Effects of 5-hydroxymethyl-2-furfural on the volume and membrane permeability of red blood cells from patients with sickle cell disease

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Key points

1. We addressed the hypothesis that the heterocyclic aldehyde 5-hydroxymethyl-2-furfural (5HMF) may act synergistically to ameliorate the complications of sickle cell disease through effects on red blood cell (RBC) membrane transport, in addition to its well-known action of increasing the oxygen affinity of HbS.

2. 5HMF was found to reduce deoxygenation-induced dehydration of RBCs, whether in response to maintained deoxygenation or cyclical deoxygenation / re-oxygenation.

3. Acting at low millimolar concentrations, 5HMF reduced the activity of the deoxygenation-induced cation conductance (sometimes termed P_{sickle}), an effect which correlated with reduction in sickling. 5HMF similarly inhibited deoxygenation-induced activation of the Ca^{2+}-activated K^+ channel (or Gardos channel), an effect not seen following pharmacologically-mediated increases in intracellular Ca^{2+} via the ionophore A23187. Deoxygenation-induced phosphatidylserine exposure, which is associated with Ca^{2+} entry via P_{sickle}, was also inhibited by 5HMF

4. By contrast, effects of 5HMF on the K^+-Cl^- cotransporter (KCC) were modest, with slight inhibition following treatment with *N*-ethylmaleimide (NEM) to abolish activity of its regulatory protein kinases, but stimulation in RBCs untreated with NEM.

5. It would therefore appear that an important beneficial action of 5HMF, in addition to effects on HbS oxygen affinity, is reduction in P_{sickle}-mediated Ca^{2+} entry following RBC sickling, thereby inhibiting the deleterious sequelae of Gardos channel activation, RBC dehydration and also lipid scrambling.

Abstract

The heterocyclic aldehyde 5-hydroxymethyl-2-furfural (5HMF) interacts allosterically with HbS in red blood cells (RBCs) from patients with sickle cell disease (SCD), thereby increasing oxygen affinity and decreasing HbS polymerisation and RBC sickling during hypoxia. We hypothesise that should 5HMF also inhibit the main cation pathways implicated in dehydration of RBCs from SCD patients - the deoxygenation-induced cation pathway (P_{sickle}), the Ca^{2+}-activated K^+ channel (the Gardos channel) and the K^+-Cl^- cotransporter (KCC) - it would have a synergistic effect in protection against sickling, directly through interacting with HbS, and indirectly through maintaining hydration and reducing [HbS]. This study was therefore designed to investigate the effects of 5HMF on RBC volume and K^+ permeability *in vitro*. 5HMF markedly reduced the deoxygenation-induced dehydration of RBCs whether in response to maintained deoxygenation or to cyclical deoxygenation / re-oxygenation. 5HMF was found to inhibit P_{sickle}, an effect which correlated with its effects on sickling. Deoxygenation-induced activation of the Gardos channel and exposure of phosphatidylserine were also inhibited, probably indirectly via reduced entry of Ca^{2+} through the P_{sickle} pathway. Effects of 5HMF on KCC were more modest with a slight inhibition in *N*-ethylmaleimide (NEM, 1 mM) -treated RBCs and stimulation in RBCs untreated with NEM. These findings support the hypothesis that 5HMF may also be beneficial through effects on RBC ion and water homeostasis.

Introduction

The complications of sickle cell disease (SCD) originate from the presence and polymerisation of the abnormal form of haemoglobin (Hb), HbS, in patients' red blood cells (RBCs). Compared to the normal adult HbA, HbS shows a single amino acid substitution at the β6 position, whereby glutamic acid is replaced by valine (Perutz & Mitchison 1950; Bunn & Forget 1986). Following the deoxygenation-induced conformational change of Hb, the loss of these negative charges allows neighbouring HbS molecules to aggregate and form long, rigid polymers which distort RBC shape. Other deleterious sequelae include altered rheology, RBC fragility, solute loss and dehydration and increased stickiness (Bunn & Forget 1986). The consequent clinical complications fall into two groups: first, a chronic anaemia consequent upon increased red blood cell (RBC) destruction; and second, acute ischaemic signs following blockage of the microvasculature (Steinberg 1999; Nagel & Platt 2001; Rees *et al.* 2010). The precise clinical signs depend on the organ(s) involved but can be numerous. Severity varies markedly between individuals, however, though the reason for this is not understood (Steinberg 1999).

There is at present no specific treatment for SCD (Steinberg 1999; Rees *et al.* 2010) although hydroxyurea has received considerable attention (Charache *et al.* 1987; Charache *et al.* 1992; Platt 2008; Rees 2011). The mechanisms of action of hydroxyurea remain uncertain but are probably mostly due to increased expression of fetal Hb, HbF. As HbF is not incorporated into HbS polymers and also serves to dilute the intracellular concentration of HbS, its expression reduces the tendency for HbS to polymerise and for HbS-containing RBCs to sickle. Hydroxyurea, however, is not without risks, being potentially teratogenic especially in the long term, showing variable response between individuals, and also having problems with non-compliance (Platt 2008). Such factors have restricted its use to more severely affected individuals (Rees 2011).

Investigations continue into the development of other effective therapies. A promising alternative approach has been the development of compounds which directly interpolate with the HbS molecules and thereby reduce polymerisation upon deoxygenation. The most encouraging of these have been the aromatic aldehydes such as vanillin (Zaugg *et al.* 1977; Abraham *et al.* 1991; Abdulmalik *et al.* 2005) and similar substituted benzaldehydes like 12C79 (valerosol) and 589C80 (tucaresol) (Kneen & White 1981; Beddell *et al.* 1984). These aldehydes form Schiff bases with HbS, allosterically increasing its oxygen affinity, and reducing polymerisation and RBC sickling. Despite effectively reducing sickling and increasing RBC hydration, none of these compounds proved useful clinically (eg Fitzharris *et al.* 1985; Keidan *et al.* 1986; Arya *et al.* 1996). More recently naturally occurring heterocyclic derivatives of furanic acid have been tested (Safo *et al.* 2004; Abdulmalik *et al.* 2005). These are active at lower concentrations and appear better tolerated. One of them, 5-hydroxymethyl-2-furfural (5HMF, also known as Aes-103), is currently in phase two clinical trials in SCD patients in USA and UK (NIH 2013).

Several abnormal, or abnormally regulated, cation transporters participate in the pathogenesis of SCD (Joiner 1993; Gibson & Ellory 2002; Lew & Bookchin 2005). These include the well-established pathways of the K^+-Cl^- cotransporter (or KCC) and the Ca^{2+}-activated K^+ channel (or Gardos channel), together with a third pathway, sometimes termed P_{sickle}. Unlike KCC and the Gardos channel, the molecular identity of P_{sickle} is unknown but it appears to be a cation conductance pathway activated by deoxygenation, HbS polymerisation and RBC shape change (Joiner *et al.* 1988; Joiner 1993; Ma *et al.* 2012). P_{sickle} mediates both entry of Ca^{2+} (Rhoda *et al.* 1990; Etzion *et al.* 1993) and exit of Mg^{2+} (Ortiz *et al.* 1990; Willcocks *et al.* 2002), with subsequent activation of the Gardos channel and also perhaps KCC. Deoxygenation-induced Ca^{2+} entry through P_{sickle} is further implicated in loss of phosphatidylserine (PS) asymmetry in RBCs from SCD patients (Lubin *et al.* 1981; Blumenfeld *et al.* 1991; Kuypers 1998; de Jong *et al.* 2001; Weiss *et al.* 2012; Cytlak *et al.* 2013), subsequent to activation of a Ca^{2+}-dependent scrambling process (Haest 2003).

Together the three cation pathways interact to mediate solute loss (Gibson 2001; Lew & Bookchin 2005), thereby concentrating HbS, which greatly reduces the lag time for polymerisation upon deoxygenation (Eaton & Hofrichter 1987). PS exposure also increases RBC stickiness (Kuypers 2008). These processes thereby increase the likelihood of sickling and vascular occlusion of the microvasculature.

Through its effects on HbS polymerisation, it is possible that 5HMF also modulates the cation permeability of RBCs from SCD patients. Should 5HMF reduce solute loss and maintain better hydration of the RBCs, HbS polymerisation would be inhibited by two potentially synergistic mechanisms – a direct interaction of 5HMF with HbS stabilising the oxygenated conformation, and a second mechanism through reduction in [HbS] thus increasing the lag time to sickling on deoxygenation. This hypothesis is investigated in the present study. Findings show that RBC volume is better preserved in response to maintained deoxygenation and also to cyclical deoxygenation / re-oxygenation. Radioactive tracer methodologies were used to investigate the effects of 5HMF on K^+ permeability, and specifically the activities of P_{sickle}, the Gardos channel and KCC in RBCs from SCD patients. FITC-labelled lactadherin was also used to measure PS exposure. Results show that 5HMF modulates all three cation transport pathways and PS scrambling, as well as affecting HbS polymerisation and sickling. These additional actions of 5HMF may be of significant consideration when assessing its clinical use and also in the design of similar more efficacious compounds to ameliorate the complications of SCD.

Materials and methods

Chemicals

Bumetanide, 5-hydroxymethyl-2-furfural (5HMF), 3-[N-morpholino] propane sulfonic acid (MOPS), *N*-ethylmaleimide (NEM), ouabain and salts were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Clotrimazole and A23187 were purchased from Calbiochem (Nottingham, UK). $^{86}Rb^{+}$ was supplied by Perkin Elmer (Beaconsfield, UK). Fluorescein isothiocyanate-conjugated lactadherin (LA-FITC) came from Haematologic Technologies Inc. (VT, USA) supplied via Cambridge Bioscience (Cambridge, UK) and phycoerythrin (PE)-conjugated anti-glycophorin A from Becton Dickinson Biosciences (CA, USA).

Sample collection and handling

Blood samples were taken for routine tests according to clinical indications, from patients homozygous (HbSS) for sickle cell disease (SCD) into the anticoagulant EDTA. Once routine testing had been completed, discarded, anonymised blood was analysed. The use of discarded blood was approved by the local ethics committee following guidelines set out in the Declaration of Helsinki. Samples were kept at 4 ^{o}C until use within 48 h.

Solutions and tonometry

The standard saline (Cl-MBS) comprised (in mM): 145 NaCl, 1.1 $CaCl_2$, 5 glucose and 10 MOPS, (pH 7.4 at 37 ^{o}C; 290 $\pm$ 5 mosmol.kg^{-1} H_2O). For experiments in which Cl^{-} dependence of K^{+} influx was examined, NO_3^{-}-containing salts replaced those containing Cl^{-} (N-MBS). To prevent the rapid RBC shrinkage which would otherwise occur following maximal stimulation of the Gardos channel in experiments in which intracellular Ca^{2+} was directly raised by incubation with the Ca^{2+} ionophore A23187, a high-K^{+}- and low-Ca^{2+}-containing saline was used with Ca^{2+} buffered with EGTA, comprising (in mM): 80 KCl, 70 NaCl, 2 $CaCl_2$, 0.15 $MgCl_2$, 2 EGTA, 5 glucose and 10 MOPS (HK-MBS) with a free $[Ca^{2+}]_o$ of 10 μM. The wash solution to remove unincorporated $^{86}Rb^{+}$ comprised isotonic $MgCl_2$ (107 mM), buffered with MOPS (10 mM), pH 7.4 at 4 ^{o}C (Mg-MBS). Stock solutions of bumetanide (10 mM) were prepared in 100 mM Tris base and used at a final concentration of 10 μM. Stock solutions of ouabain (10 mM) were prepared in distilled water and used at a final concentration of 100 μM. Stocks of clotrimazole (CLT; 5 mM) were prepared in DMSO and used at a final concentration of 5 μM. In most experiments, whole blood was washed five-times in N-MBS to remove Cl^{-}, plasma and buffy coat. RBC suspensions were then pre-incubated at 15 % haematocrit (Hct) in eppendorf tubes with or without 5HMF (5 mM) for 30 min at 37 ^{o}C and then placed in tonometers (Eschweiler, Kiel, Germany) to equilibrate at the requisite O_2 tension before flux measurement (still in N-MBS). Tonometers were flushed with warm, humidified gas mixtures, supplied at the appropriate O_2 tension using a Wösthoff gas mixing pump (Speake *et al.* 1997). For flux measurements, RBC suspensions were then diluted 10-fold into flux tubes, still equilibrated at the required O_2 tension. Where its effects were investigated, tonometers and flux tubes also contained 0.1-5 mM 5HMF. For experiments involving A23187, the ionophore (6 μM final) was added to warm saline at 37 ^{o}C in test tubes whilst vortexing. For experiments involving NEM, NEM (1 mM) was present during pre-incubation with 5HMF after which aliquots were again diluted 10-fold into flux tubes. For CLT, dissolved in DMSO, appropriate controls were all treated with the same concentration of solvent (0.1 % final).

Measurements of RBC sickling

To assess morphological sickling, RBCs were incubated in tonometers at 2 % Hct for 20 min after which samples were fixed in the same solution as that used during incubation but with the addition of 0.3 % glutaraldehyde. Control experiments showed that this protocol was sufficient to maintain RBC shape for several weeks. Sickling was assessed by light microscopy. Several hundred RBCs (typically 300-400) were counted using an Improved Neubauer haemocytometer. Control experiments examined the effect of 5HMF (5 mM) on sickling at an O_2 tension of 10 mmHg. These showed that the action of 5HMF was not immediate but that some pre-incubation was required, probably due to the time taken for aldehydes like 5HMF to enter RBCs and form adducts with Hb (Abraham *et al.* 1991; Abdulmalik *et al.* 2005). Thus in a representative experiment at 10 mmHg O_2, inhibition of sickling by 5HMF over 20 min was 20 % without pre-inucbation, increasing to inhibitions of 47 %, 68 %, 71 % and 79 % after 15, 30, 45 and 60 min pre-incubation. In subsequent experiments, 5HMF was therefore added to RBC suspensions at 37 ^{o}C for 30 min prior to flux and volume assays, and 60 min where phosphatidylserine exposure was measured.

Measurement of RBC water content

The effect of different oxygen tensions (with RBCs maintained under full oxygenation or deoxygenated at 0 or 15 mmHg O_2, or during cyclical deoxygenation / re-oxygenation), all for 60 min at 37 ^{o}C, was investigated on RBC volume. For oxy / deoxy cycles, gas delivered by the Wösthoff pump was switched between an oxygen tension of 0 and 100 mmHg every 10 min. RBC water content was then measured by the wet weight – dry weight method (Borgese *et al.* 1991). In brief, after incubation for 60 min at 5 % Hct, RBCs were pelletted by centrifugation at 12,000 g for 15 min at 4 ^{o}C. The extruded pellet was weighed immediately (to an accuracy of 0.01 mg) and again after drying for 18 h at 95 ^{o}C. Water content was expressed as ml water per g dry cell solids (ml / g d.c.s.).

K^+ flux measurements

To determine the activity of the K^+ transport pathways, K^+ influx was measured at 37 ^{o}C using $^{86}Rb^+$ as a congener for K^+ (Dunham & Ellory 1981; Hannemann *et al.* 2011). RBCs washed in N-MBS were equilibrated in tonometers for 20 min then diluted 10-fold into saline, pre-equilibrated at the appropriate O_2 tension, at 260 mOsm.kg^{-1} and pH 7. Hypotonic swelling and low pH were chosen in order to stimulate the K^+-Cl^- cotransporter (KCC). $^{86}Rb^+$ was added in 150 mM KNO_3 to give a final [K^+] of 7.5 mM in all experiments except those with HK saline and A23187-treated RBCs. Typically, three flux conditions were used: (i) Cl-MBS, (ii) Cl-MBS with clotrimazole and (iii) N-MBS with clotrimazole. After incubation with radioisotope for 10 min, RBCs were washed five-times in ice-cold Mg-MBS to remove extracellular $^{86}Rb^+$. Ouabain (100 μM) and bumetanide (10 μM) were present in all experiments to obviate any K^+ transport through the Na^+/K^+ pump and the Na^+-K^+-2Cl^- cotransporter, respectively. KCC activity was assayed as Cl^--dependent K^+ influx (using flux conditions ii & iii); Gardos channel activity as the CLT-sensitive (5 μM) K^+ influx (using conditions i & ii); and P_{sickle} as the deoxygenation-induced, clotrimazole-independent K^+ influx measured in the absence of Cl^- (condition iii). When measuring Gardos channel activity in the presence of A23187, a K^+ uptake measurement was carried out with serial samples of RBCs taken at 1, 3, 5, 7 and 10 min after addition of $^{86}Rb^+$, with aliquots added directly into ice-cold Mg-MBS and then washed a further four times. Either microhaematocrit determination or the cyanohaemoglobin method was used to measure the Hct with appropriate samples for this taken before the start of each experiment.

Labelling of phosphatidylserine exposure

To investigate the effect of 5HMF on deoxygenation-induced phosphatidylserine (PS) exposure, RBC suspensions (0.5 % Hct) were placed in a 24-well plate (Nunclon) in Cl-MBS with 1 mM vanadate, and continuously shaken at 270 rpm in a hypoxic Galaxy R incubator (Model 300, RS Biotech, CandM Scientific Ltd, Livingston, UK) for up to 4 hours. Oxygen tension was set to 20 mmHg. To measure PS exposure, 5 µl aliquots (10^5 RBCs) of each sample were placed in 250 µl of LA-FITC binding buffer (16 nM LA-FITC in standard saline with 1 mM vanadate) and incubated in the dark at room temperature for 10 min. RBCs were then pelleted by centrifugation for 10 s at 16,100 g, washed once in saline to remove unbound LA-FITC and kept on ice until flow cytometry analysis. Control unlabelled RBCs treated with 5HMF (5 mM) established the absence of aldehyde-dependent self-fluorescence.

FACS acquisition and analysis

Externalised PS was measured with FITC using an excitation wavelength of 488nm in the FL1 channel with an emission wavelength of 519 nm on a fluorescence-activated flow cytometer (FACSCalibur, Becton Dickinson, BD) and analysed with the BD CellQuest Pro software using a protocol published previously (Cytlak *et al.* 2013). Measurements were taken using a logarithmic gain. Forward scatter (FSC , size) and side scatter (SSC, granularity) gates for RBCs were identified in control experiments using anti-glycophorin A-PE labelled RBCs. The positive fluorescent gate was set using RBCs unlabelled with FITC-LA. For each measurement 10,000 events were gated. PS positive cells were defined as all events falling within the preset FSC, SSC and positive fluorescent gates.

Statistics

Results are mostly presented as means ± S.E.M., but ± S.D. in Figure 2, and as individual points in Figure 4b, of n observations in RBC samples taken from different individuals. Where appropriate, comparisons were made using paired student t tests. When transport activities were measured over a range of oxygen tensions, t-tests followed by Holm-Šídák corrections for multiple comparisons were used (Graphpad Prism 6; CA, USA). Correlations in Figure 3b were made using the Pearson correlation test. The level of significance used was $P < 0.05$.

Results

The effects of 5HMF on sickling in RBCs from SCD patients

In the first series of experiments, the effects of 5HMF on sickling were confirmed (Figure 1). In the absence of 5HMF, sickling in fully deoxygenated RBC approached about 80 % of the total, compared to about 10 % in fully oxygenated RBCs. The latter is probably indicative of irreversibly sickled cells (ISCs) which sometimes accumulate in the circulation. 5HMF had no significant effect on percentage sickling at the two extremes of oxygen tension. At intermediate oxygen tensions (10-40 mmHg), however, 5HMF reduced sickling by about 50 %, consistent with previous reports (Safo *et al.* 2004; Abdulmalik *et al.* 2005).

The effect of 5HMF on volume of RBCs from SCD patients

The effects of 5HMF on RBC volume were then addressed (Figure 2). In fully oxygenated RBCs, RBC volume was unaffected by addition of 5HMF. Under low oxygen tension (≤15 mmHg) RBC volume declined significantly in control RBCs. By contrast in RBCs also incubated with 5HMF, volume was unchanged compared with those held under fully oxygenated conditions and was significantly increased above control RBCs lacking 5HMF. The effect of cyclical deoxygenation / re-oxygenation was also tested. In this case, the presence of 5HMF again produced an increase in RBC volume compared to RBCs incubated in its absence. These effects of 5HMF would be consistent with inhibition of the cation efflux pathways which are upregulated in both oxygenated and deoxygenated RBCs from SCD patients (Lew & Bookchin 2005). The mechanisms were therefore investigated further.

The effect of 5HMF on the activity of P_{sickle} in RBCs from SCD patients

The non-specific deoxygenation-induced conductance termed P_{sickle} is thought to be activated upon reduction of oxygen tension, HbS polymerisation and morphological sickling (Mohandas *et al.* 1986). Consistent with this postulate, 5HMF reduced P_{sickle} activity (Figure 3a), with an inhibition of about 40 % at 20 and 10 mmHg oxygen, and 15 % at 0 mmHg. Sickling and P_{sickle} activity correlated both in control RBCs (Pearson correlation $r = 0.885$, $p < 0.0001$) and those treated with 5HMF ($r = 0.789$, $p < 0.0001$), as shown in Figure 3b.

The effect of 5HMF on the activity of the Gardos channel in RBCs from SCD patients

The Gardos channel is also activated by deoxygenation, HbS polymerisation and intracellular Ca^{2+} accumulation mediated probably via P_{sickle}. Reducing sickling and P_{sickle} activity might be expected to inhibit the Gardos channel activity indirectly through reduction in Ca^{2+} entry. This pattern was observed (Figure 4a). Inhibition of deoxygenation-induced activation of the Gardos channel was about 70 % at 20 and 10 mmHg oxygen and 30 % at 0 mmHg, a larger effect than apparent with P_{sickle}. A possible direct effect of 5HMF on the Gardos channel independent of any effects on Ca^{2+} entry was also tested. In these experiments, RBCs were treated with A23187 and a free extracellular Ca^{2+} of 10 μM (buffered with 2 mM EGTA) to produce near maximal activation of the Gardos channel. K^+ flux approached about 300 mmol.(l cells.h)$^{-1}$ and some evidence of saturation of $^{86}Rb^+$ accumulation was observed by about 7 min (Figure 4b). The similarity of the K^+ uptake curves in the

absence (filled symbols) and presence (open symbols) of 5HMF in ionophore-treated RBCs, however, argues against a direct effect of 5HMF on Gardos channel activity.

The effect of 5HMF on deoxygenation-induced phosphatidylserine exposure in RBCs from SCD patients

Ca^{2+} entry into RBCs following HbS polymerisation and activation of P_{sickle} upon deoxygenation is also associated with increased externalisation of phosphatidylserine (PS) mediated via the Ca^{2+}-dependent scrambling process (Weiss *et al.* 2012; Cytlak *et al.* 2013). Inhibition of P_{sickle}, as well as inhibiting the Gardos channel, would be expected to reduce deoxygenation-induced PS exposure. This was investigated by incubating RBCs from SCD patients in 24-well plates whilst shaking in a hypoxic incubator (20 mmHg O_2) over four hours. Results are shown in Figure 5. Deoxygenation increased PS exposure and externalisation was significantly reduced by treatment with 5HMF.

The effect of 5HMF on activity of KCC in RBCs from SCD patients and normal individuals

Unlike the situation in RBCs from normal individuals, in RBCs from SCD patients KCC activity is substantial and also observed in both oxygenated RBCs and following deoxygenation (Gibson *et al.* 1998), probably reflecting differences in the regulatory pathways in RBCs from these two genotypes (Merciris *et al.* 2001). KCC activity is shown in untreated RBCs from SCD patients and in the presence of 5HMF (Figure 6). Under fully oxygenated conditions and also at oxygen tensions below the P_{50} for HbS, 5HMF produced a significant stimulation of KCC activity, although the magnitude of this effect declined with oxygen tension. In fully deoxygenated RBCs, 5HMF had no significant effect on KCC activity.

KCC activity is regulated by a cascade of protein kinase and phosphatase enzymes with increased activity associated with dephosphorylation of regulatory serine-threonine residues (Cossins *et al.* 1994; Gibson & Ellory 2003). The effect of 5HMF could therefore be mediated either directly on the transporter or indirectly via these regulatory enzymes. These possibilities were investigated by pre-treating RBCs with *N*-ethylmaleimide (NEM), an alkylating reagent which acts on –SH groups, and which activates KCC and prevents inhibition by inhibitors of protein phosphatases, together with any effect by modalities such as volume and oxygen tension which require their activity (Lauf 1983; Cossins *et al.* 1994). As expected, KCC activity was elevated by treatment with 1 mM NEM (Figure 7). In the presence of NEM, addition of 5HMF produced a modest inhibition of KCC activity, in contrast to the stimulation observed in the absence of NEM treatment.

KCC is also observed in oxygenated RBCs from normal individuals albeit at lower activities. 5HMF was therefore also tested in normal RBCs. KCC activity in control conditions increased from 0.32 ± 0.05 mmol.(l cells.h)$^{-1}$ to 0.49 ± 0.07 (means ± S.E.M., n = 3, $p < 0.02$). NEM also increased KCC activity in these RBCs but in this case addition of 5HMF had no significant effect.

Dose dependence of 5HMF in RBCs from SCD patients

Previous studies on HbS solutions, whole blood or in sickle transgenic mice *in vivo* have used concentrations of 5HMF in the low millimolar range (Safo *et al.* 2004; Abdulmalik *et al.* 2005), similar to the concentration of Hb in RBCs. Dose dependence of 5HMF for increased O_2 affinity and reduction of sickling in these previous studies indicate an IC_{50} of about 1 mM. It is also important to establish whether lower concentrations of the reagent are effective on the transport pathways

studied here. This was investigated in the experiments shown in Figure 8. Significant levels of inhibition of P_{sickle}, the Gardos channel and PS exposure were apparent at concentrations of 5HMF of 1, 0.1 and 2 mM, respectively. In these deoxygenated RBCs, stimulation of KCC was significant by 5 mM.

Discussion

The present results demonstrate that 5HMF prevented dehydration of RBCs from SCD patients during maintained deoxygenation and during cyclical deoxygenation / re-oxygenation regimes. 5HMF was further found to inhibit two of the main cation pathways which contribute to dehydration. Thus as well as reducing morphological sickling in response to hypoxia, 5HMF inhibited both the deoxygenation-induced cation conductance (P_{sickle}) and the Ca^{2+}-activated K^+ channel (the Gardos channel), likely through inhibiting P_{sickle}-mediated Ca^{2+} entry. Consistent with the latter, deoxygenation-induced phosphatidylserine (PS) exposure, associated with Ca^{2+} entry through P_{sickle}, was also reduced. The third pathway which participates in solute loss, the KCl cotransporter (KCC), was stimulated, probably through an effect on its regulatory phosphorylation cascade.

5HMF is a naturally occurring five-membered heterocyclic aromatic aldehyde shown to interact allosterically with HbS to increase oxygen affinity and reduce sickling *in vitro* (Safo *et al.* 2004). 5HMF combines with HbS to form high affinity Schiff bases, at least with the N-terminal α valine and probably at other additional sites (Safo *et al.* 2004). 5HMF is found in several food products such as honey as well as being present along with other furanic compounds in traditional Chinese medicines given for SCD (Rehmanniae Radix) (Lin *et al.* 2008). Its low toxicity profile, better even than the aromatic aldehyde vanillin, makes it likely to be well tolerated *in vivo*. *In vivo* experiments with a transgenic mouse model of SCD showed that 5HMF was found to have good pharmacokinetic properties (Abdulmalik *et al.* 2005). As well as reducing RBC sickling, 5HMF also prolonged the survival time of these sickle mice under severe hypoxia. Similar findings were later observed in non-sickle mice under acute hypobaric hypoxia (Li *et al.* 2011). 5HMF appeared well tolerated in normal healthy volunteers also producing significant increases in Hb oxygen affinity (Stern *et al.* 2012). It has now progressed to phase 2 clinical trials in patients with SCD (NIH 2013).

The current results also demonstrate powerful effects on volume and K^+ permeability of RBCs from SCD patients. As expected from its morphological effects on sickling, 5HMF reduced activation of P_{sickle}, a pathway thought to be activated by HbS polymerisation and the sickling shape change (Mohandas *et al.* 1986). P_{sickle} activity elevates intracellular Ca^{2+} as well as depleting Mg^{2+} (Ortiz *et al.* 1990; Rhoda *et al.* 1990; Willcocks *et al.* 2002). That the Ca^{2+}-activated K^+ channel or Gardos channel activity was also inhibited by 5HMF is consistent with these effects, with its lack of effect following Ca^{2+} loading of RBCs with ionophore suggesting the absence of a direct action of 5HMF on the channel. In a similar way, 5HMF also reduced deoxygenation-induced PS exposure, which is probably also mediated via Ca^{2+} entry and activation of the Ca^{2+}-dependent scrambling process (Weiss *et al.* 2012; Cytlak *et al.* 2013). These findings and the correlation between percentage sickling and P_{sickle} activity (Figure 3) suggest that the main action of 5HMF on K^+ permeability would appear to be linked to its effects on HbS polymerisation. These observations are consistent with the main effects of HbS on RBC cation permeability being mediated via its polymerisation (Mohandas *et al.* 1986). A direct inhibitory effect of 5HMF on P_{sickle}, however, cannot be excluded.

The effects of 5HMF on KCC are more complicated. In control RBCs, KCC activity was moderately increased and this effect was more evident at higher oxygen tensions. Following treatment with NEM, by contrast, 5HMF was found to modestly inhibit transporter activity. These findings are consistent with an action of 5HMF via the regulatory phosphorylation cascade which controls KCC activity (Cossins *et al.* 1994; Gibson & Ellory 2003). It is possible that Mg^{2+} depletion, secondary to P_{sickle} activity, may stimulate KCC through inhibition of protein phosphorlyation (Delpire & Lauf 1991; Muzyamba *et al.* 2006). If this was the case, however, a stimulatory effect of 5HMF would be expected to be more pronounced following deoxygenation and this was not the case. The exact mechanism by which 5HMF interacts with KCC requires further investigation. In this context, it is worth noting that 5HMF was previously reported to be without effect on Na^+ transport pathways (Na^+/K^+ pump, Na^+/H^+ exchange and Na^+-K^+-$2Cl^-$ cotransporter) (Safo *et al.* 2004).

The present results demonstrate a protective action of 5HMF against RBC dehydration under continuous hypoxia and also during cyclical deoxygenation / re-oxygenation. By contrast, the insignificant effect of 5HMF on the volume of fully oxygenated RBCs suggests that even though KCC activity was probably increased, the effect was negligible. It is worth pointing out that although KCC activity was increased by 5HMF, the absolute magnitude of the fluxes were relatively small.

In conclusion, the hypothesis that 5HMF may reduce sickling through synergistic effects directly on HbS polymerisation and indirectly through reduction in solute loss and increased hydration appear valid. The effect of 5HMF on K^+ permeability and RBC volume would be useful measurements in clinical trials in SCD patients.

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Conflict of interest

There are no conflicts of interest.

Authors contributions

The study was planned by JSG, AH, DCR and ST; experiments and data analysis were carried out by AH and UMC; ms writing was carried out by JSG and AH, with additional input from DCR and ST; samples were supplied by DCR and ST. All authors have read and approved the final version of this manuscript.

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Figure legends

Figure 1. The effect of 5-hydroxymethyl-2-furfural (5HMF) on sickling of red blood cells (RBCs) from patients with sickle cell disease (SCD). RBCs (15 % haematocrit, Hct) were pre-incubated with 5HMF (5 mM) for 30 min after which they were equilibrated in Eschweiler tonometers at the indicated oxygen tension for 20 min. Aliquots were then fixed with glutaraldehyde and RBC shape assessed by light microscopy. The percentage of sickled RBCs at each oxygen tension was normalised to that at 0 mmHg O_2 in the absence of 5HMF, which had a value of 84 ± 1.4% (range from 63 - 88%). Histograms represent means ± S.E.M., n = 6-7. * $p < 0.05$ comparing control RBCs in the absence of 5HMF with those incubated in its presence.

Figure 2. Effect of 5HMF on cell volume of RBCs from patients with SCD. Cell volume was measured by wet weight – dry weight and expressed as ml water per g dry cell solids (ml.(g d.c.s.)$^{-1}$) in RBCs maintained for 60 min under fully oxygenated conditions (Oxy), or under continual deoxygenation (≤15 mmHg O_2), or under cycles of deoxygenation / re-oxygenation (Oxy / deoxy), in which oxygen tension was changed from 100 mmHg to 0 mmHg every 10 min. Histograms represent means ± S.D., n = 3-7, and in all cases were paired experiments carried out in the absence and presence of 5HMF. # $p < 0.05$ comparing control oxygenated RBCs to control RBCs under the different deoxygenation protocols. * $p < 0.05$ comparing deoxygenated RBCs in the absence of 5HMF with those incubated in its presence. N.S. comparing oxygenated and deoxygenated RBCs in the presence of 5HMF.

Figure 3. Effect of 5HMF on the activity of P_{sickle} in RBCs from patients with SCD. RBCs were treated as described in the legend to Figure 1 except that after equilibration at different oxygen tensions in tonometers aliquots were diluted 10-fold into test tubes for measurement of K^+ influx (calculated as mmol.(l cells.h)$^{-1}$ and measured at an extracellular [K^+] of 7.5 mM), all in the absence or presence of 5HMF (5 mM). P_{sickle} is defined as the K^+ influx in Cl^--free saline in the presence of ouabain (100 μM), bumetanide (10 μM) and clotrimazole (5 μM). (a) Activity of P_{sickle} at each oxygen tension was normalised to that at 0 mmHg O_2 in the absence of 5HMF, which had a value of 1.51 ± 0.27 mmol.(l cells.h)$^{-1}$ (range from 0.66 - 2.45 mmol.(l cells.h)$^{-1}$). Histograms represent means ± S.E.M., n = 6-7. * $p < 0.05$ comparing control RBCs in the absence of 5HMF with those incubated in its presence. (b) Correlation of sickling and activity of P_{sickle} in RBCs from patients with SCD. Percentage sickling and activity of P_{sickle}, from Figures 1 and 3a, both normalised to values in control RBCs at 0mmHg, were correlated in RBCs pre-incubated with 5HMF (5 mM) or handled similarly but in the absence of this heterocyclic aldehyde. Pearson correlations were calculated as 0.885 in untreated RBCs ($p < 0.0001$) and 0.789 in the presence of 5HMF ($p < 0.0001$).

Figure 4. Effect of 5HMF on the activity of the Gardos channel in RBCs from patients with SCD. (a) RBCs were treated as described in the legend to Figure 3a, all in the absence or presence of 5HMF (5 mM). Gardos channel activity is defined as the K^+ influx (calculated as mmol.(l cells.h)$^{-1}$ and measured at an extracellular [K^+] of 7.5 mM) in the absence or presence of clotrimazole (5 μM). Ouabain (100 μM) and bumetanide (10 μM) were included in all experiments. Activity of the Gardos channel at each oxygen tension was normalised to that at 0 mmHg O_2 in the absence of 5HMF, which had a value of 3.1 ± 0.77 mmol.(l cells.h)$^{-1}$ (range from 0.65 - 5.96 mmol.(l cells.h)$^{-1}$). Histograms represent means ± S.E.M., n = 6-7. * $p < 0.05$ comparing control RBCs in the absence of 5HMF with those incubated in its presence. (b) Effect of 5HMF on K^+ uptake in Ca^{2+}-loaded RBCs from patients with SCD. Total K^+ uptake (given as mmol.(l cells)$^{-1}$ and measured at an extracellular [K^+] of 80 mM) was measured over 10 min in RBCs treated with the ionophore A23187 (6 μM) in high K^+-containing saline, at a free extracellular [Ca^{2+}] of 10 μM (clamped with 2 mM EGTA), in the absence or presence of 5HMF (5 mM). Under these conditions, K^+ uptake in the absence of ionophore was < 0.5 mmol.(l cells)$^{-1}$ over 10 min. Ouabain (100 μM) and bumetanide (10 μM) were included in all experiments. Symbols represent paired data points from 4 different experiments, filled for controls in the absence of 5HMF, unfilled with 5HMF.

Figure 5. Effect of 5HMF on phosphatidylserine exposure in RBCs from patients with SCD. After a 60 minute pre-incubation, RBC suspensions in Cl-MBS were deoxygenated (O_2 of 20 mmHg) in shaken 24-well plates in a low oxygen incubator for up to four hours in the absence and presence of 5HMF (5 mM). Aliquots were removed at intervals and % RBCs positive for phosphatidylserine exposure measured using FACS. Histograms represent means ± S.E.M., n = 4. # $p < 0.05$ comparing control RBCs at time 0 and after 240 min. * $p < 0.05$ comparing control RBCs in the absence of 5HMF with those incubated in its presence after 240 min.

Figure 6. Effect of 5HMF on the activity of the K^+-Cl^- cotransporter (KCC) in RBCs from patients with SCD. RBCs were treated as described in the legend to Figure 3a, all in the presence or absence of 5HMF (5 mM). KCC activity is defined as the K^+ influx (calculated as mmol.(l cells.h)$^{-1}$ and measured at an extracellular [K^+] of 7.5 mM) in the absence or presence of Cl^- (substituted with NO_3^-). Ouabain (100 μM), bumetanide (10 μM) and clotrimazole (5 μM) were included in all experiments. Activity of KCC at each oxygen tension was normalised to that at 100 mmHg O_2 in the absence of 5HMF, which had a value of 1.7 ± 0.31 mmol.(l cells.h)$^{-1}$ (range from 0.56 - 2.54 mmol.(l cells.h)$^{-1}$). Histograms represent means ± S.E.M., n = 5-6. * $p < 0.05$ comparing control RBCs in the absence of 5HMF with those incubated in its presence.

Figure 7. Effects of *N*-ethylmaleimide (NEM) and 5HMF on the activity of KCC in RBCs from patients with SCD. RBCs were pre-incubated for 30 min with or without NEM (1 mM) and/or 5HMF (5 mM) after which they were diluted 10-fold into test tubes for measurement of K^+ influx, all in the absence or presence of 5HMF (5 mM). KCC activity is defined as the K^+ influx (calculated as mmol.(l cells.h)$^{-1}$ and measured at an extracellular [K^+] of 7.5 mM) in the absence or presence of Cl^- (substituted with NO_3^-) Ouabain (100 μM), bumetanide (10 μM) and clotrimazole (5 μM) were included in all experiments. Histograms represent means ± S.E.M., n = 5. * $p < 0.05$ comparing RBCs in the absence and presence of 5HMF.

Figure 8. Dose dependence of 5HMF on the activities of P_{sickle}, Gardos channel and KCC, and PS exposure in RBCs from patients with SCD. To measure transport activities, RBCs were treated as described in the legends to Figures 3a, 4a and 6 with transport activity measured as mmol.(l cells.h)$^{-1}$) (at an extracellular [K^+] of 7.5 mM) at 10mmHg O_2. PS exposure was measured as described in the legend to Figure 5 at 20mmHg O_2. Transport activities and PS exposure were normalised to that in the absence of 5HMF. Histograms represent means ± S.E.M., n = 4 for fluxes and n = 5 for PS exposure. * $p < 0.05$ in RBCs incubated in the presence of 5HMF compared with its absence.

Figure 1

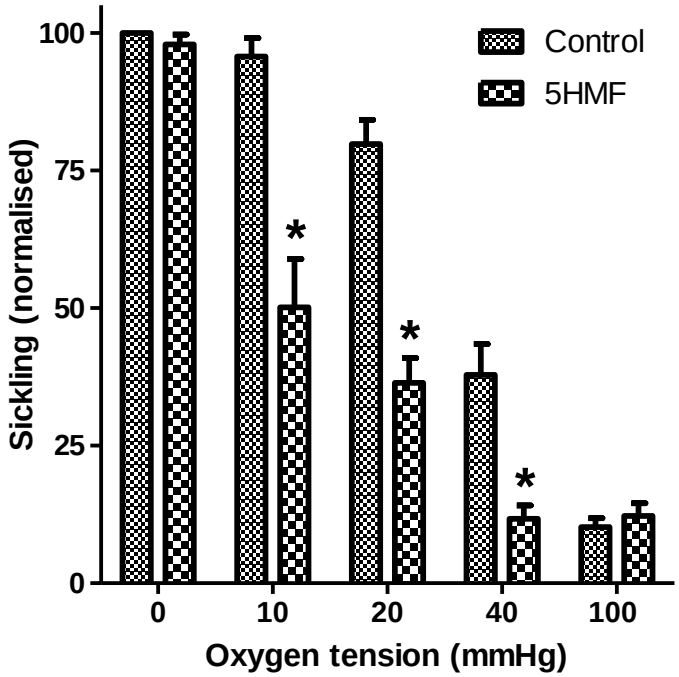

Control
5HMF
Sickling (normalised)
100
75
50
25
0
*
*
*
0
10
20
40
100
Oxygen tension (mmHg)

Figure 2

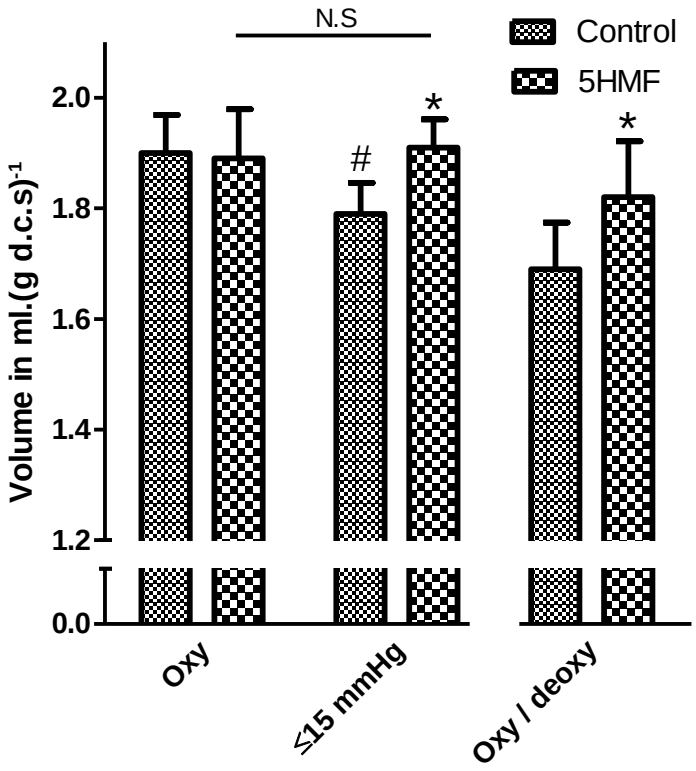

N.S
Control
5HMF
Volume in ml.(g d.c.s)-1
2.0
1.8
1.6
1.4
1.2
0.0
#
*
*
Oxy
≤15 mmHg
Oxy / deoxy

Figure 3a

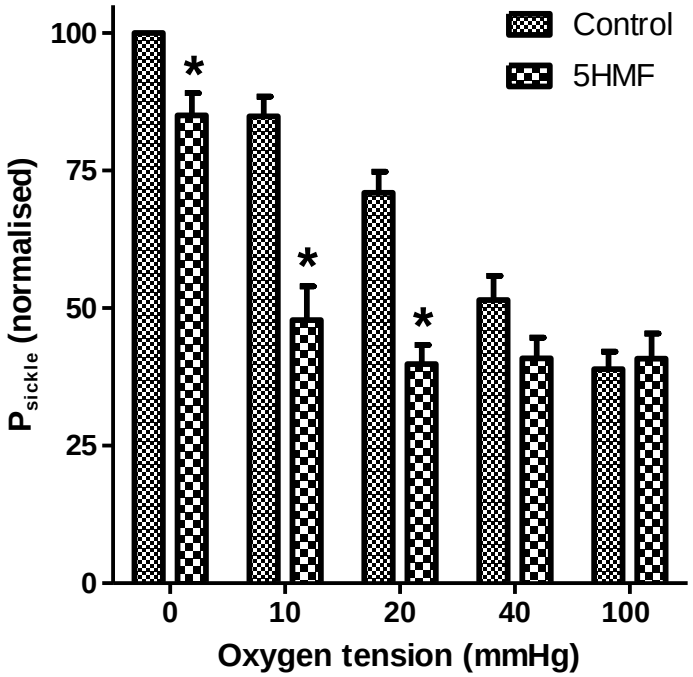

Control
5HMF
100
75
50
25
0
P_{sickle} (normalised)
*
*
*
0
10
20
40
100
Oxygen tension (mmHg)

Figure 3b

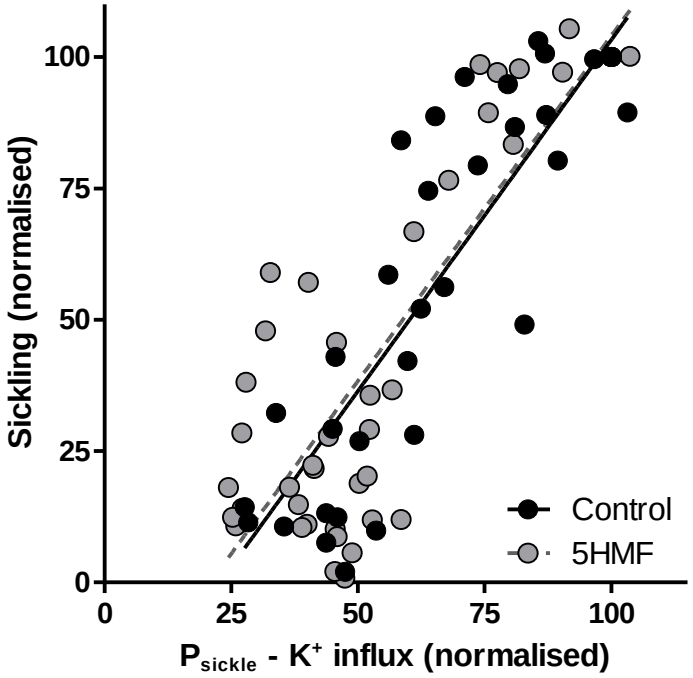

Sickling (normalised)
100
75
50
25
0
0
25
50
75
100
P sickle - K+ influx (normalised)
Control
5HMF

Figure 4a

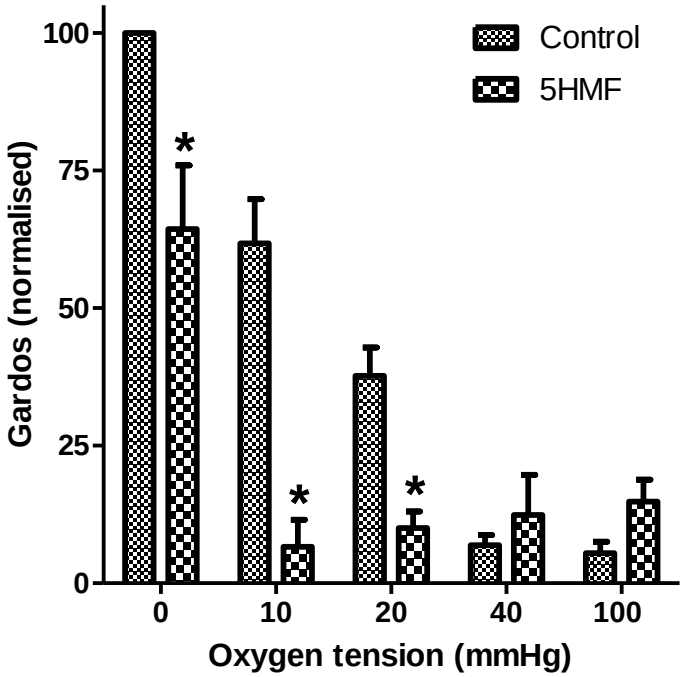

Control
5HMF
Gardos (normalised)
100
75
50
25
0
*
*
*
0
10
20
40
100
Oxygen tension (mmHg)

Figure 4b.

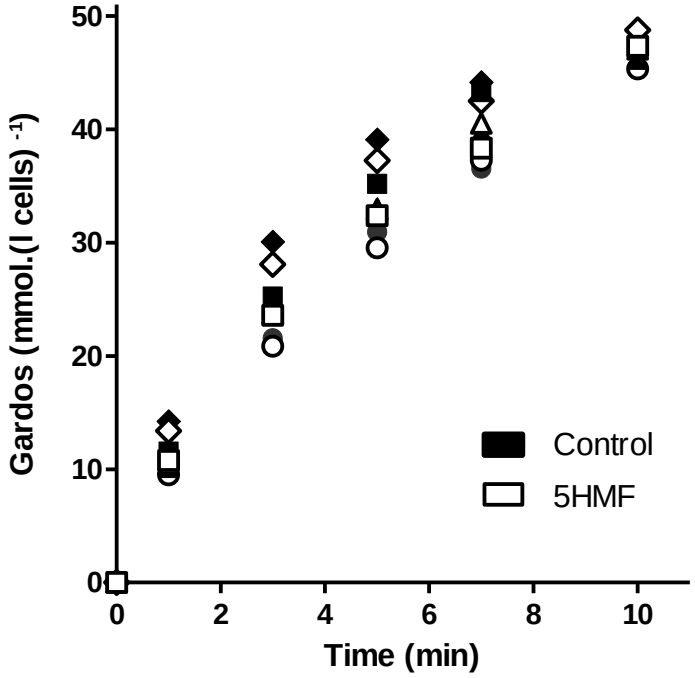

Gardos (mmol.(l cells) -1)
50
40
30
20
10
0
0
2
4
6
8
10
Time (min)
Control
5HMF

Figure 5

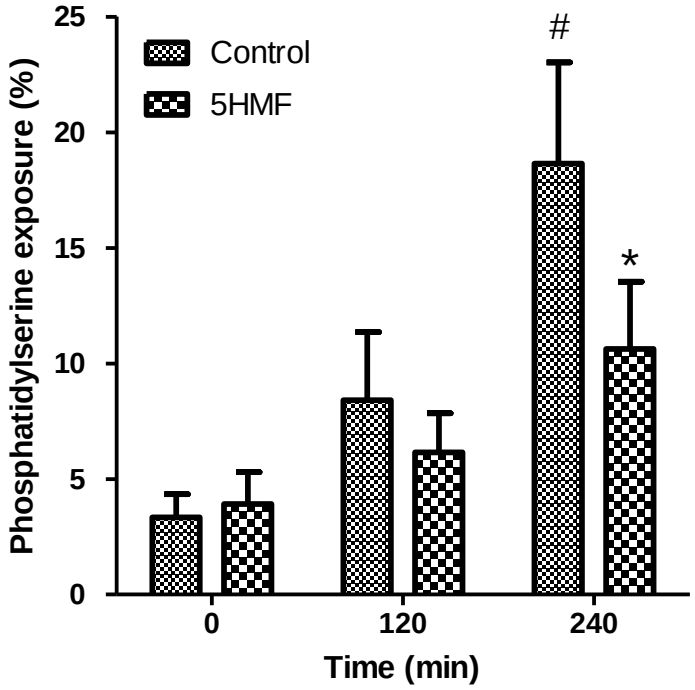

Phosphatidylserine exposure (%)
25
20
15
10
5
0
Control
5HMF
#
*
0
120
240
Time (min)

Figure 6

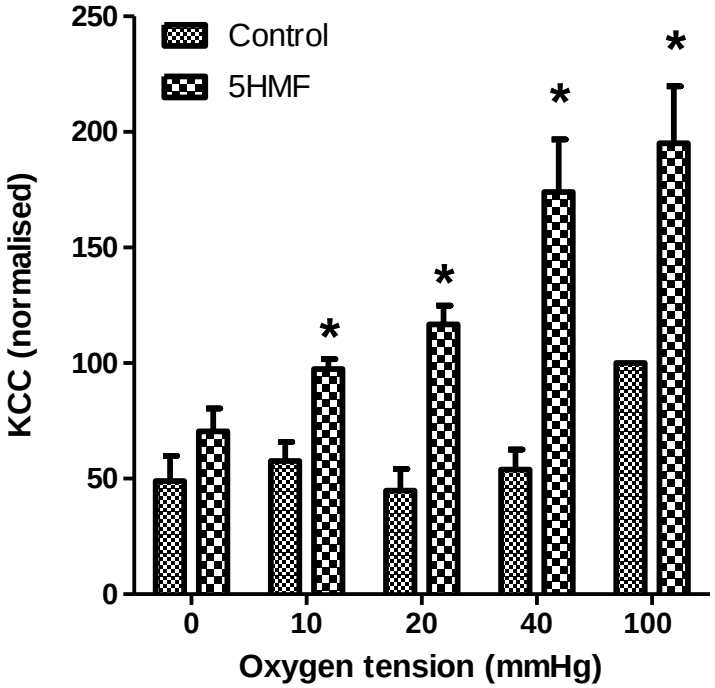

Control
5HMF
KCC (normalised)
250
200
150
100
50
0
0
10
20
40
100
Oxygen tension (mmHg)
*
*
*
*

Figure 7

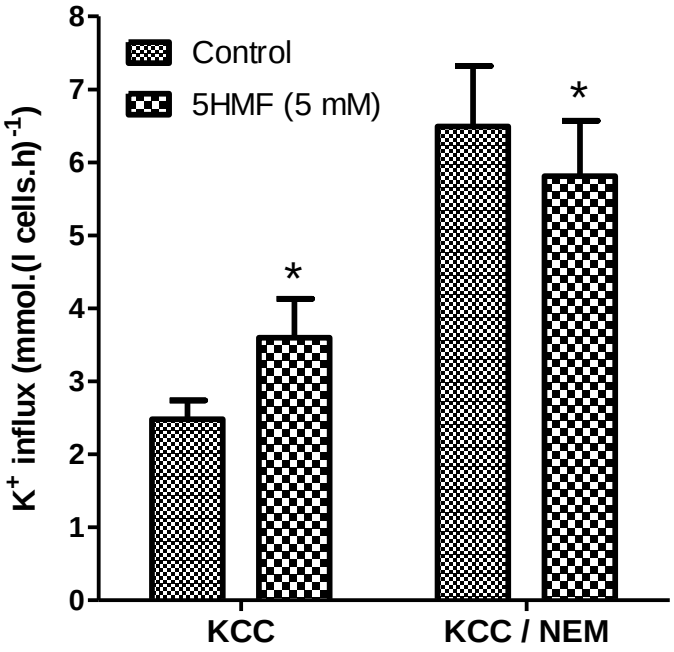
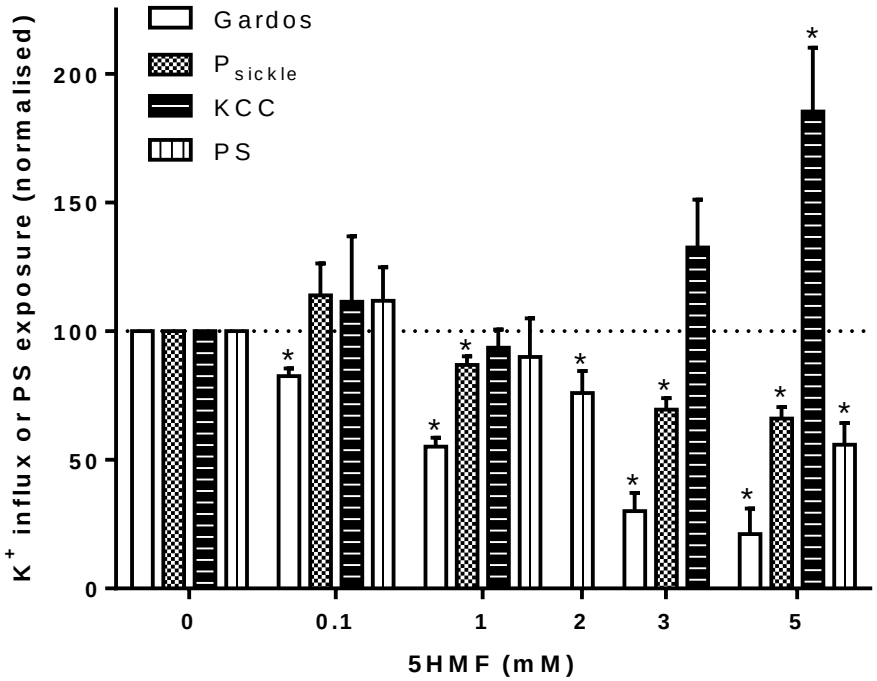

Control
5HMF (5 mM)
K+ influx (mmol.(l cells.h)-1)
0
1
2
3
4
5
6
7
8
*
*
KCC
KCC / NEM

Figure 8


Gardos
P sickle
KCC
PS
K+ influx or PS exposure (normalised)
200
150
100
50
0
0
0.1
1
2
3
5
5HMF (mM)