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Cardiac expression of HMOX1 and PGF in sickle cell mice and haem-treated wild type mice dominates organ expression profiles via Nrf2 (*Nfe2l2*)

Oluwabukola T. Gbotosho[1,2], **Samit Ghosh**[1,3], **Maria G. Kapetanaki**[2,4], **Yu Lin**[1,2], **Frances Weidert**[2], **Grant C. Bullock**[5], **Solomon F. Ofori-Acquah**[1,2,3,6], **Gregory J. Kato**[1,2]

[1]Division of Hematology-Oncology, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

[2]Department of Medicine, Pittsburgh Heart, Lung, Blood, and Vascular Medicine Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

[3]Department of Medicine, Center for Translational and International Hematology, School of Medicine, University of Pittsburgh, Pittsburgh, PA, USA

[4]Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

[5]Division of Hematopathology, Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

[6]School of Biomedical and Allied Health Sciences, University of Ghana, Accra, Ghana

Summary

Haemolysis is a major feature of sickle cell disease (SCD) that contributes to organ damage. It is well established that haem, a product of haemolysis, induces expression of the enzyme that degrades it, haem oxygenase-1 (HMOX1). We have also shown that haem induces expression of placental growth factor (PGF), but the organ specificity of these responses has not been well-defined. As expected, we found high level expression of *Hmox1* and *Pgf* transcripts in the reticuloendothelial system organs of transgenic sickle cell mice, but surprisingly strong expression in the heart ($P < 0.0001$). This pattern was largely replicated in wild type mice by intravenous injection of exogenous haem. In the heart, haem induced unexpectedly strong mRNA responses for *Hmox1* (18-fold), *Pgf* (4-fold), and the haem transporter *Slc48a1* (also termed *Hrg1*; 2.4-fold). This was comparable to the liver, the principal known haem-detoxifying organ. The NFE2L2 (also termed NRF2) transcription factor mediated much of the haem induction of *Hmox1* and *Hrg1* in

Correspondence: Gregory J. Kato, Division of Hematology–Oncology, Department of Medicine, Heart, Lung and Blood Vascular Medicine Institute, University of Pittsburgh, 200 Lothrop Street, Pittsburgh, PA 15261, USA. katogj@upmc.edu.

Author contributions

OTG and GJK designed the research project, analysed, interpreted data, and drafted the manuscript. OTG performed the experiments. SG, MGK, YL and FW assisted with the experiments. GCB and SO-A designed and supervised experiments. All authors critically reviewed and approved the final version.

Conflicts of interest

The authors declare no competing financial interest in relation to the study.

Supporting Information

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all organs, but less so for *Pgf*. Our results indicate that the heart expresses haem response pathway genes at surprisingly high basal levels and shares with the liver a similar transcriptional response to circulating haem. The role of the heart in haem response should be investigated further.

Keywords

haemolysis; sickle cell disease; haem; *Hmox1*; *Pgf*

Introduction

Advancements in medicine have contributed to a longer life span for patients with sickle cell disease (SCD), mainly in developed countries (Crocker *et al*, 1990), with increased development of age-related progressive end-organ injury, including kidney disease, pulmonary hypertension and diastolic heart failure. Even transplanted organs in patients with SCD have high risks of damage and failure circumstantially related to haemolysis and sickling (Huang *et al*, 2013; Kerins & Ooi, 2017).

Products of intravascular haemolysis contribute to impaired nitric oxide (NO) bioavailability, endothelial dysfunction and organ damage in SCD (Kato *et al*, 2007; Kato *et al*, 2017; Vichinsky, 2017). In addition, excess haem (iron protoporphyrin IX) released from damaged sickle red blood cells also acts as a damage-associated molecular pattern (DAMP), thereby activating inflammatory pathways (Ghosh *et al*, 2013; Belcher *et al*, 2014; Gladwin & Ofori-Acquah, 2014). Haem is well-documented to induce haem oxygenase-1 (HMOX1) in diverse cell types, a response that detoxifies haem (Vercellotti *et al*, 2016; Ingoglia *et al*, 2017). *Hmox1* generates equimolar amounts of carbon monoxide and bilirubin, which exerts antioxidant and anti-inflammatory effects (Wagener *et al*, 2001; Otterbein *et al*, 2003; Wagener *et al*, 2003). SLC48A1 (also termed HRG1), a haem transporter, is important for haem transport from the phagolysosome of macrophages during erythrophagocytosis (Rajagopal *et al*, 2008; White *et al*, 2013). *Hmox1* and *Hrg1* expression is regulated by Nuclear factor (erythroid derived 2)-like 2 (NFE2L2, also termed NRF2), a key cytoprotective transcription factor that also regulates the basal and inducible expression of multiple antioxidant proteins and detoxification enzymes (Alam *et al*, 2003; Motohashi & Yamamoto, 2004). Recent studies in mice have shown a protective effect of NRF2 in attenuating complications of SCD, such as inflammation, acute lung injury, anaemia (Ghosh *et al*, 2016; Ghosh *et al*, 2018) and spleen damage (Zhu *et al*, 2018).

Our laboratory has shown that haem also induces Placental Growth Factor (PGF) (Wang *et al*, 2014), which is an angiogenic growth factor secreted by proliferating erythroblasts during normal development (Tordjman *et al*, 2001.). *Pgf* promotes the expression of the vasoconstrictor endothelin-1 by endothelial cells, associated with pulmonary hypertension in patients with SCD (Perelman *et al*, 2003; Sundaram *et al*, 2010) and thalassaemia intermedia (Kelaidi *et al*, 2018). In non-haemolytic disease, such as chronic kidney disease, elevated PGF is associated with increased left ventricular mass index (Peiskerova *et al*, 2013) and other cardiovascular events (Heeschen *et al*, 2003; Matsui *et al*, 2015).

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Organ damage in SCD is a multifactorial process and different organs may be affected in distinct ways by primary events, such as haemolysis and circulating non-haemoglobin bound haem. Because haemolysis contributes to organ damage in SCD, we investigated organ-specific expression patterns of *Hmox1* and *Pgf* in sickle cell mice and modelled this in wild type mice by increasing circulating extracellular haem. Furthermore, we investigated the role of *Nfe2l2* (also termed *Nrf2*) in the organ-specific transcriptional response pattern of *Hmox1*, *Pgf* and *Hrg1*.

Methods

Mice

Male and female Townes' knocked-in transgenic sickle mouse (SS) and strain controls expressing normal human haemoglobin (AA mice), C57BL/6J (*Nrf2*$^{+/+}$) and *Nrf2*$^{-/-}$ mice were used. C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA; stock number 000664) while SS, AA and *Nrf2*$^{-/-}$ mice were obtained from a colony maintained by Dr Solomon Ofori-Acquah's laboratory in our institution. Mouse genotypes were confirmed by polymerase chain reaction (PCR). Haemin [Fe(III)PPIX, Sigma-Aldrich, St. Louis, MO] was first dissolved in 0.25 mol/l NaOH and then adjusted to pH 7.5 with HCl before filter sterilization. The haemin solution was protected from light and injected into the tail vein of 14- to 16-week-old mice. The haemin dose was 50 μmol/kg body weight for SS and AA mice, and 120 μmol/kg body weight for *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice. Some of the mice received sterile vehicle (0.25 mol/l NaOH adjusted to pH 7.5 with HCl used in preparation of haemin) as a control. A range of doses and times were tested; 3 h after injection produced consistent survival with no adverse effects on all strains of mice in this study.

Isolation of haematopoietic progenitors from bone marrow and spleen

Mouse bone marrow cells were isolated from the femur and tibia with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin and 2 mmol/l EDTA from mice untreated and treated with vehicle or haemin for 3 h. The bone marrow suspension was filtered through a 70-μm-pore size cell strainer (Fisherbrand, Pittsburgh, PA, USA; Catalogue number 22363548) centrifuged at 300 ***g*** for 5 min. Splenocytes were separated from stromal elements by passing spleen tissue through 70-μm filters. The isolated splenocytes were incubated in 5 ml of red blood cell lysis buffer (155 mmol/l NH_4Cl, 14 mmol/l $NaHCO_3$ and 127 mmol/l EDTA) for 5 min at 8°C and washed twice with PBS.

Real-time PCR

Genes of interest (Table SI) were evaluated using the TaqMan® Gene expression assay and the TaqMan® RNA-to-C_t^{TM} 1-Step Kit (both from ThermoFisher Scientific, Pittsburgh, PA, USA) according to the manufacturer's instructions. Whole organs were harvested from mice immediately after death or 3 h after haemin injection. Freshly isolated organs (300 mg) were snap-frozen and kept at 80°C until use. Organs were homogenized in Qiazol lysis reagent using the Next Advance Bullet Blender (Next Advance, Inc. Troy, NY, USA). Homogenized samples were centrifuged at 18 800 ***g*** for 10 min to obtain clear lysates. All tissue processing was carried out at 4°C. Total RNA was extracted from the tissue lysates using the miRNeasy Mini Kit (Catalogue number 217004, Qiagen, Germantown, MD, USA) and quantified using

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the Nanodrop 8000 micro-volume spectrophotometer (ThermoFisher Scientific). Real-time PCR reactions were set-up with 50 ng of RNA in duplicates. Genes of interest were evaluated using the TaqMan® Gene expression assay and the TaqMan® RNA-to-C_t^{TM} 1-Step Kit (both from ThermoFisher) according to the manufacturer's instructions. Relative quantification was calculated with the standard C_t method; amplification signals from target gene transcripts were normalized to those from beta-glucuronidase (*Gusb*) transcripts. Relative fold induction was calculated by further normalization to gene transcripts from vehicle-treated animals. *Gusb* gene expressions were similar across all mouse strains used and across all organs within a given mouse strain (Figure S1A–C). *Gusb* gene expression in organs from control mice was similar to that from the corresponding organs from haemin-injected mice.

Haem quantification

Mice were perfused with PBS under anaesthesia. Harvested organs (300 mg) were homogenized in radio-immunoprecipitate assay buffer using the Next Advance Bullet Blender (Next Advance, Inc.). Homogenized samples were centrifuged at 18 800 ***g*** for 10 min to obtain clear lysates. All tissue processing was carried out at 4°C. Total haem in tissue lysates was quantified using a colourimetric assay kit (QuantiChrom haem assay kit; Bioassay Systems, Hayward, CA, USA) as described in previous studies (Ghosh *et al*, 2018). The bicinchoninic acid (BCA) assay kit (ThermoFisher Scientific, Catalogue number 23225) was used to quantify total protein in the lysates. Total haem values were first normalized to total protein, and then the haem values from vehicle-injected control organs were subtracted from the corresponding haem-injected organ values to determine the increase in haem in each organ after haemin injection.

HMOX1 and PGF protein quantification

Heart HMOX1 and PGF concentration were measured using the mouse HMOX-1 enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Farmingdale, NY, USA) and the PGF ELISA kit (Sigma-Aldrich) following the manufacturer's instructions. Total protein was quantified using the BCA method.

Statistical analysis

GraphPad Prism 7 software (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical analyses. Results are reported as mean ± standard error of the mean. Group means were compared using parametric tests, such as *t*-test (for two groups) and one-way analysis of variance for more than two conditions. Statistical significance was set at P values of <0.05.

Study approval

The Institutional Animal Care and Use Committee at the University of Pittsburgh approved all experimental procedures performed on mice (Protocol number 16099101).

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Results

Prominent cardiac expression of *Hmox1* and *Pgf* in sickle cell mice

We observed differential expression of *Hmox1* transcripts in untreated SS mice with highest expression level in the spleen, liver and kidney ($P < 0.0001$, Fig 1A). We hypothesized that uptake of haem from haemolysis induces this heterogeneous expression. To investigate this hypothesis, we increased circulating haem in SS mice with intravenous injection of haemin. Haem injection produced a sharp increase in *Hmox1* gene expression in all organs analysed after 3 h. *Hmox1* transcripts significantly increased, as expected, by about 20-fold in the kidney, 8-fold in the liver, 6-fold in the spleen, 5-fold in the lung and 4-fold in the BM after haemin injection compared to vehicle injected controls (Fig 1). Surprisingly, the heart showed 18-fold induction of *Hmox1*, implying an unexpected capacity of the heart to take up haem and induce its degradation pathway. The spleen showed little increase in *Hmox1* expression following haemin injection, probably because its basal expression was already quite high in the spleens of the untreated SS mice (Fig 1A), presumably due to the high rate of splenic macrophage turnover of sickle erythrocytes. All the organs of AA strain control mice were similarly affected by haemin injections. A second surprise was that the haemin-induced liver *Hmox1* transcript level was significantly lower in SS mice than in AA mice (Fig 1C). This result implies that SS mice may have an impaired reserve capacity for induction of *Hmox1*, a vital enzyme for detoxifying and clearing haem.

Previous work from our laboratory (Sundaram *et al*, 2010; Wang *et al*, 2014) and others (Heeschen *et al*, 2003; Perelman *et al*, 2003; Peiskerova *et al*, 2013; Matsui *et al*, 2015; Gu *et al*, 2018; Kelaidi *et al*, 2018) has shown a link between haem, elevated PGF and vasculopathy in diseases with chronic haemolytic anaemia, such as SCD and thalassaemia. Therefore, we evaluated the expression of *Pgf* in the organs of SS mice, and determined that there was heterogeneity ($P < 0.0001$, Fig 1E) in basal *Pgf* expression in these mice, surprisingly with the highest expression in the heart (DeltaC_t 2.9 ± 0.3), lung (3.1 ± 0.2) and kidney (5.0 ± 0.6). With haemin injection, cardiac *Pgf* transcript showed a robust 4-fold further increase compared to vehicle-treated control mice (Fig 1F, G). Cardiac expressions of PGF and HMOX1 in SS mice are surprisingly high, and induced to even higher levels by circulating haem.

Strong cardiac inducibility of *Hmox1* and *Pgf* in haemin-treated wild type mice

We hypothesized that the haemin treatment of mice would be sufficient to replicate the organ-specific variability in *Hmox1* and *Pgf* expression seen in SS mice. $Nrf2^{+/+}$ mice were injected intravenously with vehicle or a higher dose of haemin (120 μmol/kg) and organs were harvested 3 h later. *Hmox1* mRNA levels were significantly increased ($P < 0.0001$, Fig 2A) in all organs from $Nrf2^{+/+}$ mice that received haemin compared with vehicle controls. Most importantly, different organs demonstrated variable degrees of *Hmox1* inducibility with an increase of 207-fold in the liver, 201-fold in the heart, 101-fold in the kidney, 69-fold in the lung and 7-fold in the spleen (Fig 2B). The strong inducibility of *Hmox1* expression by haem in liver and kidney was expected, but comparable level of haem inducibility of *Hmox1* in the heart is a novel observation. For *Pgf* expression, we observed the highest basal expression in the heart, followed by lung and kidney ($P < 0.0001$, Fig 2C).

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Following haem exposure, heart tissue had the highest elevation of *Pgf* expression (Fig 2C). Interestingly, haemin treatment of *Nrf2*$^{+/+}$ mice largely recreated the organ-specific expression pattern of *Pgf* in untreated SS mice (Fig 1E compared to Fig 2C). This suggests that in sickle cell mice, haem exposure from haemolysis is a factor regulating organ-specific *Pgf* expression (Fig 2C). The high basal and haem-induced expression of *Hmox1* and *Pgf* in the heart is remarkable.

Organ-specific haem induction of *Hmox1* is largely *Nrf2* dependent

The *Nrf2* gene regulates the basal and inducible expression of *Hmox1* and to understand the mechanisms and molecular pathways involved in the haemin-induced organ-specific *Hmox1* expression, we utilized *Nrf2* null mice. Similar to results from other strains described above, we observed organ-specific heterogeneity in haemin-induced *Hmox1* expression in *Nrf2*$^{-/-}$ mice (Fig 2B), with the kidney (86-fold), heart (49-fold) and liver (44-fold) showing relatively higher induction of *Hmox1*. Most importantly, haemin induction of *Hmox1* was largely *Nrf2*-dependent, with 59–78% lower response in *Nrf2*$^{-/-}$ mice in every organ except kidney. The 101-fold haem-induction of *Hmox1* in the kidney was essentially *Nrf2*-independent (Fig 2B). Interestingly, *Pgf* expression in the organs of *Nrf2*$^{-/-}$ mice (Figure S2) were similar to those of *Nrf2*$^{+/+}$ mice (Fig 2C), with the basal expression of *Pgf* highest in the heart (2.15 DeltaC_t), followed by lung and kidney (Figure S2). As observed in *Nrf2*$^{+/+}$ mice, heart and lung had the highest expression of *Pgf* post-haemin exposure in *Nrf2*$^{-/-}$ mice. Haemin injections resulted in significant *Pgf* induction in both *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice (Figure S3) with no significant change between the two strains, suggesting that haem-induced *Pgf* expression in the organs examined was *Nrf2* independent.

HMOX1 and PGF proteins are highly inducible by haem and *Nrf2*-dependent

We confirmed at the protein level the strong cardiac inducibility of HMOX1 and PGF expression in the heart tissue lysates 3 h after haem injection. We further analysed HMOX1 protein as a percentage of total heart protein in both vehicle and haemin treated animals. In agreement with our mRNA measurements above, HMOX1 protein increased by approximately 49% ($P < 0.05$), 57% ($P < 0.001$), and 33% ($P < 0.05$) in SS, *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice respectively (Fig 3A). HMOX1 protein expression in the heart lysates was *Nrf2*-dependent in both vehicle-treated ($P < 0.001$) and haemin-injected mice ($P < 0.05$, Fig 3A), also supporting the mRNA measurements described above. In the same heart lysates, PGF protein as a percentage of total heart protein increased by about 22% ($P < 0.01$), 29% ($P < 0.05$), and 18% ($P < 0.01$) in SS, *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice respectively (Fig 3B). However, the percentage of cardiac PGF was not significantly different between *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice (Fig 3B).

Haem levels in different organs after haem injection correlates with *Hmox1* induction

Our previous results consistently demonstrated a heterogeneous *Hmox1* response to haemin among different organs that were examined, suggesting variability in haem degradation or haem distribution. To investigate this further, we measured intracellular haem in *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice that received vehicle or haemin. The organs were perfused to remove circulating red blood cells before processing for haem quantification. There was variability in haem biodistribution 3 h after intravenous injection of haemin (Fig 4A). Our results

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indicate that haem levels at 3 h remained significantly increased by about 67% ($P < 0.05$) in the spleen and 53% ($P < 0.05$) in the liver of *Nrf2*$^{+/+}$ animals while the other organs were largely unaffected. Showing a remarkable degree of correlation, the organs with the highest haemin induction of *Hmox1* mRNA showed the lowest levels of intracellular haem, suggesting a more rapid clearance of haem by enhanced *Hmox1* expression in the heart, liver, kidney and lung (Pearson $R^2 = 0.82$, $P = 0.03$, Fig 4B).

Nrf2 deficiency altered the haem distribution in haemin-injected mice. In haemin-injected *Nrf2*$^{-/-}$ animals, organ-specific haem levels were significantly higher by about 47% ($P < 0.05$) in the spleen, 58% ($P < 0.01$) in the kidney and 68% ($P < 0.05$) in the liver compared to haemin-injected *Nrf2*$^{+/+}$ mice. Interestingly, vehicle-treated *Nrf2*$^{-/-}$ mice showed mild but statistically significant haem accumulation in the heart ($P < 0.01$) and lung ($P < 0.05$) compared to *Nrf2*$^{+/+}$ mice, presumably due to basal state effects of *Nrf2* deficiency on chronic metabolism of endogenous haem. After haemin injection, the *Nrf2*$^{-/-}$ mice accumulated significantly higher levels of haem in the heart ($P < 0.01$) and kidney ($P < 0.05$) compared to haemin-injected *Nrf2*$^{+/+}$ mice. This may be due to impaired inducible haem clearance in the heart and kidney of the *Nrf2*-deficient mice compared to *Nrf2*$^{+/+}$ mice (Fig 2B). The liver and lung haem concentrations were not significantly different between the *Nrf2*$^{-/-}$ and *Nrf2*$^{+/+}$ mice.

Haem clearance is associated with haem transporter expression

Our results above suggested that extracellular haem induced the expression of *Hmox1* and *Pgf* genes in an organ-specific manner. Therefore, we hypothesized that differences in haem trafficking might contribute to this phenotype. Because *Hrg1* functions as a haem transporter (Rajagopal *et al*, 2008; White *et al*, 2013), we evaluated *Hrg1* mRNA expression in these organs. Haemin injection significantly induces the expression of *Hrg1* gene in most organs ($P < 0.05$), which was strongly dependent on *Nrf2* in most organs (Fig 5A). Depending on the organ, *Hrg1* induction by haemin was between 54% and 75% lower in *Nrf2*$^{-/-}$ mice. Compared to AA controls, SS mice showed significantly higher ($P < 0.05$) haemin induction of *Hrg1* in the spleen (~3-fold), liver (~3-fold) and kidney (~2-fold) (Fig 5B). *Hrg1* induction closely paralleled the same organ-specific induction pattern as *Hmox1* in relation to haem content, probably reflecting similar regulatory mechanisms ($R^2 = 0.82$, $P = 0.004$, Fig 5C).

Discussion

Sickle cell disease is no longer the ‘disease of childhood’ as described in the 1960’s (Maglione *et al*, 1993). Recent studies have shown that over 90% of the patients live to adulthood, especially in developed countries (Maglione *et al*, 1993; Platt *et al*, 1994). The improvement in survival is due to several interventions, such as new-born screening, use of penicillin prophylaxis and pneumococcal vaccination, hydroxycarbamide therapy and advancement in research and translational studies (Platt *et al*, 1994; Nouraie *et al*, 2013). These advances in medicine have contributed to better prognosis and quality of care for patients with SCD compared to five decades ago. Despite this progress in medicine and treatment of SCD patients, mortality in adult patients remains high (Platt *et al*, 1994). Organ

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failure due to accumulated injury from persistent haemolysis (Gardner *et al*, 2016) and sickling remains one of the major causes of death in patients with SCD (Vichinsky, 2017). The current study assessed organ-specific haem induction of *Pgf* and *Hmox1*, as a way to identify the most at-risk organs in SCD during elevation of circulating extracellular haem. The SCD pattern of *Hmox1* and *Pgf* expression is largely replicated by haem injection, mimicking acute haemolysis.

HMOX1 is a cytoprotective enzyme that metabolizes haem. Its deletion in mice amplified the severity of cerebral (Paulukonis *et al*, 2016) and noncerebral malaria (Seixas *et al*, 2009), ischaemia-reperfusion injury (Maitra *et al*, 2017), inflammation and vascular injury in different organs in SCD (Belcher *et al*, 2006; Belcher *et al*, 2010). Our data support and extend the prior work of Belcher *et al.* (2006, 2010), which showed *Hmox1* expression and haem-inducibility in some organs of transgenic sickle mice. We have characterized the expression of *Hmox1* in organs from naïve and haemin-treated Townes sickle mice. We observed wide heterogeneity in the induction of transcript levels of *Hmox1* in different organs, but, more importantly, we noticed a significant but very distinct haemin induction pattern depending on the organ. Spleen and liver showed the highest expression of *Hmox1* as expected, as these are key organs in the reticuloendothelial system, involved in turnover of old and senescent red blood cells (RBCs). Exposure to extracellular haem further increased the transcript level of *Hmox1* in these organs, with an unexpected strongest induction in the kidney and the heart of SS mice, which had lower baseline expression. The role of *Hmox1* in the heart is controversial. Cardioprotective roles of *Hmox1* in the heart includes reduction in oxidative stress (van Berlo *et al*, 2013), improved mitochondrial function (Hull *et al*, 2016), prevention of vascular remodelling (Mito *et al*, 2008), inhibition of apoptosis (Wang *et al*, 2010; Vichinsky, 2017) and prevention of atherosclerosis. Increased *Hmox1* expression and activity is also linked to diabetes-induced oxidative stress in the heart via accumulation of iron (Farhangkhoee *et al*, 2003). *Hmox1* expression in the heart of SS mice comprises part of the previously reported atheroprotective effect of SCD in SS/apolipoprotein E-deficient mice (Wang *et al*, 2013).

Interestingly, our result also suggests a previously unreported impaired inducible reserve capacity of *Hmox1* induction in the liver (Fig 1C). Our finding is consistent with the published effectiveness of augmented *Hmox1* expression as a protective therapy through gene transfer (Belcher *et al*, 2010) or through *Nrf2* activators (Ghosh *et al*, 2016; Ghosh *et al*, 2018) in sickle cell mice. In agreement with the current literature (Boyle *et al*, 2011; Loboda *et al*, 2016), our data offers novel insights regarding the organ-specific *Hmox1* expression *in vivo*. Although *Nrf2* depletion ubiquitously blunted haemin-induced *Hmox1* levels, the magnitude of the *Hmox1* reduction varied in the different organs, suggesting the presence of additional unknown mechanisms that control *Hmox1* expression. These mechanisms deserve future investigation.

Similar to *Hmox1*, we have investigated *Pgf* haemin-induction in organs of SS, $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice. For the first time, we describe high-level expression of *Pgf* in the heart of sickle cell mice. *Pgf* is expressed in several organs including placenta, erythroid cells, heart and skeletal muscle (Pamplona *et al*, 2007). We observed the highest *Pgf* expression in the heart, followed by the lung and kidney in organs isolated from na€ve adult SS mice. On

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exposure to extracellular haem in the circulation, we found a surprisingly high increase in *Pgf* transcript and total protein levels in the heart. Similarly, *Pgf* heart levels in haemin injected *Nrf2*$^{+/+}$ animals rose higher than all other organs. Elevated circulating level of PGF protein is associated with worsening cardiac outcomes in patients with chronic kidney disease (Heeschen *et al*, 2003; Matsui *et al*, 2015), atherosclerosis (Cao *et al*, 1997) and asthma (Maglione *et al*, 1993). We have previously reported the association between Pgf, inflammation, pulmonary hypertension (PH) and high mortality in patients with SCD (Perelman *et al*, 2003; Sundaram *et al*, 2010). This present result, showing the highest basal expression of *Pgf* in the heart followed by the lung in SS and *Nrf2*$^{+/+}$ mice, is unexpected and in contrast to previous reports of low expression levels in these organs (Alam *et al*, 2003; Liu *et al*, 2005; Pamplona *et al*, 2007). This apparent inconsistency might be due to differences in mouse strains, although our own results were consistent across four different strains of mice. Additionally, our group and others have shown that erythroid hyperplasia, hypoxia and erythropoietin in SCD contribute to elevated circulating PGF levels in both humans and mice with SCD (Tordjman *et al*, 2001; Perelman *et al*, 2003; Sundaram *et al*, 2010; Wang *et al*, 2014). High expression of *Pgf* in the heart is associated with angiogenesis and ischaemia (Maglione *et al*, 1993), though its role in cardiovascular disorders is somewhat controversial. Kolakowski *et al*., (2006) reported that direct intramyocardial injection of PGF following a large myocardial infarction attenuated adverse ventricular remodelling and improved myocardial function. On the other hand, elevated serum PGF is an independent risk factor for mortality (Matsui *et al*, 2015) and left ventricular diastolic dysfunction in patients with chronic kidney disease (Peiskerova *et al*, 2013). PGF contributes to cardiac hypertrophy (Dewerchin & Carmeliet, 2012, Eiymo Mwa Mpollo *et al*, 2016, Roncal *et al*, 2010), through intermediate paracrine growth factors such as interleukin 6 (Accornero *et al*, 2011; Dewerchin & Carmeliet, 2012), which is known to be elevated in the serum of SCD patients (Taylor *et al*, 1995; Qari *et al*, 2012; Sarray *et al*, 2015).

The third gene that we evaluated was the haem transporter *Hrg1*, which is known to facilitate haem export from the phagolysosome into the cytosol during RBC recycling in macrophages (Rajagopal *et al*, 2008; White *et al*, 2013). Our results describe, for the first time, how *Hrg1* is regulated *in vivo* by the addition of an extracellular haem analogue with or without *Nrf2*. Similar to *Hmox1* and *Pgf*, HRG1 expression in *Nrf2*$^{+/+}$ mice was stimulated by haemin injections in an organ specific manner. Furthermore, the absence of *Nrf2* in the *Nrf2* null animals eradicated the haemin effect in all organs in agreement with a previous publication reporting the role of NRF2 in HRG1 regulation (Alam *et al*, 2003). Interestingly, *Hrg1* induction by haemin strongly correlates with intracellular haem content in haemin-injected mice. Moreover, intracellular haem content strongly correlates with *Hmox1* expression in the same mice. The relationship of haem transport to haem clearance requires further investigation.

It is interesting that haemin injection in wild-type mice largely recreates the profile of *Hmox1* and *Pgf* expression by organ seen in untreated SS mice. This suggests that sickling, vaso-occlusion or anaemia is not specifically required for this *Hmox1* and *Pgf* pattern. Further stimulation of the SS mice with additional haem simply amplifies this pattern, but retains the dominance of liver and heart expression of *Hmox1* and *Pgf*. Although the change in expression of haem-regulated genes is not surprising in liver and spleen, organs involved

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in haem clearance, we report previously underappreciated *Hmox1* basal and inducible expression in the heart.

We found prominent organ-specific *Hmox1* and *Pgf* expression patterns, which could promote variable protective and maladaptive responses among these organs. A recent investigation of blockade of PGF suggested beneficial effects in SS mice (Gu *et al*, 2018). The remarkable regulation of *Hmox1*, *Pgf* and *Hrg1* in the heart by haem offers a particular new avenue for investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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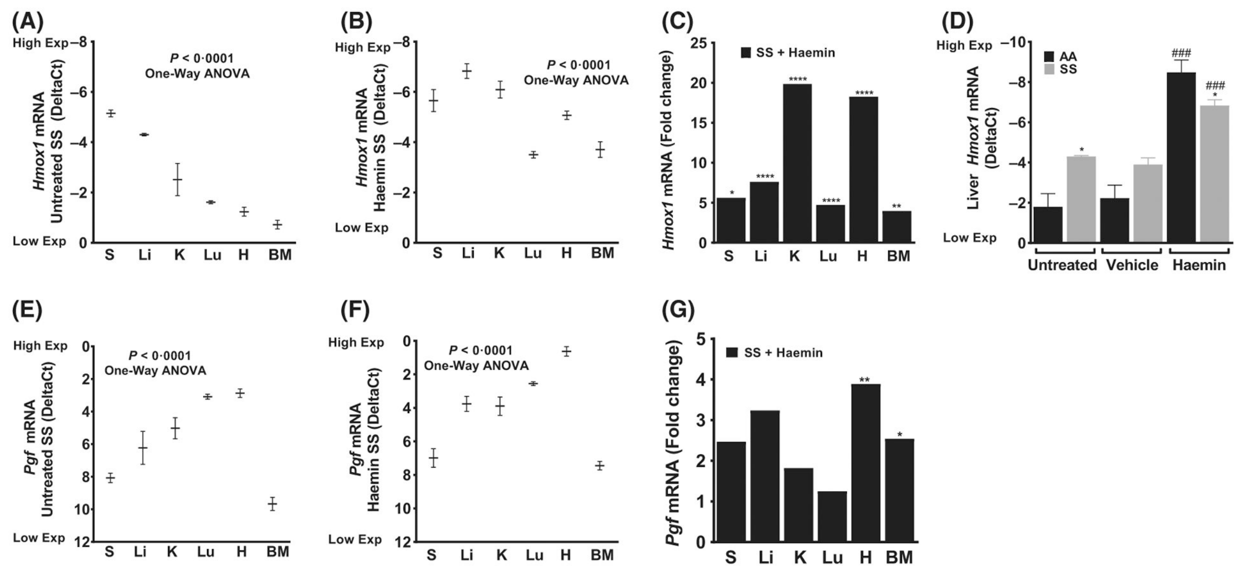

Fig 1.

Heterogeneity in basal and haem-induced *Hmox1* and *Pgf* expression in organs from sickle cell mice. (A) Endogenous *Hmox1* expression in untreated SS mice ($n = 3–5$). Haem-induced *Hmox1* expression in SS mice presented as (B) DeltaC_t and (C) Relative fold change ($n = 7–10$). (D) Impaired *Hmox1* expression in the liver of SS mice compared to AA mice ($n = 3–10$, *$P < 0.05$ *AA compared to SS and $^{###}P < 0{\cdot}001$ $^{\#}$vehicle compared to haemin within strain). (E) Endogenous *Pgf* expression in SS mice ($n = 3–5$). Haemin induced *Pgf* expression presented as (F) DeltaC_t and (G) Relative fold change ($n = 7–10$). For DeltaC_t, lowest value = highest expression and highest value = lowest expression. Target gene transcripts were normalized to *Gusb* for all mRNA expression level. *Gusb* expression was similar in all mice strains used and in all of these organs in animals injected with either vehicle or haemin. For relative fold change, samples were further normalized to vehicle gene transcripts. One-Way ANOVA or Student's *T*-test; All values are mean ± SEM, *compared to vehicle. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ****$P < 0{\cdot}0001$. ANOVA, analysis of variance; BM, bone marrow; Exp, Expression; H, heart; K, kidney; Li, liver; Lu, lung; S, spleen.

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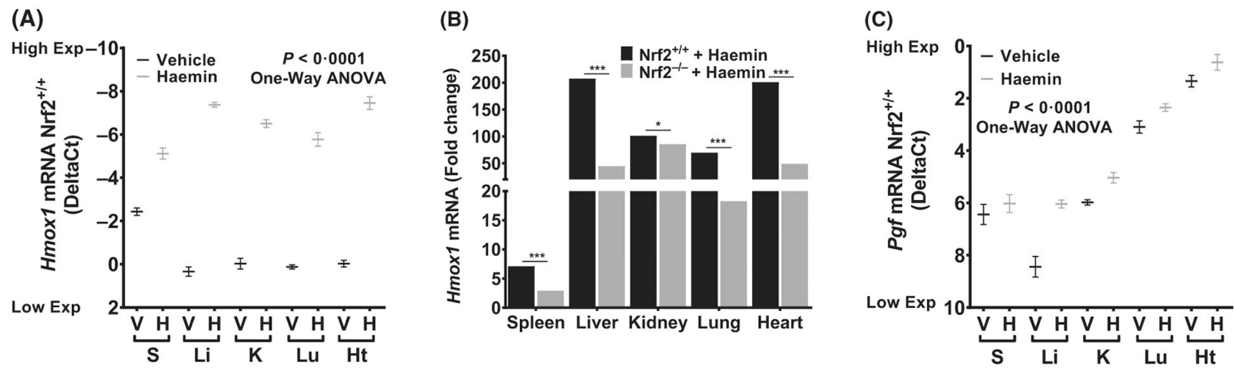

Fig 2.

Organ-specific variability in *Hmox1* inducibility is *Nrf2* dependent. (A) *Hmox1* expression in wild type mice injected with vehicle or haemin (DeltaC_t, $n = 8–11$, $P < 0.0001$ vehicle vs haemin in all organs shown). (B) *Hmox1* expression is *Nrf2* dependent in wild type mice injected with haemin ($n = 6–11$). (C) Haemin induced *Pgf* expression in wild type mice mimics the pattern seen in sickle cell mice ($n = 8–11$, $P < 0.05$ vehicle vs haemin in all organs shown except spleen). For DeltaC_t, lowest value = highest expression and highest value = lowest expression. Target gene transcripts were normalized to *Gusb* for all mRNA expression level. *Gusb* expression was similar in all mice strains used and in all of these organs in animals injected with either vehicle or haemin. For relative fold change, samples were further normalized to vehicle control gene transcripts. One-Way ANOVA or Student's *t*-test; All values are mean ±SEM, *compared to vehicle. *$P < 0·05$, **$P < 0·01$, ***$P < 0·001$, ****$P < 0·0001$. ANOVA, analysis of variance; BM, bone marrow; Exp, Expression; H, haemin; Ht, heart; K, kidney; Li, liver; Lu, lung; S, spleen; V, vehicle.

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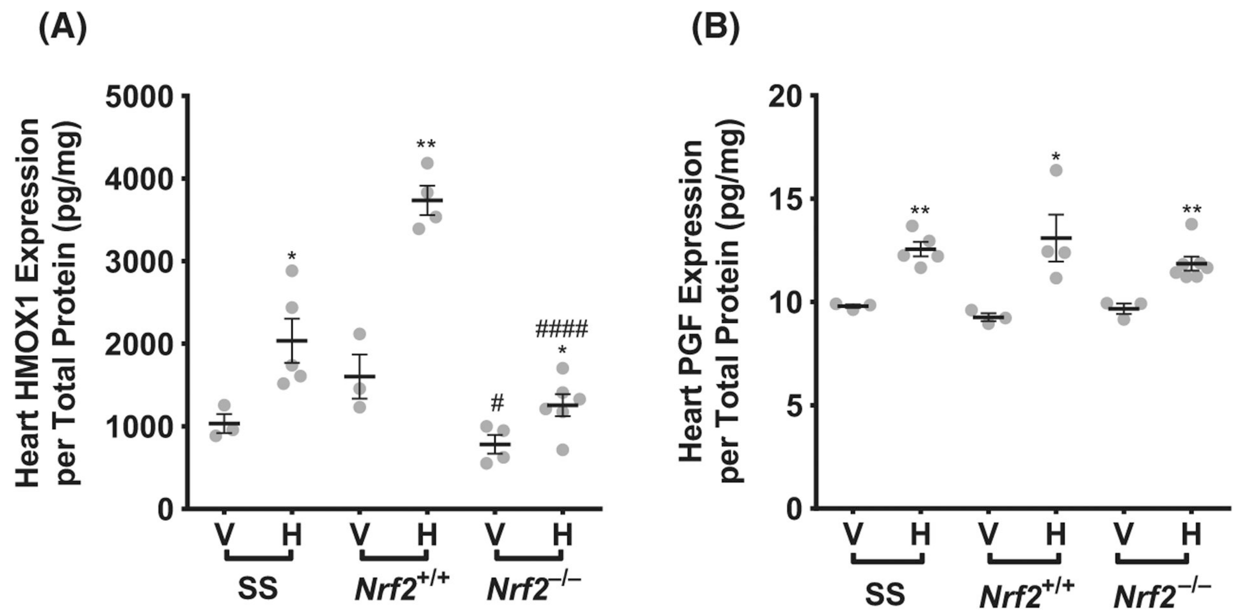

Fig 3.

HMOX1 and PGF proteins are highly inducible by haem and *Nrf2*-dependent. (A) Vehicle-treated and haemin induced HMOX1 protein in the heart as a percentage of total protein concentration ($n = 3–7$). (B) Vehicle-treated and haemin induced PGF and protein in the heart as a percentage of total protein concentration ($n = 3–7$). Student's t test, *Vehicle-treated compared to haemin-treated within strain, # Vehicle/haemin-treated $Nrf2^{+/+}$ compared to $Nrf2^{-/-}$. All values are mean ± SEM. *$P < 0·05$, **$P < 0·01$, ***$P < 0·001$, #$P < 0·05$, ####$P < 0001$. H, haemin; V, vehicle.

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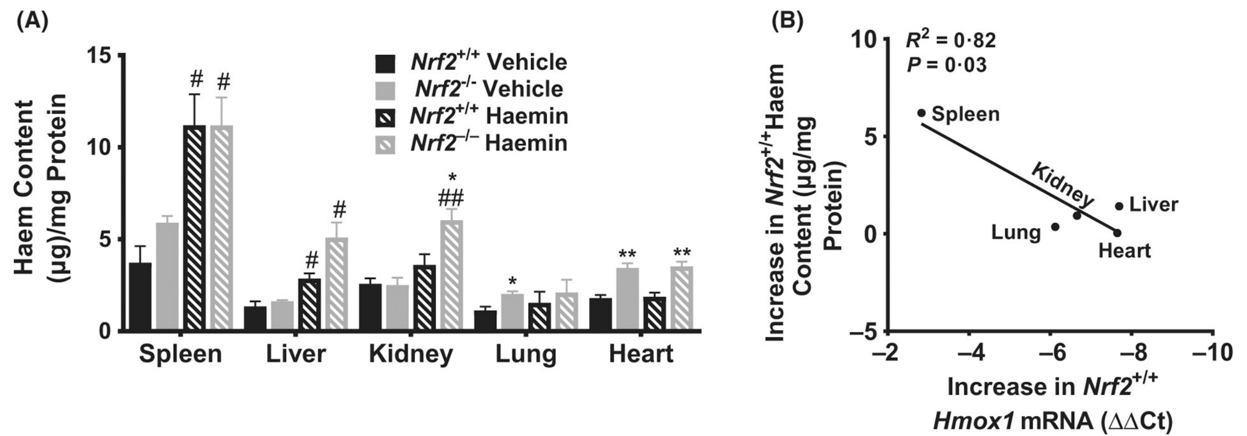

Fig 4.

Biodistribution of intracellular haem content in mice. (A) Haem content was quantified as a ratio of total protein from tissue lysate in organs from *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice 3 h after haemin injection ($n = 3$–6). (B) Change in haem content is correlated with haemin-induced *Hmox1* expression in wild type mice ($n = 3$–11). For DeltaC_t, lowest value = highest expression and highest value = lowest expression. Target gene transcripts were normalized to *Gusb* for all mRNA expression level. *Gusb* expression was similar in all mice strains used and in all of these organs in animals injected with either vehicle or haemin. All values are mean ± SEM. Student's *T*-test, **Nrf2*$^{+/+}$ compared to *Nrf2*$^{-/-}$ within same treatment, # Haemin compared to Vehicle within strain. $^{\#}P < 0{\cdot}05$, $^{\#\#}P < 0{\cdot}01$, $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$.

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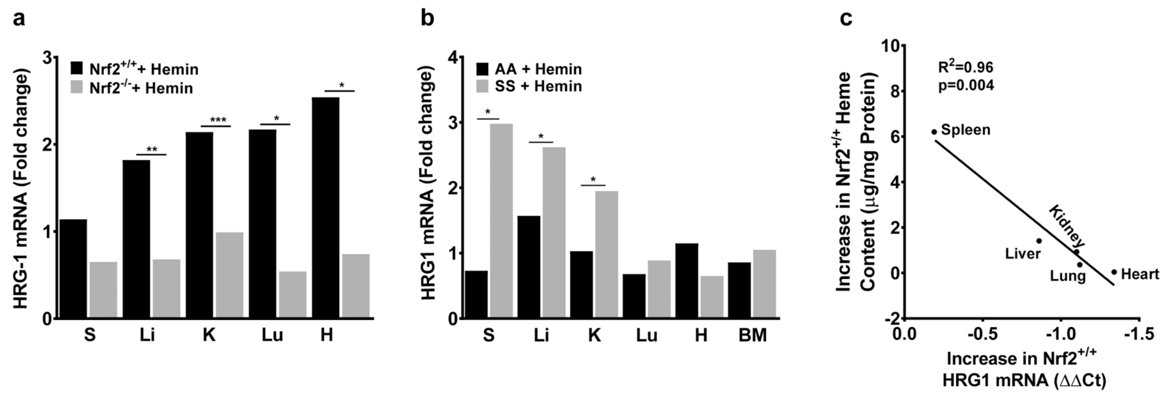

Fig 5.

Haem clearance is positively associated with *Hmox1* induction and haem trafficking. (A) *Nrf2*-dependent, organ specific induction of haem importer Hrg1 ($n = 4$). (B) Haem importer *Hrg1* expression is more highly induced by haem in liver, kidney and spleen of sickle cell mice ($n = 3$–10). (C) Change in haem content is correlated with haemin-induced *Hrg1* expression in wild type mice ($n = 3$–4). For DeltaC_t, lowest value = highest expression and highest value = lowest expression. Target gene transcripts were normalized to *Gusb* for all mRNA expression level. *Gusb* expression was similar in all mice strains used and in all of these organs in animals injected with either vehicle or haemin. For relative fold change, samples were further normalized to vehicle control gene transcripts. R^2 was calculated with Pearson correlation. All values are mean ±SEM. *$P < 0·05$, **$P < 0·01$, ***$P < 0.001$. BM, bone marrow; H, heart; K, kidney; Li, liver; Lu, lung; S, spleen.