Sodium nitrite therapy attenuates the hypertensive effects of HBOC-201 via nitrite reduction

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Safety concerns coupled with a lack of supply of donated RBCs (red blood cells) have led to a great deal of interest in the development of blood substitutes as alternative resuscitative agents. Such resuscitative agents would need to be capable of simultaneously improving vascular function and tissue perfusion/oxygenation. The current generation of so-called HBOCs (haemoglobin-based oxygen carriers) comprise haemoglobins from different species (human and bovine) that have been chemically modified to improve structural stability (thereby extending circulatory half-life and minimizing renal toxicity) and to modulate oxygen affinity (to counter the higher oxygen affinity of cell-free haemoglobin compared with erythrocytic haemoglobin) [1,2]. Despite extensive efforts in their development, previous studies have indicated there are major additional concerns with the clinical use of HBOCs [3–8]. These concerns stem from the ability of cell-free haemoglobins to stimulate pro-oxidative reactions and scavenging NO [3–8], and an endogenously produced free radical that plays critical roles in vascular homeostasis [9,10]. In this way, HBOCs may promote hypertensive and pro-oxidative responses and thereby exacerbate underlying tissue injury.

NO is instrumental in vascular homeostasis mechanisms controlling blood flow, regulating mitochondrial respiration and maintaining an anti-inflammatory and anti-thrombotic environment [9,10]. The critical role for NO is indicated by the association between loss of, or aberrant, NO-dependent signalling and the pathogenesis of a variety of diseases including hypertension, atherosclerosis and ischemia-reperfusion injury [11]. Cell-free haemoglobin, in either its oxygenated or deoxygenated state, rapidly reacts with NO (k = ~10^7 M^−1 s^−1) [12], which coupled with the relatively high concentrations of haem (at the mM level) that arise from HBOC administration during resuscitative therapies [8], results in a significant inhibition of NO signalling that manifests acutely with a hypertensive response. Whereas this may be of benefit in certain traumatic injuries e.g. traumatic brain injury, NO scavenging is generally considered to be detrimental and this property remains a significant problem in the therapeutic development of HBOCs [13].

Recent concepts suggest that the inorganic anion nitrite represents a reservoir for NO bioactivity during hypoxia/ischemia, both in tissues and the vasculature [14–18]. In this paradigm, nitrite is reduced by one electron to form NO via mechanisms that are activated during hypoxia/ischemia. Nitrite-derived NO restores NO signalling which leads to cytoprotection in a variety of pathologies (e.g. ischemia/reperfusion injury, haemorrhagic stroke, hypoxic pulmonary hypertension and hypertension) and stimulates NO-dependent physiological responses (e.g. angiogenesis) [13,16–19]. How this occurs may involve parallel or additional separate mechanisms that require further study.

Key words: haemorrhage, nitric oxide, nitrite reductase activity, resuscitation, shock.
nitrite is reduced to NO during hypoxia remains an active area of investigation with the precise mechanism probably depending upon the specific tissue and degree of hypoxia. In the vascular compartment previous studies have suggested that the reaction of nitrite with deoxyhaemoglobin is important [20–26]. Specifically, this involves the combination of hypoxia (or more precisely hypoxaemia), deoxygenated RBCs or haemoglobin and nitrite stimulated NO-dependent vasodilatation via activation of soluble guanylate cyclase and formation of cGMP [21]. This so-called nitrite reductase activity of deoxyhaemoglobin should be contrasted with the nitrite oxidase activity of oxyhaemoglobin as shown in the following equations:

$$\text{Hb}[\text{Fe}^2+] + \text{NO}_2^- + \text{H}^+ \rightarrow \text{Hb}[\text{Fe}^3+] + \text{OH}^-$$

$$4\text{Hb}[\text{Fe}^2+] + 4\text{NO}_2^- + 4\text{H}^+ \rightarrow 4\text{NO}^- + 4\text{Hb}[\text{Fe}^3+] + \text{O}_2 + 2\text{H}_2\text{O}$$

where Hb is haemoglobin.

Moreover, the nitrite reductase activity of deoxyhaemoglobin is under allosteric control over the haemoglobin oxygen affinity [27]. The latter is indicated in part by the observation that the initial rate of nitrite reduction shows a bell-shaped dependence on haemoglobin fractional saturation, being maximal near the haemoglobin p50 (the partial pressure at which haemoglobin is 50% saturated). This may allow for a coupled response by which haemoglobin-based oxygen sensing results in NO formation at sites of tissue hypoxia [21,23]. Interestingly, nitrite alone can stimulate vasodilatation of isolated aorta [24,28–31] and this is inhibited by addition of cell-free oxyhaemoglobin, consistent with a role for NO in nitrite-dependent vasodilatation [24,29]. However, cell-free deoxyhaemoglobin has no effect, despite being equally competent at inhibiting NO-dependent vasodilatation [24,32]. This would be expected from similar rates of NO scavenging by oxy- and deoxyhaemoglobin as shown in the following equations:

$$\text{Hb}[\text{Fe}^2+] + \text{O}_2 \rightarrow \text{Hb}[\text{Fe}^3+]\text{(metHb)} + \text{NO}_3^-$$

$$\text{Hb}[\text{Fe}^3+] + \text{NO} \rightarrow \text{Hb}[\text{Fe}^3+]\text{NO}$$

where metHb is methaemoglobin.

Together, these results suggest that in the presence of nitrite, the nitrite reductase activity of deoxyhaemoglobin creates a balance between NO formation and haem-based NO scavenging that dictates the degree to which NO can stimulate signalling and vasodilation.

The potential for nitrite-derived NO formation by interaction with deoxyhaemoglobin raises an intriguing possibility that co-administration of nitrite with an HBOC may result in NO formation in tissues where HBOCs are significantly deoxygenated. Such co-administration may therefore counteract the NO scavenging effects and thereby attenuate hypertension. In this scenario, therapeutic nitrite can be viewed as an NO-donor. Although many NO-donor compounds have been developed, the advantages of nitrite in this scenario is that nitrite-derived NO would be coupled to HBOC deoxygenation and therefore produce NO at sites where increased blood flow is required. Other NO-donors would lack the site-specific release of NO. Consistent with this concept, recent studies by Yu et al. [33,34] show that inhaled NO, which increases circulating nitrite concentrations, and reagent nitrite itself can attenuate the hypertensive effects of either murine cell-free tetrameric haemoglobin or HBOC-201 (a glutaraldehyde cross-linked bovine haemoglobin) in a top-load murine model of resuscitation. Moreover, Lui et al. [35] have shown that polyethylene glycol conjugation of α-chain cross-linked haemoglobin, which results in an HBOC with suppressed vasoactivity, increased the rate constant for deoxyhaemoglobin-mediated nitrite reduction. This suggests that current strategies used to produce stable HBOC may also be used to modulate its nitrite reductase activity and that increasing such activity may be of clinical benefit in diminishing the pressor effects of HBOC.

In the present study we evaluated the nitrite reductase activity of HBOC-201 (haemoglobin-based oxygen carrier 201; Hemopure®), a purified glutaraldehyde-polymerized bovine haemoglobin, and tested the potential for nitrite therapy to attenuate HBOC-201-mediated hypertension in a murine model of trauma-haemorrhage and resuscitation. HBOC-201 is ‘T’-state stabilized haemoglobin with a higher p50 (i.e. a lower oxygen affinity) of ∼38 mmHg at 37°C, pH 7.4, compared with the native cell-free haemoglobin with a p50 of ∼10 mmHg at 37°C, pH 7.4. HBOC-201 also has an extended shelf-life, does not require cross matching or cold storage, is non toxic and has been shown to be an efficacious resuscitative agent in diverse experimental models of trauma and haemorrhagic shock [36–38]. However, it is also known to cause significant hypertension secondary to scavenging of endogenous NO [36–38].

**EXPERIMENTAL**

For a detailed description of the materials and methods used see the Supplementary Experimental section available at http://www.BiochemJ.org/bj/422/bj4220423add.htm. All procedures using animals were performed under standard aseptic conditions and according to the University of Alabama at Birmingham institutional animal care committee approved protocols.

**Vessel bioassay studies**

For all vessel experiments thoracic aortas from male Sprague–Dawley rats were used as described previously in [24]. Aortic rings in KHB (Krebs–Henseleit buffer) [24] were pretreated with 5 μM indomethacin and 100 μM L-NMMA (Nω-monomethyl L-arginine) and equilibrated with 95, 21, 2 or 0% O2 gas mixtures containing 5% CO2 and balanced with N2. Following precontraction with PE (L-phenylephrine) the vasodilatory effects of nitrite or MNO (Mahma NONOate) were determined in the absence or presence of either 20 μM hHb (human haemoglobin) or 20 μM HBOC-201 (all concentrations listed are based on haem), which were added before initiating the dose–response. Vasodilatory effects of the cumulative MNO or nitrite additions were determined by measuring the delta tension and expressing this as a percentage of relaxation with respect to the maximal PE constriction. Cumulative dose-dependent relaxation curves were fitted to a sigmoidal function using GraphPad (GraphPad Software), from which the EC50 was obtained.

During preliminary vasodilation studies with HBOC-201 it was observed that in the presence of nitrate, but not MNO, HBOC-201 concentrations decreased over time. Concomitant with this decrease HBOC-201 protein precipitates were noticed in the vessel bioassay chambers. As a varying haem concentration will affect both NO scavenging and NO formation processes, and hence the vessel response to vasoactive stimuli, in a subset of experiments we assessed the vasodilatory effect of single doses of nitrite in the absence and presence of HBOC-201. For these experiments, vessels were pre-contracted with PE and pre-equilibrated with the desired O2-containing gas. HBOC-201 was then added and 2 min was allowed for HBOC-201 mixing/equilibration. Then a single dose of nitrite was added and changes in vessel tension recorded. Using this protocol no significant loss of HBOC-201 was observed (see the Results section). This process was repeated using different...
vessel segments and different nitrite doses to assimilate a dose-dependence of nitrite-dependent vasodilation in the presence and absence of HBOC-201. No significant loss of HBOC-201 was observed in the MNO-dependent vasodilation experiments, nor was any loss of hHb observed in any experiment.

Assessment of hHb or HBOC-201 concentration and redox/ligation state

Aliquots of hHb or HBOC-201 were collected from vessel bioassay chambers, after equilibration with different O2 containing gases, immediately before and after addition of either nitrite or MNO. Redox and ligation state was determined by fitting measured, visible spectra to the previously acquired base spectra for oxy-, deoxy-, met-, ferryl-, nitrosyl- and metnitrite forms of both HBOC-201 and hHb, which are all species that may be populated during reaction with nitrite, using a least squares method as described previously in [21–24]. Preparation of reference spectra is described in the Supplementary Experimental section available at http://www.BiochemJ.org/bj/422/bj4220423add.htm (see Figure 1(A) for HBOC-201 spectra).

Determination of the nitrite reductase activity of HBOC-201

Rates of deoxyhaem loss at 37°C were measured to assess the nitrite reductase activity of HBOC-201. A progressive degassing of 30 μM HBOC-201 in PBS, pH 7.4, containing 100 μM DTPA (diethylenetriaminepenta-acetic acid) was performed under a stream of helium or N2 to obtain a range of O2 fractional saturations. The resulting solutions were transferred anaerobically to sealed spectrophotometer cuvettes and the visible spectra from 450 to 700 nm were collected immediately before and every 30 s after the addition of 5 mM sodium nitrite (i.e. excess nitrite) for 15 min. Concentrations of deoxyHBOC-201 as a function of time were determined by spectral deconvolution using reference spectra to the different HBOC-201 redox and ligation states listed above and as described in [21–23]. Rate constants were calculated by dividing the slope of the initial (10%) linear portion of the curve by the initial concentration of deoxyHBOC and nitrite. Concentrations of nitrosylHBOC-201 were used as an index of NO production and were determined by spectral deconvolution after 720 s of nitrite addition. All nitrite stock solutions were prepared in degassed PBS.

NO detection by chemiluminescence

Direct formation of NO was monitored by ozone-based chemiluminescence using a 280i NO analyser (GE Sievers). A mixture containing 1 mM sodium nitrite in PBS, pH 7.4, with 100 μM DTPA and 50 μl of antifoam (GE Sievers) was equilibrated at 37°C under anoxic conditions in a sealed chamber directly connected to the analyser before the addition of HBOC-201. Nitric oxide formation was confirmed by the addition of the NO scavenger cPTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide].

cGMP measurement

Rat aortic rings in KHB were equilibrated with gas mixtures containing 0 and 95 % O2, supplemented with 5 % CO2, at 37°C and pH 7.45 in the presence of 5 μM indomethacin, 100 μM L-NMMA, 100 μM IBMX (isobutylmethylxanthine), 100 nM PE and 0.00067% (v/v) SE-15 antifoam (Sigma–Aldrich). Then 20 μM HBOC-201 was added and allowed to equilibrate for 1 min before the addition of 25 μM nitrite. Rings were collected after 10 min, blotted dry, weighed and frozen in liquid nitrogen. cGMP was measured in ring homogenates by ELISA according to manufacturer’s instructions (Cayman Chemical).

Trauma-haemorrhage and resuscitation model

C57Bl/6 male mice were anaesthetized by inhalation of 5% isoflurane in air. The concentration of isoflurane was then reduced to the minimal concentration for maintenance (<1%). The abdomen and groins were shaved and washed with 10% povidone-iodine. A 2 cm midline laparotomy was performed to induce soft-tissue trauma. The incision was closed in two layers (fascia/muscle and skin) and bathed in 1% lidocaine for analgesia. Both femoral arteries were cannulated with catheters (Braintree Scientific). Systemic arterial pressure was continuously monitored through one arterial line while haemorrhage and resuscitation was performed via the other. Mice were bled over 30 min to a MAP (mean arterial pressure) of 25 ± 5 mmHg. This blood pressure was maintained for a further 60 min by additional bleeding as required. At the end of the 90 min haemorrhagic shock period, animals were resuscitated over 30 min with either LR (lactated Ringer’s solution) or HBOC-201 formulation equal to total blood volume (approx. 60% of total blood volume). Then, 100 μl of nitrite was administered intravenously in a bolus form (nitrite stock solutions were 0.1 mM, 0.3 mM, 1 mM or 100 mM) immediately prior to the initiation of HBOC-201 infusion. A further higher dose of nitrite (100 μl of 1 M stock solution) was also evaluated in preliminary experiments (n = 2) but this induced death within the resuscitation phase and was therefore excluded from our analysis (results not shown). All experiments were carried out for at least 120 min post-resuscitation. Arterial blood samples were obtained at designated intervals for nitrite measurement and assessment of paco2 (arterial partial pressure of CO2), fractional saturation and methaemoglobin.

Measurement of NO metabolites

Plasma nitrite, S-nitrosothiols and N-nitroso compounds were measured as described previously in [39]. Upon collection, RBCs and plasma were immediately separated by centrifugation at 2000 g for 1 min. Plasma was then mixed 1:1 with a solution containing a final concentration of 1mM NEM (N-ethylmaleimide) and 100 μM DTPA. The mixture was incubated at room temperature (22°C) for 2 min prior to snap freezing in liquid nitrogen. Samples were then thawed on ice and in the dark within 4 h of collection and nitrite, S-nitrosothiol and N-nitroso species measured.

Statistical analysis

Dose-dependent vessel relaxation responses were fitted to sigmoidal curves and the results were analysed by 2-way repeated measures ANOVA with a Bonferroni correction post-test. Changes in circulating nitrite levels during haemorrhagic shock and resuscitation with HBOC-201, with or without exogenous nitrite addition, were analysed by 2-way ANOVA with a Bonferroni correction post-test. To evaluate if nitrite therapy attenuated HBOC-201-mediated hypertension during haemorrhagic shock and resuscitation, repeated-measures ANOVA was determined for each of the three experimental periods (haemorrhage, shock and resuscitation). Statistical significance in vessel bioassays and biochemical experiments was assessed by either repeated-measures ANOVA or one-way ANOVA followed by Bonferroni corrections post-test. P values less than 0.05 were considered significant in all cases and analyses were performed using GraphPad Prism Software.

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RESULTS
Nitrite reduction by HBOC-201

We first tested whether HBOC-201 could reduce nitrite and whether this process is regulated by O2 fractional saturation. Nitrite was added to HBOC-201 at different O2 saturations and changes in visible absorbance spectra were followed as a function of time. Figure 1(B) shows a representative experiment performed at 60 % O2 saturation. Time-dependent changes in deoxyHBOC-201 were calculated to assess the nitrite reductase activity (Figure 1C) from experimentally measured spectra by least squares deconvolution (shown in Figure 1A). Figure 1(D) shows the initial rate and calculated rate constant for nitrite reduction (using initial nitrite and deoxyhaemoglobin concentrations) as a function of O2 fractional saturation. Unlike hHb which shows a bell-shaped dependence of initial rate against fractional saturation [21,23], the initial rate for nitrite-reduction by HBOC-201 shows a significant inverse and linear relationship against fractional saturation. This is reflected in a similar profile for the rate constant, which also significantly increases as fractional saturation decreases. Consistent with nitrite-reduction, NO was a product of nitrite deoxy-HBOC-201 reactions as determined by formation of nitrosyl-HBOC-201 (Figure 1E), which increased at lower fractional saturations, and by the formation of free NO under anoxic conditions (Figure 1F). The latter was confirmed by loss of signal in response to the NO scavenger cPTIO as shown in Figure 1(F).

Effects of hHb and HBOC-201 oxygen fractional saturation on NO- and nitrite-mediated vasodilation of aortic rings

To assess if nitrite-reduction by HBOC-201 regulates NO signalling we employed the experimental model of vasodilation of isolated aortic rings as described previously in [24]. This approach, which provides a sensitive read-out of NO-dependent signalling (i.e. vasodilation) is amenable to performing experiments at different oxygen tensions and hence HBOC-201 fractional saturations. Figure 2 shows that the fractional saturation of either HBOC-201 or cell-free human haemoglobin attained in vessel bioassay chambers equilibrated with 95, 21, 2 and 0 % O2 ranged from ~95 to 30 % and ~100 to 65 % respectively. Consistent with HBOC-201 having a higher p50 value, HBOC-201 was deoxygenated compared with hHb at oxygen tensions ≤21 %. To allow comparison of HBOC-201 and hHb we chose to study the effects of HBOC-201 and hHb at 95, 21, 2 and 0 % O2 to provide a range of overlapping fractional saturations for each haemoglobin preparation. To evaluate if the O2 fractional saturation of HBOC-201 affects NO scavenging, we tested the effects of HBOC-201 on vasodilation elicited by the NO-donor...
Results are means ± S.E.M. (n = 5–9).

MNO. Figures 3(A)–3(D) show that NO-dependent vasodilation was inhibited at all fractional saturations by both HBOC-201 and hHb as indicated by significant right-shifts in MNO dose-dependent vasodilation. These results indicate that, similar to hHb, both oxygenated and deoxygenated HBOC-201 are competent in inhibiting NO-dependent signalling in the vascular compartment.

We next tested the effects of HBOC-201 and hHb on nitrite-dependent vasodilation. Consistent with previous studies [24,29] showing a role for NO in nitrite-mediated vasodilation, hHb inhibited vasodilation at high (2, 21 and 95 %) O2 fractional saturations (see Supplementary Results and Supplementary Figure S1 at http://www.BiochemJ.org/bj/422/bj4220423add.htm). Under deoxygenated conditions, however, hHb failed to inhibit nitrite-dependent vasodilation. We have shown previously that this failure to inhibit NO-dependent effects at lower fractional saturations can be explained by the nitrite reductase activity of deoxyhaemoglobin, with the resultant NO formation countering NO scavenging [24]. Supplementary Figure S1 shows results from experiments testing the effects of HBOC-201 on nitrite-dependent vasodilation. However, in the course of these experiments it was noted that in the presence of nitrite, the concentration of HBOC-201 was decreasing, particularly at higher oxygen tensions. A systematic evaluation of HBOC-201 concentration as a function of time demonstrated that the HBOC-201 concentration decreased ~40% during the course of nitrite-dependent vasodilation experiments at both 95 and 21 % O2 (see Supplementary Figure S2A at http://www.BiochemJ.org/bj/422/bj4220423add.htm). Interestingly, nitrite had no effect on the concentration of hHb (see Supplementary Figure S2A) and the loss of HBOC-201 did not occur in the presence of MNO (see Supplementary Figure S2B), suggesting a specific interaction between nitrite, higher oxygen tensions and HBOC-201 that leads to HBOC-201 decomposition. The changing HBOC-201 concentration during these experiments precludes interpretation of effects on nitrite-dependent vasodilation. We therefore employed a modified protocol in which the vasodilatory effects of single doses of nitrite were assessed in the presence or absence of HBOC-201. Using this protocol, which resulted in nitrite/HBOC-201 incubation times of 3.9 ± 0.4 min (mean ± S.E.M.; n = 7) compared with 38.1 ± 4.1 min (mean ± S.E.M.; n = 17) for nitrite-dose cumulative studies (see Supplementary Figure S1), no significant loss of HBOC-201 occurred at any oxygen tension (results not shown). Figure 4 shows that using this approach HBOC-201 significantly inhibited nitrite-dependent vasodilation at 95 and 21 % O2, but did not affect nitrite-dependent vasodilation at either 2 or 0 % O2. Consistent with these results, nitrite increased cGMP levels to similar extents both in the absence and presence of HBOC-201 at 0 % O2 (Figure 5).

Nitrite administration attenuates HBOC-201-mediated hypertension during trauma-haemorrhage

We next tested if nitrite therapy could prevent HBOC-201-mediated hypertension in a murine model of trauma-haemorrhagic shock and resuscitation. Consistent with previous studies [33,36], resuscitation with HBOC-201 increased MAP above prehaemorrhage levels, underscoring its hypertensive effects (Figures 6A and 6B). However, in a dose-dependent manner, one bolus administration of nitrite immediately before resuscitation prevented HBOC-201-mediated hypertension (Figure 6A), with MAP returning to prehaemorrhage levels with 30 nmol nitrite (Figures 6B and 6E). Figure 6(C) shows that at the end of resuscitation with LR alone, MAP remains approx. 15 mmHg below prehaemorrhage levels and that bolus addition of 30 nmol nitrite immediately before resuscitation did not have any further effect on MAP relative to LR alone. Figure 6(D) shows that at the end of resuscitation with 30 nmol nitrite and HBOC-201, circulating nitrite was significantly increased by approx. 2–3-fold compared with HBOC-201 alone, and returned to basal levels at 2 h post-resuscitation. No significant changes in circulating S-nitrosothiols or N-nitroso compounds were observed under this condition (results not shown). Importantly, compared with the effects of HBOC-201 alone, 30 nmol nitrite did not change fractional saturation, paO2 nor paCO2 post-resuscitation (Table 1). This indicated that nitrite can improve haemodynamics without affecting the O2 and CO2 carrying functions of haemoglobin (Table 1). To assess the safety of nitrite administration we measured changes in MAP, methaemoglobin and serum levels of HBOC-201. Figure 6(E) shows a paired analysis of the effects of different doses of nitrite on MAP at the end of resuscitation relative to prehaemorrhage. MAP increased significantly with HBOC-201 alone and with HBOC-201 and 10 nmol nitrite. However, with 10 μmol nitrite a significant decrease in MAP was observed. No significant changes in MAP were observed with 30 nmol or 100 nmol nitrite. Supplementary Figure 3 (available at http://www.BiochemJ.org/bj/422/bj4220423add.htm) shows that with HBOC-201 alone methaemoglobin increased to approx. 2–3% at 2 h post-resuscitation. Nitrite at concentrations of ≤100 nmol did not affect this increase in methaemoglobin. Only at the highest dose tested (10 μmol) did methaemoglobin increase significantly beyond that observed with HBOC-201 alone. Finally, as the vessel bioassay studies indicated an interaction of relatively high concentrations of nitrite and oxyHBOC-201, resulting in the decomposition of the HBOC-201, we measured serum haemoglobin, indicating the amount of HBOC-201, at approx. 3–4 h post resuscitation in the HBOC-201 alone and HBOC-201 and 30 nmol nitrite groups. Table 1 shows that nitrite administered at this dose, which was able to normalize hypertension, did not cause any loss of HBOC-201.

DISCUSSION

Previous developments have highlighted the potential for NO formation from haemoglobin and nitrite in mediating vascular function and offering a novel therapeutic strategy to attenuate hypertensive effects of HBCOs [13,20,21,25,33–35,40–43]. HBOC-201 was chosen as a model HBOC for the present work because it has been extensively studied. The aim was to evaluate the nitrite reductase activity of HBOC-201 and test if NO derived from this process is capable of maintaining NO-dependent vasodilation
Effects of hHb and HBOC-201 on cumulative NO-dependent vasodilation at different oxygen tensions

NO-dependent vasodilation was assessed in the absence or presence of either 20 μM HBOC-201 or 20 μM hHb at either (A) 95%, (B) 21%, (C) 2% or (D) 0% O₂. Lines show the best fit using sigmoidal fitting algorithms. Results represent means ± S.E.M. (n = 2–3). In some cases errors are smaller than symbol size. ***P < 0.001, **P < 0.01, *P < 0.05 for HBOC-201 and hHb relative to control. #P < 0.01, ψP < 0.05 for HBOC-201 relative to hHb. P-values calculated by two-way repeated-measures ANOVA with Bonferroni corrections post-test.

Effects of HBOC-201 on bolus nitrite-dependent vasodilation at different oxygen tensions

To avoid time-dependent loss of HBOC-201 in nitrite-dependent vasodilation experiments, aortic segments were exposed to single additions of nitrite at the indicated concentrations in the presence (white bars) or absence (black bars) of 20 μM HBOC-201 and at (A) 95%, (B) 21%, (C) 2% or (D) 0% O₂ as described in the Experimental section. Results represent means ± S.E.M. (n = 3–8). Indicated P-values were calculated by two-way repeated-measures ANOVA.

The differential reactivities of oxyhaemoglobin and deoxyhaemoglobin with nitrite have been acknowledged for many years [27], with oxyhaemoglobin oxidizing nitrite to nitrate and deoxyhaemoglobin reducing nitrite to NO. Recent insights into the mechanisms of this oxygen-linked reactivity of haemoglobin have shown that nitrite reduction is modulated via allosteric regulation of haemoglobin conformation states (R- or T-states) [21–23]. Specifically, the rate of nitrite reduction by haemoglobin...
Nitrite co-administration attenuates hypertensive effects of HBOC-201

Figure 5 Effects of HBOC-201 on bolus nitrite-dependent increases in cGMP

Rat aortic segments were equilibrated in KHB at pH 7.4 and 37 °C with a gas mixture containing 0% O₂ and 5% CO₂. Vessels were pretreated with 5 μM indomethacin, 100 μM l-NMMA, 100 μM IBMX and 0.00067% SE-15 antifoam in the presence or absence of 20 μM HBOC-201 before the addition of either 25 μM sodium nitrite or PBS vehicle controls. Vessel rings were collected after 10 min and tissue cGMP levels determined by enzyme-linked immunoanalysis. Results are means ± S.E.M. (n = 3–4). P < 0.0001 as determined by one-way ANOVA, with P-values shown determined by applying Bonferroni’s correction post-test.

Table 1 Effects of nitrite on paO₂, paCO₂ and blood O₂ fractional saturation during haemorrhagic shock and resuscitation

<table>
<thead>
<tr>
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<th>HBOC-201</th>
<th>HBOC-201 + 30 nmol nitrite</th>
<th>P-value (n)</th>
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<tbody>
<tr>
<td>paO₂ (mmHg)</td>
<td>80.2 ± 16.8</td>
<td>80.1 ± 15.3</td>
<td>0.99 (7)</td>
</tr>
<tr>
<td>paCO₂ (mmHg)</td>
<td>43 ± 4.5</td>
<td>47.9 ± 2.4</td>
<td>0.35 (7)</td>
</tr>
<tr>
<td>Oxygen fractional saturation (%)</td>
<td>73.5 ± 5.3</td>
<td>69.3 ± 5.5</td>
<td>0.58 (7)</td>
</tr>
<tr>
<td>Serum HBOC-201 (g/dl)</td>
<td>4.95 ± 0.24</td>
<td>4.71 ± 0.13</td>
<td>0.43 (8)</td>
</tr>
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...shows a bell-shaped dependence on fractional saturation, with the maximum around the haemoglobin p50. This property has been demonstrated with cell-free and erythrocytic hHb [21,23], rabbit [44], murine and most recently adult and fetal sheep haemoglobin [45]. The importance of p50 in nitrite reduction

Figure 6 Effects of nitrite on HBOC-201-induced hypertension during haemorrhagic shock and resuscitation

(A) Changes in MAP expressed as a percentage change from prehaemorrhage levels. The time course is separated into three phases of haemorrhage, shock and resuscitation as described in the Experimental section. Nitrite was administered 15–30 s before HBOC-201, which was administered continuously over 30 min during the resuscitation phase. Nitrite doses indicate amount added. For clarity, only data at select times are shown and represent means ± S.E.M. (n is indicated on the Figure and the values refer to individual mice). * P < 0.0001 by two-way ANOVA for resuscitation phase.

(B) The change in MAP post-resuscitation (averaged from 120 to 124 min) with respect to its prehaemorrhage (0 min) value. Results are means ± S.E.M. (n = 5–17). * P < 0.03 by one-way ANOVA.

(C) The change in MAP post-resuscitation (averaged from 120 to 124 min) with respect to its prehaemorrhage (0 min) value after resuscitation with LR or LR and nitrite administered immediately before the resuscitation phase. Results are means ± S.E.M. (n = 4–7). P-values were calculated by an unpaired t-test. (D) Plasma nitrite levels during haemorrhage, shock and resuscitation with HBOC-201 alone (C) or HBOC-201 and 30 nmol nitrite (D), prehaemorrhage; ii, end of shock phase and immediately before addition of nitrite; iii, 30 min after HBOC-201 or HBOC-201 and nitrite resuscitation; iv, 120 min after completion of resuscitation. Results are means ± S.E.M. (n = 4–6). * P < 0.05 compared with HBOC-201 alone, at the indicated time point, by two-way ANOVA with Bonferroni’s correction post-test. (E) MAP prehaemorrhage (black bars) and 30 min post resuscitation (grey bars). Results show means ± S.E.M. (n = 5–17). P-values were calculated by paired t tests for prehaemorrhage and post-resuscitation levels. Nitrite doses are indicated.
can be explained by considering that the rate is controlled by firstly, the availability of deoxyhaemoglobin for nitrite binding and secondly, by the average intrinsic haem redox potential. A more negative redox potential, associated with R-state haemoglobin, facilitates electron transfer and results in increased rate constants for nitrite reduction. Additional factors have been suggested to control the nitrite reductase activity, including steric factors that regulate haem accessibility for nitrite [46] and mechanisms that regulate how nitrite may enter the RBC [32]. Consistent with the hypothesized faster rates of nitrite reduction by R-state deoxyhaem, fetal haemoglobin, which is more R-state compared with adult haemoglobin, reduces nitrite approx. 2-fold faster than adult haemoglobin [45]. Increasing additions of PEG [poly(ethylene glycol)] chains to cross-linked haemoglobin also increases the rate constant for nitrite reduction in a manner proportional to the R-state character [35].

HBOC-201 represents an interesting haemoglobin with respect to nitrite reduction as it is T-state stabilized, resulting in a higher p50, a therapeutic goal that improves oxygen delivery [47]. Extrapolating from the results shown in Figure 1(D), the rate constant for nitrite reduction by fully deoxygenated HBOC-201 is 0.25 M$^-1$ s$^{-1}$ at 37°C. Reported rate constants [40] for either deoxygenated cell-free hHb or cell-free sheep haemoglobin at 25°C are 0.25 M$^-1$ s$^{-1}$ and 0.28 M$^-1$ s$^{-1}$ respectively. Considering that these numbers were obtained at 25°C, it is reasonable to expect that these reaction rates will be higher at 37°C. As a result we propose that the rate constant for nitrite reduction by HBOC-201 is lower compared with either deoxygenated cell-free hHb or cell-free sheep haemoglobin, consistent with T-state character of HBOC-201. This is further reflected in the dependence of the rate constant and initial rate of nitrite reduction on HBOC-201 O$_2$ fractional saturation. For native haemoglobin the rate constant for nitrite reduction increases with fractional saturation parallelising R-state configuration [21,23]. With HBOC-201 however the rate constant was linear and inversely proportional to the O$_2$ fractional saturation, a relationship that was also observed with the initial rate (Figure 1D). Whereas the increased initial rate is expected based on deoxyhaem concentrations, the mechanistic basis for the observed increase in the rate constant for nitrite reduction with HBOC-201 deoxygenation remains unclear. We have not measured the nitrite reductase kinetics of native bovine haemoglobin precluding comparison of HBOC-201 with the unmodified, and non-T-state stabilized, control. It also should be noted that we cannot exclude the possibility that steric effects, introduced by glutaraldehyde modification, could be responsible. However, the results presented underscore the potential to modulate the nitrite reductase activity of any given HBOC by both controlling deoxyhaem concentrations (p50) and the intrinsic rate constant for nitrite reduction (R- or T-state character).

Importantly, we also observed NO formation from the nitrite–deoxyHBOC-201 reaction (Figures 1E and 1F). Recent insights suggest that nitrite reduction to NO occurs via the intermediate formation of N$_2$O$_3$, secondary to methaem–nitrite reactions with NO [48]. We have not explored this mechanism in the present study as we are focusing specifically on the ability of HBOC-201 to produce NO from nitrite and evaluate the potential of this process in mediating vascular NO-dependent signalling. The latter was assessed by using isolated aortic ring bioassays. We employed an experimental design that allows the testing of how the interplay between nitrite, NO and HBOC-201 at different fractional saturations affect the nitrite-dependent vasodilation. Consistent with our previous study [24], hHb inhibited MNO-dependent vasodilation at all the tested fractional saturations due to rapid haem-based NO scavenging. These observations were repli...
its high nitrite reductase activity). However, we speculate that due to slower nitrite reductase (i.e. it has a lower rate constant for the affinity and/or the intrinsic nitrite reductase activity. Relative to of the next generation of HBOCs.

scavenging and hypertensive effects. These results further support a property that would further limit potential toxicity of nitrite administration.

In summary, we present results that support the use of nitrite as an adjunct therapy to attenuate HBOC-dependent hypertension. We propose that HBOC-mediated nitrite reduction counters NO scavenging and hypertensive effects. These results further support the notion that the effectiveness of using any HBOC as a nitrite reductase in vivo can be affected by either altering the oxygen affinity and/or the intrinsic nitrite reductase activity. Relative to other α-chain cross-linked haemoglobins [35], HBOC-201 is a slower nitrite reductase (i.e. it has a lower rate constant for the nitrite reductase activity). However, we speculate that due to its high p50, sufficient deoxygenation occurs in vivo to sustain significant nitrite reductase activity. These results underscore that the potential flexibility afforded by the ability to influence nitrite reductase activity, by altering intrinsically redox potential and/or oxygen affinity, may be critical in the therapeutic development of the next generation of HBOCs.

AUTHOR CONTRIBUTION
Cilina Rodriguez, Dario Vitturi, Jin He, Marianne Vandromme and Angela Brandon performed the research. Cilina Rodriguez, Dario Vitturi, Marianne Vandromme, Anne Hutchings, Jeffrey Kerby and Rakesh Patel designed the research and analysed data. Cilina Rodriguez, Dario Vitturi, Marianne Vandromme, Loring Rue, III, Jeffrey Kerby and Rakesh Patel wrote the paper.

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REFERENCES


SUPPLEMENTARY ONLINE DATA
Sodium nitrite therapy attenuates the hypertensive effects of HBOC-201 via nitrite reduction

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EXPERIMENTAL

All reagents were purchased from Sigma–Aldrich except MNO and L-NMMA, which were purchased from Axxora Platform. Sodium nitrite was dissolved in PBS, pH 7.4. Indomethacin was dissolved in 100% ethanol, which was then diluted in the vessel relaxation experiments to a final solvent concentration of 0.1% (v/v). Antifoam SE-15 was dissolved in mixtures containing 5% DMSO and added to a final solvent concentration of 0.0067% in the vessel bath. No effects of these solvents were observed on vasoactive responses at these concentrations (results not shown). hHb was purified from healthy volunteers according to the institutional review board-approved protocols as described previously in [38]. Male Sprague–Dawley rats (200–250 g) were purchased from Harlan. HBOC-201 was obtained from Biopure.

Vessel relaxation studies

For all vessel experiments thoracic aorta from male Sprague–Dawley rats were used as described previously in [38]. To assess the effects of O2 on nitrite- or MNO-mediated vasodilation, vessels were preconstricted with 5, 21, 2 or 0% O2 gas mixtures containing 5% CO2 and balanced with N2. Gas was delivered by mass-flow controllers (Sierra Instruments) set to 0.15 l/min, a requirement found to be necessary to achieve reproducible haemoglobin and HBOC fractional saturations between experiments. In all experiments vessels were pretreated with 5 μM indomethacin and 100 μM L-NMMA, to block cyclooxygenase and endothelial NO synthase respectively. Vessels with 5% oxygen were preconstricted to approx. 50–75% of maximal KCl constriction with 100 μM PE. Upon lowering O2 tensions, vessel tone can fluctuate before reaching a stable tone, as indicated previously in [43]. For all experiments reported in the present study nitrite or MNO dose-responses were initiated after reaching a stable contractile tone. Nitrite- or MNO-dependent vasodilation was assessed as described in the Experimental section of the main paper.

Assessment of hHb or hHBOC-201 concentration and redox/ligation state

Aliquots of haemoglobin or HBOC-201 were collected as described previously in [38] using gas-tight syringes and transferred to sealed gas-equilibrated cuvettes. UV-visible spectra of samples were recorded from 450–700 nm and concentrations of hHb or HBOC-201, together with redox and ligation state, were assessed by fitting the measured spectra to the previously acquired base spectra (from oxy-, deoxy-, met-, ferryl-, nitrosyl- and nitrite forms of both HBOC-201 and hHb) using a least squares method as described previously in [26,36–38]. Standard spectra of hHb and HBOC-201 were generated and used for deconvolution of respective haemoglobins.

Preparation of reference spectra for deconvolution

All reference spectra from 450–700 nm were obtained from HBOC-201 stocks prepared in PBS at pH 7.4, unless otherwise specified, and expressed as the corresponding extinction coefficients. OxyHBOC-201 was prepared by equilibrating a stock solution under a 100% O2 stream in a sealed quartz cuvette. DeoxyHBOC-201 was prepared by the addition of excess sodium dithionite to reduce and consume all oxygen in a sealed cuvette. NitrosylHBOC-201 was prepared by adding a 2:1 excess of MNO to the previously prepared deoxyHBOC-201 solution in 0.1M phosphate buffer. Met-HBOC-201 was prepared by the addition of excess potassium ferricyanide to a HBOC-201 stock and metnitriteHBOC-201 was then obtained by the addition of excess sodium nitrite to oxidized HBOC-201. Finally ferryl-HBOC-201 was prepared by treatment of metHBOC-201 with excess H2O2 for 5 min followed by separation in a PD10 Sephadex column (GE Healthcare). Standard spectra for deconvolution of cell-free human haemoglobin were acquired as described previously in [38].

Trauma-haemorrhage and resuscitation model

All experiments were carried out for at least 120 min post-resuscitation and, at the end of the study period, mice were anesthetized by inhalation of 5% isofluorane in air, and were killed by cardiac puncture and cervical dislocation, in accordance with the University of Alabama at Birmingham institutional animal care committee guidelines. For all experiments MAP was continuously recorded using a BPA400 Analyzer (Micro-Med). Arterial blood samples were obtained from the femoral arterial catheter at designated intervals for measurement.

Measurement of NO metabolites

In order to determine plasma nitrite, S-nitrosothiols and N-nitroso compounds, 150 μL of blood was collected at: (i) pre-initiation of haemorrhage; (ii) 90 min after initiation of experiment (i.e. at end of shock period); (iii) 105–110 min after initiation of experiment (corresponding to 20 min post addition of nitrite and onset of

1 We dedicate the present study to the memory of Anne Hutchings, who passed away during the course of the work.
2 These authors contributed equally to this work.
3 Jeffrey Kerby and Rakesh Patel are co-inventors on a National Institutes of Health/University of Alabama at Birmingham provisional patent application for use of nitrite salts with haemoglobin-based oxygen carriers.
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Nitrite-dependent vasodilation was assessed in the absence or presence of either 20 μM HBOC-201 or 20 μM hHb at the indicated oxygen tensions. Lines show the best fit using sigmoidal fitting algorithms and results represent means ± S.E.M. (n = 4–9). In some cases errors are smaller than symbol size. *P < 0.001, †P < 0.01, hHb relative to control. P-values were calculated by two-way repeated measures ANOVA with a Bonferroni correction post-test.

Figure S2 Effects of nitrite or MNO on HBOC-201 and hHb concentrations during vessel bioassay experiments

Aliquots of either HBOC-201 or hHb were taken from vessel bioassay chambers perfused with various oxygen tensions as indicated. Total haem (heme) was determined by UV–visible spectroscopy coupled with spectral deconvolution. Samples were taken both prior to initiation (initial) with (A) nitrite or (B) MNO dose-dependent vasodilation studies and also at the end of the cumulative dose–response (end). Data are presented as percentage haem relative to the respective initial concentration. Initial haem concentrations were 21.6 ± 0.5 μM and 21.2 ± 0.2 μM for the MNO and nitrite experiments respectively. The average time between initial and end sampling for MNO experiments was 28.1 ± 3.4 min (mean ± S.E.M.; n = 9) and for nitrite experiments was 38.1 ± 1.4 min (mean ± S.E.M.; n = 17).

RESULTS

Effects of HBOC-201 and hHb in nitrite-dependent vasodilation

hHb inhibited nitrite-dependent vasodilation at all oxygen tensions that resulted in hHb oxygenation (95, 21 and 2% O₂). This result can be explained by NO scavenging as previously reported [12]. However, when hHb was significantly deoxygenated, with a fractional O₂ saturation of approx. 25%, at 0% O₂ hHb did not affect the nitrite-dependent vasodilation. We have suggested previously [24] that this effect may be explained by deoxyhaemoglobin-mediated nitrite reductase activity resulting in NO generation, which counters the NO scavenging processes. HBOC-201 showed a similar reactivity at O₂ tensions that result in deoxygenation. Surprisingly, however, at 95 and

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Figure S3 Effects of nitrite on methaemoglobin formation during resuscitation with HBOC-201 in a model of trauma-haemorrhage

Methaemoglobin (methHb) levels pre-haemorrhage (black bars) and post-resuscitation (grey bars). Results show means ± S.E.M. (n = 2–7).

21% O₂, where oxygenated HBOC-201 is the predominant ligation state populated, nitrite-dependent vasodilation was not affected (Supplementary Figure S1). It was noted during vasodilation experiments that the concentration of HBOC-201 was decreasing specifically in the presence of nitrite, and that this was particularly observed at higher oxygen tensions. Supplementary Figure S2 quantifies this effect and demonstrates that the HBOC-201 concentration decreases by approximately 40% during the course of nitrite-dependent vasodilation experiments at both 95 and 21% O₂ (Supplementary Figure S2A). This decrease in haem will in turn decrease the rate of both the NO scavenging and the nitrite oxidation kinetics. It also may explain the apparent lack of inhibition of nitrite-dependent vasodilation by oxyHBOC-201. Supplementary Figure S2(A) also shows that no loss of hHb is observed during these studies. Supplementary Figure S2(B) shows that no loss of HBOC-201 occurs during MNO-vasodilation experiments.