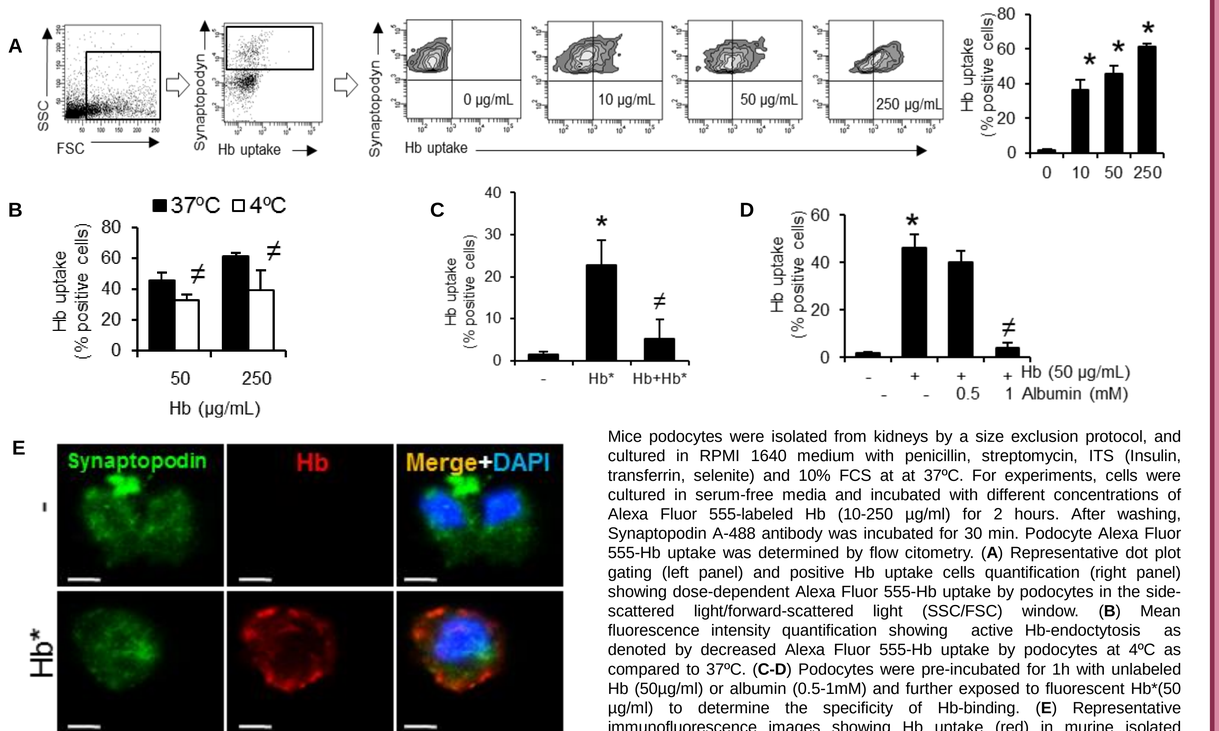
Hemolysis was induced in 12-week-old C57BL/6 and Nrf2 knockout mice by intraperitoneal administration of phenylhydrazine (200mg/kg of body weight, Sigma-Aldrich). Mice were euthanized 24 hours after phenylhydrazine injection (n=5 per group) and blood and urine samples were collected for biochemistry; and dissected kidneys for RNA and protein expression, flow cytometry analysis, immunofluorescence, and transmission electronic microscopy (**A**) Representative picture of the kidneys and the experimental model scheme. (**B**) Biochemical characteristics of Nrf2+/+ and Nrf2-/- mice treated or not with phenylhydrazine. (**C**) Representative confocal microscopy images showing co-localization (white arrows) of Hb (green), HO-1 (green), Ferritin (green) with the podocyte marker nephrin (red) in Phe-mice, scale bar 25 µM. Nuclei were stained with DAPI (blue). (**D**) Representative images of transmission electronic microscopy showing fusion of podocyte foot processes in phenylhydrazine-treated mice (Phe). Results are expressed as mean ± SE. * p<0.05 vs control mice. Animal studies were in accordance with the Directive 2010/63/EU of the European Parliament and were approved by the Institutional Animal Care and Use Committee (IIS-Fundacion Jimenez Diaz).

Nrf2 knockout mice have increased podocyte death and altered expression of key proteins for podocyte function



(**A**) Representative immunofluorescence images showing co-localization of M30 (green), a marker of apoptosis, with the podocyte marker nephrin (red) in saline or Phenylhydrazine (Phe)-treated mice (n=5 per group). Scale bar 25 µM. (**B**) Semi-quantification of M30 staining in glomerular cross sections. (**C**) Representative immunofluorescence of Nephrin (red), WT-1 (green), and DAPI (blue) in glomeruli from both control- and Phe-mice, scale bar 25 µM. (**D**) Quantitative analysis of podocyte number (WT-1 positive cells) in glomeruli from hemolytic-mice. (**E-F**) Synaptopodin (Syn) and Nephrin (Neph) protein and mRNA expression measured by western blot and RT-qPCR, respectively. Results (mean ± SE) are expressed as fold time increase as compared to control mice.

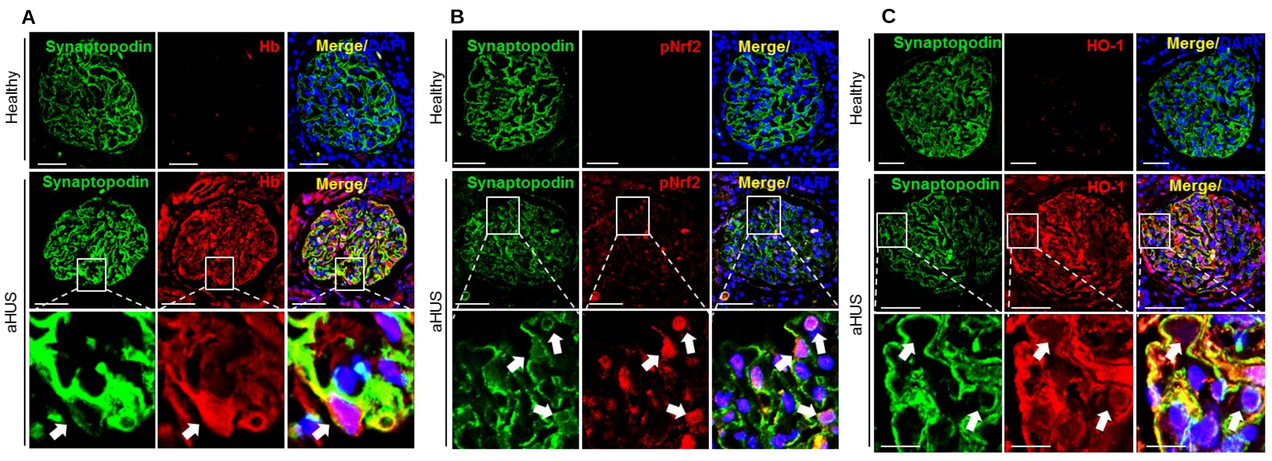
Murine Podocytes bound and endocytosed Hb in an energy- and active receptor-dependent way



Mice podocytes were isolated from kidneys by a size exclusion protocol, and cultured in RPMI 1640 medium with penicillin, streptomycin, ITS (Insulin, transferrin, selenite) and 10% FCS at at 37ºC. For experiments, cells were cultured in serum-free media and incubated with different concentrations of Alexa Fluor 555-labeled Hb (10-250 µg/ml) for 2 hours. After washing, Synaptopodin A-488 antibody was incubated for 30 min. Podocyte Alexa Fluor 555-Hb uptake was determined by flow citometry. (**A**) Representative dot plot gating (left panel) and positive Hb uptake cells quantification (right panel) showing dose-dependent Alexa Fluor 555-Hb uptake by podocytes in the side-scattered light/forward-scattered light (SSC/FSC) window. (**B**) Mean fluorescence intensity quantification showing active Hb-endoctytosis as denoted by decreased Alexa Fluor 555-Hb uptake by podocytes at 4ºC as compared to 37ºC. (**C-D**) Podocytes were pre-incubated for 1h with unlabeled Hb (50µg/ml) or albumin (0.5-1mM) and further exposed to fluorescent Hb*(50 µg/ml) to determine the specificity of Hb-binding. (**E**) Representative immunofluorescence images showing Hb uptake (red) in murine isolated podocytes by confocal microscopy. Synaptopodin (green) and DAPI (blue) were used to determine podocyte cells and nuclei, respectively.

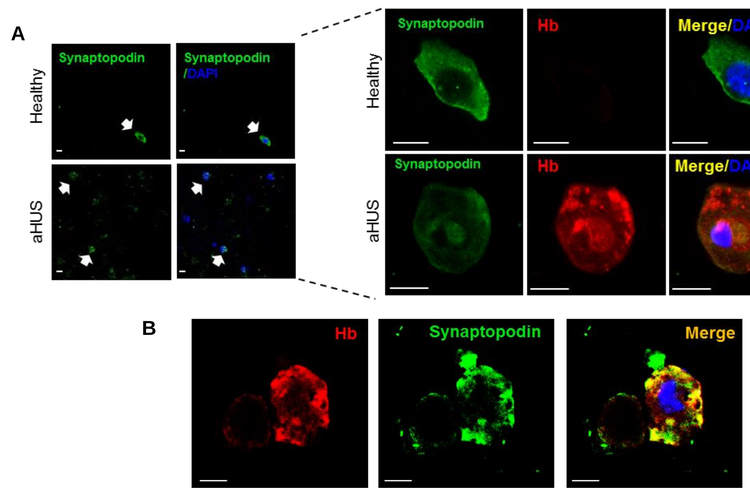
Data are expressed as mean±standard deviation. *p<0,05 vs Control, ≠p<0,05 vs Hemoglobin 50µg/mL)

Podocytes were stained for Hb, pNrf2 and HO-1 in renal sections of patients with massive intravascular hemolysis



Representative confocal microscopy images showing co-localization (white arrows) of Hb (red) (**A**), pNrf2 (**B**) and HO-1 (red) (**C**) with the podocyte marker synaptopodin (green) in patients with aHUS and healthy controls, scale bars 50 µM. Nuclei were stained with DAPI (blue). The rectangle shows the region of interest

Podocytes were stained for Hb in urinary sediments of patients with massive intravascular hemolysis



(**A**) Urine sediments from both healthy individuals and aHUS patients were centrifuged onto polylysine-coated slides using a cytospin and immunofluorescence studies were performed. The left panel shows the presence of podocytes (nucleated cells stained with synaptopodin, green, white arrows). High-magnification images are shown in the right panels where Hb (red) is located inside podocytes (synaptopodin, green) from urine sediments of aHUS patients, as determined by confocal microscopy. DAPI (blue) was used to stain nuclei. Scale bars 10µm. (**B**) Representative confocal microscopy images of urine sediments from patients with paroxysmal nocturnal hemoglobinuria showing the presence of Hb (red) inside podocytes (nucleated cells stained with synaptopodin, green), scale bars 10 µM.

Conclusion

In conclusion, our study identifies podocytes as new targets of intravascular hemolysis. Moreover, Nrf2 activation may be a potential therapeutic target to prevent loss of renal function in patients with severe intravascular hemolytic crisis. These findings provide new insights into novel aspects of Hb-toxicity and may have important pathogenic and therapeutic implications for intravascular hemolysis related diseases.

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Melania Guerrero Hue
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