bjh short report

Augmented NRF2 activation protects adult sickle mice from lethal acute chest syndrome

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Summary

Acute chest syndrome (ACS) mortality in sickle cell disease (SCD) rises sharply in young adult patients and mechanism-based prophylaxis is lacking. In SCD, haem oxygenase-1 (HO-1) declines with age and ACS is associated with low HO-1. To test if enhanced HO-1 can reduce ACS mortality, young SCD mice were treated with D3T (3H-1,2-dithiole-3-thione), an activator of nuclear-factor erythroid 2 like 2, which controls HO-1 expression, for 3 months. Following haem-induced ACS, all vehicle-treated mice succumbed to severe lung injury, while D3T-treated mice had significantly improved survival. Blocking HO-1 activity abrogated the D3T effect. Thus HO-1 may be targeted to reduce ACS severity in adult patients.

Keywords: sickle cell disease, acute chest syndrome, haem, nuclear factor erythroid 2, haem oxygenase-1.

Sickle cell disease (SCD) is a monogenic blood disorder characterized by a multisystem morbidity and low median survival. The survival of children with SCD has improved dramatically over the last 30 years, reaching up to 99·0% at 18–20 years in multiple birth cohorts (Hamideh & Alvarez, 2013). Remarkably, the death rate among patients 20 years and older is alarmingly high, even in the West (Yanni *et al*, 2009). In the United States, the adult (>19 years of age) mortality rate among SCD patients has increased by 1% ($P < 0·001$) between 1979 and 2005 (Lanzkron *et al*, 2013). Acute chest syndrome (ACS) is a leading cause of death among young adult SCD patients. The mortality rate for ACS is ~10-fold higher in patients 20 years and older compared to those 9 years and younger (Vichinsky *et al*, 2000). Although the precise mechanism of ACS pathogenesis is not fully understood, genetic association studies suggest haem oxygenase-1 (HO-1), the rate-limiting haem degradation enzyme, plays a modulating role (Bean *et al*, 2012). In agreement, steady-state plasma levels of haem, the obligate HO-1 substrate, are significantly lower in children with no history of ACS compared to those with multiple ACS episodes (Adisa *et al*, 2013). Moreover, direct evidence from studies of transgenic SCD mice (SS) supports a key pathogenic role for extracellular haem in ACS (Ghosh *et al*, 2013).

During periods of stress, nuclear factor erythroid 2 like 2 (NRF2, also termed NFE2L2), a ubiquitously expressed basic leucine zipper transcription factor, coordinates the expression of multiple anti-oxidant response genes, including HO-1 (Thimmulappa *et al*, 2006). Emerging evidence indicates the capacity of SS mice to scavenge haem declines with age; this anomaly is accelerated in SS mice lacking non-haematopoietic NRF2 activity (Ghosh *et al*, 2016). Conversely, NRF2 activation slows down progression of haemolytic anaemia and organ dysfunction with aging in SS mice (Ghosh *et al*, 2016). In agreement with these findings, constitutive activation of NRF2 via partial ablation of its negative regulatory KEAP1 significantly reduces plasma haem concentrations in SS mice (Keleku-Lukwete *et al*, 2015). Another study reported that loss of NRF2 function worsened the pathophysiology of SCD (Zhu *et al*, 2018). These observations suggest that pharmacological activation of NRF2 may attenuate ACS. Here we present data to show that global activation of NRF2 using 3H-1,2-dithiole-3-thione (D3T) reduces lethality in a haem-induced ACS model in SS mice.

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Methods

Mice

The Townes' knock-in transgenic sickle mouse was used for this study. The University of Pittsburgh Institutional Animal Care and Use Committee approved the study of these animals. For treatment with D3T, SS mice received oral gavage of vehicle (25% Glycerol and 1% Kolliphor in phosphate-buffered saline) or D3T (0·5 mmol/kg body weight) three times a week for 3 months. Mice were phlebotomized by retro orbital bleeding using a capillary tube internally coated with heparin/EDTA anticoagulant. For HO inhibition, mice were injected with tin protoporphyrin (SnPPIX, 10–250 μmol/kg; Frontier Scientific, Logan, UT, USA).

Biochemical assays

Freshly collected EDTA anti-coagulated blood was centrifuged at 1200 ***g***, 4°C for 15 min to collect plasma. Total plasma haem (TPH) in unfractionated plasma was quantified using a colourimetric assay kit (Bioassay Systems, Hayward, CA, USA) as described in previous studies (Pamplona *et al*, 2007). The HO-1 concentration in plasma was measured using and enzyme-linked immunosorbent assay (ELISA; Enzo Life Sciences, Farmingdale, NY, USA). HO activity was determined using a well-established assay based on the production rate of bilirubin from haem substrate. Total protein was measured using the bicinchoninic acid assay (Pierce, Thermo Fisher Scientific Waltham, MA, USA).

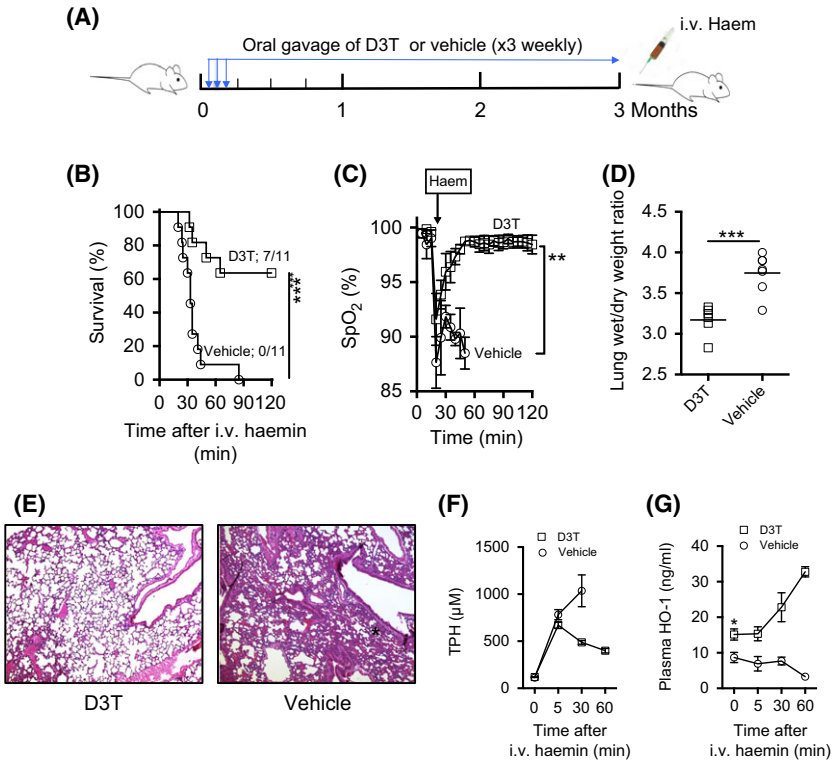

Fig 1. NRF2 activator D3T attenuates acute chest syndrome in sickle mice. (A) Schematic of experimental design. (B) Mortality in a cohort of transgenic sickle (SS) mice (n = 11) treated with vehicle or 3H-1,2-dithiole-3-thione (D3T) for 3 months followed by infusion of haemin to induced acute chest syndrome. ***P < 0·001 indicates significant differences measured by Mantel-Cox test. (C) Oxygen saturation (SpO_2), measured by pulse oximetry in D3T-treated SS mice that survived and vehicletreated SS mice that succumbed to the haem challenge (n = 3; P < 0·01). Sickle mice treated with D3T regained normal SpO_2 level after an initial drop following haem infusion. (D) Assessment of lung injury, using gravimetric analysis, in SS mice (n = 6) after indicated treatment and haem challenge (P < 0·001; Student's t-test). (E) Formalin (10% buffered) fixed paraffin-embedded lung tissue sections were stained with haematoxylin and eosin (H&E) and examined under an Olympus AX70 microscope. Representative photomicrograph of H&E-stained lung sections of D3T- and vehicle-treated SS mice challenged with haem (original magnification ×100). (F) Mice were phlebotomized by retro-orbital bleeding and freshly collected EDTA anti-coagulated blood samples were centrifuged at 1200 ***g*** for 15 min to collect plasma. Total plasma haem (TPH) was then quantified using a colourimetric assay kit (Bioassay systems). The graph shows TPH at different time intervals following haem infusion in SS mice (n = 3). Mice that received chronic D3T treatment were efficient in haem clearance. (G) Concentration of HO-1 in plasma was determined using an enzyme-linked immunosorbent assay (Enzo Life Sciences). Plasma HO-1 was significantly higher in SS mice after 3 months of D3T treatment compared to the vehicle group (*P < 0·05, Student's t-test) Rapid release of HO-1 into the plasma was evident in D3T-treated SS mice following infusion with purified haem. [Colour figure can be viewed at wileyonlinelibrary.com]

Haem induced acute chest syndrome

Lethal ACS was induced with infusion of purified haemin (Frontier Scientific; 70 μmol/kg) as previously described (Ghosh *et al*, 2013). The MouseOx™ pulse-oximeter (Starr Life Sciences, Oakmont, PA, USA) was used to measure real-time oxygen saturation (% SpO_2; percentage of functional arterial haemoglobin) in awake conditions.

Gravimetric analysis and histopathology

To assess lung oedema, lungs were weighed immediately after harvest using an isometric transducer (Harvard Apparatus, Holliston, MA, USA), dried in an oven at 80°C containing desiccant crystals for 24 h, dry weight determined and ratios calculated. For histopathology, lungs were uniformly fixed immediately after harvest in 10% buffered formalin. Sections (5 μm) of formalin-fixed paraffin-embedded tissues were de-paraffinized, rehydrated and stained with haematoxylin and eosin and examined using an AX70 microscope (Olympus, Waltham, MA, USA); images were recorded with a camera (Olympus U-CMAD3 DP70) and software (OlympusDP70/DP30 BW, ver.02.0201.147).

Statistical analysis

Results are reported as mean ± standard error of the mean. To analyse statistical significance, Mantel-Cox test and two-tailed unpaired Student's *t*-test were used as appropriate. GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA) was used for all statistical analyses. *P*-values of less than 0·05 were considered significant.

Results

Preclinical studies in transgenic SS mice indicated that NRF2 activation can protect major organs from tissue injury in SCD. To assess the efficacy of NRF2 activation to prevent acute lung injury in SCD, young SS mice (age: 1 month)

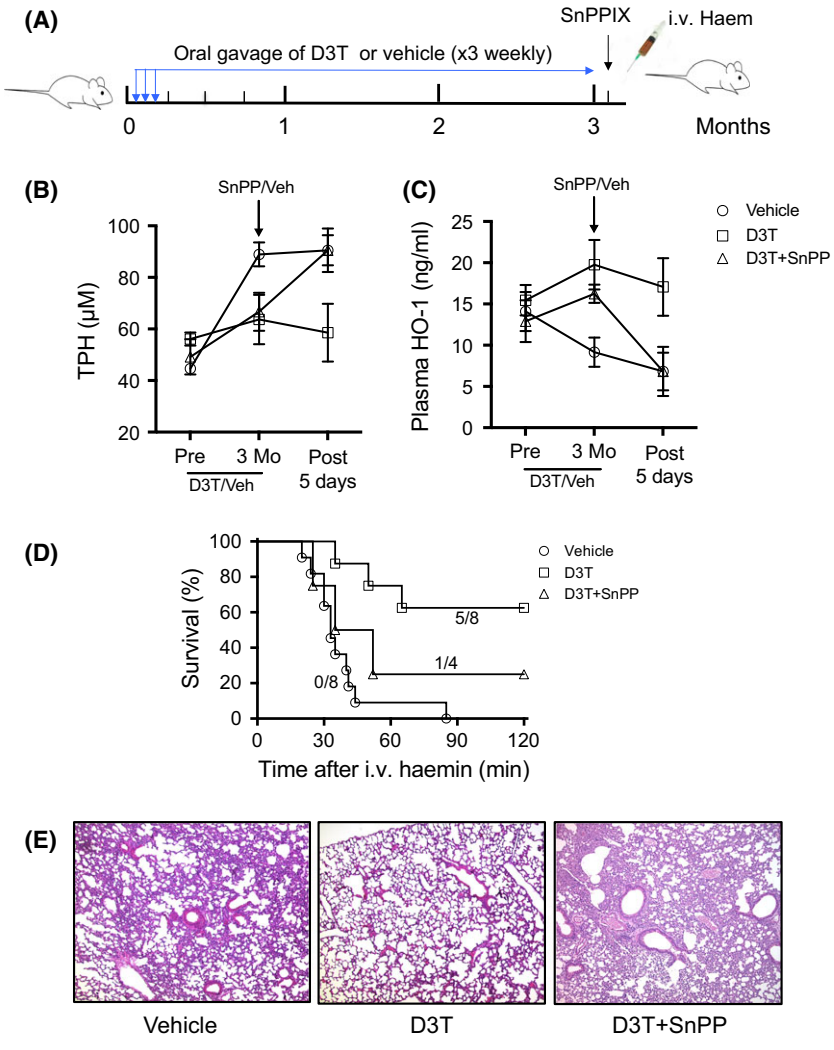

Fig 2. Haem oxygenase-1 mediates protective effect of NRF2 activation in ACS. (A) Schematic of experiment testing the effect of haem oxygenase-1 (HO-1) inhibition on the amelioration of acute chest syndrome (ACS) in SS mice treated with the NRF2 activator 3H-1,2-dithiole-3-thione (D3T). (B) Total plasma haem (TPH) and (C) Plasma HO-1 concentration at steady-state (Pre), 3 months (3 mo) after D3T and vehicle treatments and 5 days after SnPP treatment (n = 3–4). (D) Survival rate of SS mice induced to have ACS previously treated for 3 months with vehicle (n = 8), D3T (n = 8) and D3T followed by SnPP injection (n = 4). (E) Representative H&E stained lung sections, original magnification, ×100. [Colour figure can be viewed at wileyonlinelibrary.com]

received oral gavage of D3T or vehicle for 3 months. Thereafter, they were infused with purified haemin to induce ACS as previously described (Fig 1A) (Ghosh *et al*, 2013). In agreement with our previous findings, acute elevation of circulating haem triggered lethal ACS in all vehicle-treated SS mice. By contrast, D3T treatment rescued 64% of SS mice given the same bolus of purified haemin (Fig 1B). Real-time oxygen saturation measurements showed death was preceded by severe hypoxaemia (Fig 1C); these mice also had significantly higher lung wet/dry weight ratio compared to the D3T-treated mice that were rescued (Fig 1D). Histological analyses confirmed that D3T treatment ameliorated the severity of lung damage (Fig 1E).

Serial analysis of TPH showed that D3T-treatment resulted in rapid (within 1 h of onset of ACS) clearance of haem from the circulation while the concentration of haem increased steadily in the vehicle-treated SS mice, consistent with the phenomenon of extracellular haem crisis (Fig 1F). Plasma HO-1 in SS mice was significantly ($P < 0{\cdot}05$) elevated after 3-months of D3T treatment compared to vehicle-treated animals (Time 0 min). The level of plasma HO-1 rose steadily following haemin infusion (within 0–1 h) in the D3T-treated SS mice while it declined in the vehicle-treated mice, mirroring the changes in plasma haem (Fig 1G). To assess whether HO-1 activity was relevant in these observations, a cohort of young SS mice was given a single intradermal injection of SnPPIX to inhibit the enzyme. Both the activity and expression of HO-1 was significantly reduced by the fifth day of SnPPIX injection (Figure S1). Following induction of ACS, we found that the HO-1 inhibitor had blocked all the phenotypic effects associated with DT3 treatment, including the rapid haem clearance improved survival, and attenuation of tissue damage (Fig 2D and E). These results show the efficacy of D3T being reflected by the pharmacodynamic changes in circulating haem linked to HO-1 activity (Fig 2C). The pathobiology of ACS is complex and involves TLR4 signalling, adhesion molecules, and other factors that may explain why ~30% of mice did not respond to the D3T treatment or its inhibition by SnPPIX.

Discussion

The sharp rise in mortality rate in young adult SCD patients is a major clinical concern, with ACS being arguably the most common cause of death in this group. Understanding the mechanism of ACS may help to develop innovative ways to reduce this burden. Hitherto, the only severe model of ACS involves infusion of a modest amount of purified haemin that causes a lethal ALI reminiscent of ACS in SS mice but has no clinical effect in sickle trait mice. By using this model, we have uncovered a novel role for NRF2 activators in attenuating ACS in mice. Several pharmacological activators of NRF2 have entered into clinical trials, raising optimism for the development of cytoprotective drugs for SCD. A phase 1 clinical study that used sulforaphane containing broccoli sprout showed increase in NRF2 activity in whole blood of SCD patients (Doss *et al*, 2016). There are potential risks with NRF2 activation therapy, including cardiovascular events, which led to early termination of the trial with Bardoxolone methyl (Methyl 2-Cyano-3,12-dioxooleana-1,9-dien-28-oate, CDDO-me) in chronic kidney disease and type 2 diabetes mellitus (de Zeeuw *et al*, 2013). However, the success of the first electrophile-based, NRF2-activating anti-inflammatory drug strategy (dimethylfumarate, Tecfidera, Biogen, Cambridge, MA, USA)(Kawalec *et al*, 2014) has broken down barriers to US Food and Drug Administration approval of covalent-modifier drug strategies and opened up new development pathways for related drug candidates. This study has revealed a protective effect of HO-1 via NRF2 activation in ACS. While the exact mechanism of action of NRF2 activators in attenuating ACS in this model require further investigations, we suspect it involves intravascular haem degradation by HO-1 and vaso-dilation due to the intravascular release of carbon monoxide. Additional mechanistic studies are needed to define the HO-1 species involved in this process. In conclusion, we have provided a strong rationale to develop drugs that activate NRF2 and HO-1 to improve the outcome of ACS, particularly in adult SCD patients.

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Authorship contributions

SG, RH, CAI, BF and FW performed experiments and organized the data. SFOA conceived the study and wrote the manuscript with SG.

Disclosure of conflicts of interest

The authors have declared that no conflict of interest exists.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Inhibition of HO-1 activity and expression (A) Plasma HO activity determined by an assay based on production rate of bilirubin from heme substrate (B) Plasma HO-1 level, measured by Elisa in SS mice ($n = 6$) following SnPP injection (10–250 μmol/kg). (C) Comparison of HO activity inhibition in the plasma and in selected major organs of SnPP treated SS mice ($n = 6$).

References

Adisa, O.A., Hu, Y., Ghosh, S., Aryee, D., Osunkwo, I. & Ofori-Acquah, S.F. (2013) Association between plasma free haem and incidence of vaso-occlusive episodes and acute chest syndrome in children with sickle cell disease. *British Journal of Haematology*, **162**, 702–705.

Bean, C.J., Boulet, S.L., Ellingsen, D., Pyle, M.E., Barron-Casella, E.A., Casella, J.F., Payne, A.B., Driggers, J., Trau, H.A., Yang, G., Jones, K., Ofori-Acquah, S.F., Hooper, W.C. & DeBaun, M.R. (2012) Heme oxygenase-1 gene promoter polymorphism is associated with reduced incidence of acute chest syndrome among children with sickle cell disease. *Blood*, **120**, 3822–3828.

Doss, J.F., Jonassaint, J.C., Garrett, M.E., Ashley-Koch, A.E., Telen, M.J. & Chi, J.T. (2016) Phase 1 study of a sulforaphane-containing broccoli sprout homogenate for sickle cell disease. *PLoS ONE*, **11**, e0152895.

Ghosh, S., Adisa, O.A., Chappa, P., Tan, F., Jackson, K.A., Archer, D.R. & Ofori-Acquah, S.F. (2013) Extracellular haemin crisis triggers acute chest syndrome in sickle mice. *The Journal of Clinical Investigation*, **123**, 4809–4820.

Ghosh, S., Ihunnah, C., Hazra, R., Walker, A.L., Hansen, J.M., Archer, D.R., Owusu-Ansah, A.T. & Ofori-Acquah, S.F. (2016) Nonhematopoietic Nrf2 dominantly impedes adult progression of sickle cell anemia in mice. *JCI Insight*, **1**, e81090.

Hamideh, D. & Alvarez, O. (2013) Sickle cell disease related mortality in the United States (1999–2009). *Pediatric Blood & Cancer*, **60**, 1482–1486.

Kawalec, P., Mikrut, A., Wisniewska, N. & Pilc, A. (2014) The effectiveness of dimethyl fumarate monotherapy in the treatment of relapsing-remitting multiple sclerosis: a systematic review and meta-analysis. *Current Neuropharmacology*, **12**, 256–268.

Keleku-Lukwete, N., Suzuki, M., Otsuki, A., Tsuchida, K., Katayama, S., Hayashi, M., Naganuma, E., Moriguchi, T., Tanabe, O., Engel, J.D., Imaizumi, M. & Yamamoto, M. (2015) Amelioration of inflammation and tissue damage in sickle cell model mice by Nrf2 activation. *Proceedings of the National Academy of Sciences of the United States of America*, **112**, 12169–12174.

Lanzkron, S., Carroll, C.P. & Haywood, C. Jr (2013) Mortality rates and age at death from sickle cell disease: U.S., 1979–2005. *Public Health Reports*, **128**, 110–116.

Pamplona, A., Ferreira, A., Balla, J., Jeney, V., Balla, G., Epiphanio, S., Chora, A., Rodrigues, C.D., Gregoire, I.P., Cunha-Rodrigues, M., Portugal, S., Soares, M.P. & Mota, M.M. (2007) Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. *Nature Medicine*, **13**, 703–710.

Thimmulappa, R.K., Scollick, C., Traore, K., Yates, M., Trush, M.A., Liby, K.T., Sporn, M.B., Yamamoto, M., Kensler, T.W. & Biswal, S. (2006) Nrf2-dependent protection from LPS induced inflammatory response and mortality by CDDO-Imidazolide. *Biochemical and Biophysical Research Communications*, **351**, 883–889.

Vichinsky, E.P., Neumayr, L.D., Earles, A.N., Williams, R., Lennette, E.T., Dean, D., Nickerson, B., Orringer, E., McKie, V., Bellevue, R., Daeschner, C. & Manci, E.A. (2000) Causes and outcomes of the acute chest syndrome in sickle cell disease. National Acute Chest Syndrome Study Group. *New England Journal of Medicine*, **342**, 1855–1865.

Yanni, E., Grosse, S.D., Yang, Q. & Olney, R.S. (2009) Trends in pediatric sickle cell disease-related mortality in the United States, 1983–2002. *Journal of Pediatrics*, **154**, 541–545.

de Zeeuw, D., Akizawa, T., Audhya, P., Bakris, G.L., Chin, M., Christ-Schmidt, H., Goldsberry, A., Houser, M., Krauth, M., Lambers Heerspink, H.J., McMurray, J.J., Meyer, C.J., Parving, H.H., Remuzzi, G., Toto, R.D., Vaziri, N.D., Wanner, C., Wittes, J., Wrolstad, D., Chertow, G.M. & Investigators, B.T. (2013) Bardoxolone methyl in type 2 diabetes and stage 4 chronic kidney disease. *New England Journal of Medicine*, **369**, 2492–2503.

Zhu, X., Xi, C., Thomas, B. & Pace, B.S. (2018) Loss of NRF2 function exacerbates the pathophysiology of sickle cell disease in a transgenic mouse model. *Blood*, **131**, 558–562.