Normalization of hemoglobin-based oxygen carrier-201 induced vasoconstriction: targeting nitric oxide and endothelin

Taverne YJ, de Wijs-Meijler D, Te Lintel Hekkert M,
Moon-Massat PF, Dubé GP, Duncker DJ, Merkus D.

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ABSTRACT

Hemoglobin-based oxygen carrier (HBOC)-201 is a cell-free modified hemoglobin solution potentially facilitating oxygen uptake and delivery in cardiovascular disorders and hemorrhagic shock. Clinical use has been hampered by vasoconstriction in the systemic and pulmonary beds. Therefore, we aimed to 1) determine the possibility of counteracting HBOC-201-induced pressor effects with either adenosine (ADO) or nitroglycerin (NTG); 2) assess the potential roles of nitric oxide (NO) scavenging, reactive oxygen species (ROS), and endothelin (ET) in mediating the observed vasoconstriction, and 3) compare these effects in resting and exercising swine. Chronically instrumented swine were studied during rest and during exercise after administration of HBOC-201 alone or in combination with ADO. The role of NO was assessed by supplementation with NTG or administration of the eNOS inhibitor N^{ω}-nitro-L-arginine. Alternative vasoactive pathways were determined via intravenous administration of the ET_A/ET_B receptor blocker tezosentan or a mixture of ROS scavengers. The systemic and to a lesser extent the pulmonary pressor effects of HBOC-201 could be counteracted by ADO; however, dosage titration was very important to avoid systemic hypotension. Similarly, supplementation of NO with NTG negated the pressor effects but also required titration of the dose. The pressor response to HBOC-201 was reduced after eNOS inhibition and abolished by simultaneous ET_A/ET_B receptor blockade, while ROS scavenging had no effect. In conclusion, the pressor response to HBOC-201 is mediated by vasoconstriction due to NO scavenging and production of ET. Further research should explore the effect of longer-acting ET receptor blockers to counteract the side effect of hemoglobin-based oxygen carriers.

New & noteworthy

Hemoglobin-based oxygen carrier (HBOC)-201 can disrupt hemodynamic homeostasis, mimicking some aspects of endothelial dysfunction, resulting in elevated systemic and pulmonary blood pressures. HBOC-201-induced vasoconstriction is mediated by scavenging nitric oxide (NO) and by upregulating endothelin (ET) production. Pressor effects can be prevented by adjuvant treatment with NO donors or direct vasodilators, such as nitroglycerin or adenosine, but dosages must be carefully monitored to avoid hypotension. However, hemodynamic normalization is more easily achieved via administration of an ET receptor blocker.

INTRODUCTION

Hemoglobin-based oxygen carrier (HBOC)-201 is a cell- and endotoxin-free, glutaraldehyde-polymerized hemoglobin solution produced by chemical modification of hemoglobin extracted from isolated bovine red blood cells.[1] HBOCs may be used in the treatment of cardiovascular disorders, and hemorrhagic shock, in particular; however, side effects include systemic and pulmonary blood pressure elevations, plasma volume expansion, lower cardiac output and reduction in heart rate.[2,3] Despite these potentially unfavorable effects, studies in human subjects with documented coronary disease showed that HBOC-201 had no effect on left ventricular (LV) stroke work index or any of the measured coronary function parameters.[2]

The most important HBOC side-effect is systemic and pulmonary vasoconstriction. Consequently, this study first aimed to determine the possibility of reversing HBOC-201 pressor effects via simultaneous administration of adenosine (ADO), a nitric oxide (NO)-independent vasodilator, or the NO-donor nitroglycerin (NTG). The pressor effect of HBOC has been ascribed to scavenging of NO, an important endogenous vascular relaxing factor.[4-9] Free hemoglobin (Hb) undergoes rapid ($\sim 10^7$ $M^{-1}s^{-1}$)[10,11] and irreversible reaction with NO to form metHb, where Hb kinetically behaves as a dioxygenase enzyme.[12,13] In the following slower processes, iron-NO complexes are formed that may further deplete NO concentrations.[4] However, although disruption of the NO-mediated cascade may be an important contributor to transient systemic and pulmonary hypertension, it is not the only possible pathway.

Oversupplying oxygen (O_2) can stimulate vasoconstriction, to protect against the oxygen burst, but can also stimulate reactive oxygen species (ROS) formation that may result in further scavenging of NO.[14,15] By scavenging NO, conversion from pro-endothelin to endothelin (ET) is no longer inhibited, thereby increasing the release of this vasoconstrictor.[2,16,17] Also, free radicals generated by the auto-oxidation of hemoglobin may contribute to the enhanced release of ET.[18] Therefore, the second aim of this study was to address the potential roles of nitric oxide scavenging, ROS, and/or endothelin in the HBOC-201 systemic and pulmonary pressor effects.

NO has been shown to contribute to exercise-induced vasodilation in skeletal muscle, the heart, as well as the pulmonary vasculature in many[19-21], but not all studies.[22] A state of decreased NO and increased ROS and ET production resembles some aspects of endothelial dysfunction, a phenomenon that may have exaggerated effects during exercise. Hence, the third aim of this study compared the effects of HBOC-201 in resting and exercising swine.

METHODS

Animals

Studies were performed in accordance with the Council of Europe Convention (ETS123)/ Directive (86/609/EEC) for the protection of vertebrate animals used for experimental and other scientific purposes, and with approval of the Animal Care Committee at Erasmus MC, University Medical Centre Rotterdam. A total of 16 Yorkshire × Landrace swine (2–3 months old, 22 ± 1 kg at the time of surgery) of either sex (11 female and 5 male) entered the study. After completing all experimental protocols, animals were euthanized by an intravenous overdose of pentobarbitone sodium.

Surgical procedures and experimental protocol

Detailed surgical procedures are previously described.[23,24] In brief, under deep anesthesia, a thoracotomy was performed in the fourth left intercostal space. Fluid-filled catheters were placed in the aorta, pulmonary artery, left atrium and LV for measurement of pressure, infusion of drugs and blood sampling. In addition, a flow probe (Transonic Systems) was placed around the ascending aorta for measurement of cardiac output. All catheters were exteriorized at the back of the animal and filled with heparinized saline. The thorax was closed in layers, and the animal was allowed to recover for at least one week. Antibiotic prophylaxis (amoxicillin 25 mg/kg iv) was provided for 5-7 days starting immediately before surgery. Immediate postoperative analgesia was provided by buprenorphine (0.015mg/kg im), while a slow-release fentanyl patch (12 μg/h) maintained postoperative analgesia for 72 h. Studies were performed 1–3 wk after surgery, with animals resting and exercising on a motor-driven treadmill up to 85–90% of maximal heart rate. Three main protocols (as described below) were performed on different days and in random order. All chemicals were obtained from Sigma, and HBOC-201 (13 g/dl) was obtained from OPK Biotech.

Hemodynamic effects and reproducibility of HBOC-201 infusion

With swine lying on the treadmill, resting hemodynamic measurements consisting of heart rate (HR), LV pressure, first derivative of LV pressure (dP/d*t*), mean aortic pressure (MAP), pulmonary artery pressure (PAP), left atrial pressure, and cardiac output were obtained. Subsequently, swine were subjected to a five-stage exercise protocol (1–5 km/h) while hemodynamic variables were continuously recorded, and blood samples collected during the last 60 s of each 3-min exercise stage at a time when hemodynamics had reached a steady state. Blood samples were used for determination of Hb, oxygen content, and lactate using an automated blood gas analyzer (ABL210, Radiometer). After the exercise protocol was completed, animals were allowed to rest on the treadmill for 90 min, after which HBOC-201 (10 ml/kg iv) was infused over a period of 30 min. At the end of infusion, the exercise protocol was repeated. We have previously

shown excellent reproducibility of the hemodynamic response in consecutive bouts of exercise.[19,25]

Also, in three pigs, we assessed the reproducibility of the hemodynamic responses to HBOC-201 infusion by administration of three separate doses of HBOC-201 (10 ml/kg iv), separated by 5 ±1 days.

Reversal of pressor effect of HBOC-201 by nitroglycerin and adenosine

After performing a control run, six animals received HBOC-201 combined with the vasodilator ADO. Administration of ADO was started 10 min after the start of HBOC-201 infusion and continued till the end of the second run. The infusion rate of ADO (25 mg/ml) was titrated to obtain a stable MAP similar to that before HBOC administration.

Role of NO

To determine the involvement of NO in HBOC-201-induced hypertension, in six swine, the NO donor nitroglycerin (NTG) was infused starting 10 min after the start of HBOC-201 infusion. To prevent a direct interaction between the NO donor and HBOC-201, HBOC-201 and NTG were infused through separate catheters. The infusion rate of NTG (1 mg/ml) was titrated to obtain a stable MAP similar to that before HBOC administration.

To further investigate the role of endogenous NO, NO production was inhibited using the NO synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA, 20 mg/kg iv) in five swine.[19] After administration of L-NNA, swine underwent an L-NNA exercise trial. Ninety minutes later, HBOC-201 (10 ml/kg iv) was given to the animals, and they underwent a second exercise trial. As previously shown[26], L-NNA has a long-lasting effect so no additional L-NNA was administered before the second exercise protocol.

Other vasoactive pathways

Loss of NO reduces ROS scavenging and may increase the production of the potent vasoconstrictor ET. To determine the involvement of ET and ROS in HBOC-201-induced hypertension, HBOC-201 was infused after prior administration of an ET-receptor antagonist or a cocktail of ROS scavengers.

ET receptor blockade

After completing a control exercise protocol, animals were allowed to rest on the treadmill for 90 min. Then, the mixed ET_A and ET_B receptor (ET_A/ET_B) antagonist tezosentan was intravenously administered over 10 min in a dose of 3 mg/kg iv (slow bolus), followed by a continuous infusion of 6 $mg{\cdot}kg^{-1}{\cdot}h^{-1}$ iv in four swine.[24] HBOC-201 (10 ml/kg iv) was started upon completion of the tezosentan slow bolus. When HBOC-201 infusion was completed the exercise protocol was repeated.

ROS scavengers

We used a mixture of different substances to scavenge all ROS during HBOC administration. The mixture consisted of *N*-acetylcysteine (NAC, 150mg/kg iv), 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (Tempol, 30 mg/kg iv), and mercaptopropionyl glycine (MPG, 1mg/kg iv).[15]

NAC is an aminothiol and synthetic precursor of intracellular cysteine and glutathione and is thus considered an important antioxidant.[27] It is generally assumed that the antioxidant and free radical scavenging activities of NAC are attributable to increasing intracellular glutathione levels; however, NAC also possesses a reducing property through its thiol-disulfide exchange activity.[27,28] Tempol is a stable piperidine nitroxide and scavenges superoxide anions in vitro and may act as a SOD mimetic.[29,30] Tempol also reduces the formation of hydroxyl radicals either by scavenging superoxide anions or hydroxyl radicals (via the Fenton or Haber-Weiss reactions).[3,29] N-2-mercaptopropionylglycine (MPG) is a synthetic thiol compound which is not highly radical specific and scavenges different types of ROS, including $O_2^{\cdot-}$, $ONOO^-$ and OH.[31,32]

After completing a control exercise protocol, the animals were allowed to rest on the treadmill for 90 min. Then, the scavenger mixture was administered in five swine, starting 10 minutes before the HBOC-201 infusion. The administration of NAC and Tempol was completed before administration of HBOC-201, while MPG-infusion continued throughout HBOC-201 administration and the subsequent exercise protocol.

Data analysis and statistical analysis

Digital recording and offline analysis of hemodynamic variables have been described in detail elsewhere.[33-35] Systemic vascular resistance (SVR) was computed as mean aortic blood pressure divided by cardiac output. Pulmonary vascular resistance (PVR) was computed as mean pulmonary arterial pressure minus mean left atrial pressure divided by cardiac output. Body lactate production/consumption was calculated as the product of cardiac output and arterio-mixed venous lactate difference.

Statistical analysis of hemodynamic data was performed with SPSS 22 (IBM, Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY). Since no differences between male and female swine were found in the response to HBOC-201 administration alone, data from both sexes were pooled. The effects of drug treatment and exercise were compared using a two-way (ANOVA for repeated measures. When significant effects were detected, post hoc testing was performed using paired or unpaired t-test, with Bonferroni correction. Statistical significance was accepted when $P \leq 0.05$. Data are presented as mean ± SE.

RESULTS

Hemodynamic Effects and Reproducibility of HBOC-201 administration.

Administration of HBOC resulted in significant pressor effects in the systemic and pulmonary circulations with an increase in MAP (27±3 mmHg) and PAP (14±1 mmHg). These pressor responses were accompanied by a probably baroreflex-mediated decrease in HR, which together with a slight decrease in stroke volume, resulted in a decrease in cardiac output (Table 1). These pressor effects were the result of significant systemic and pulmonary vasoconstriction, as evidenced by significant increases in SVR and PVR. There was no sign of anaerobic metabolism, as arterial and mixed venous lactate levels and body lactate consumption (not shown) were maintained. The hemodynamic responses to HBOC-201 administration occurred during the first 10 min of HBOC-201 administration after which they stabilized. Moreover, a second and third HBOC administration with 5-7 days washout in between yielded hemodynamic responses similar to the first administration (Figure 1).

Table 1. Hemodynamics effecfs of HBOC-201 at rest and during exercise.

		Rest	Exercise level (km/h)				
	Treatment	Lying	1	2	3	4	5
Systemic hemodynamics							
HR (bpm)	Control	124 ± 5	170 ± 9*	177 ± 9*	188 ± 8*	218 ± 10*	244 ± 10*
	HBOC-201	100 ± 3†	138 ± 5†*	148 ± 6†*	161 ± 5†*	192 ± 7*	221 ± 8*
MAP (mmHg)	Control	89 ± 3	83 ± 3	83 ± 3	83 ± 2	84 ± 2	87 ± 3
	HBOC-201	113 ± 3†	105 ± 3†	105 ± 2†*	103 ± 2†*	104 ± 2†*	104 ± 2†*
SV (ml/beat)	Control	38 ± 2	43 ± 2*	43 ± 2	43 ± 2	40 ± 2	39 ± 2
	HBOC-201	42 ± 2	46 ± 2	44 ± 2	44 ± 2	40 ± 2	40 ± 2
CO (l/min)	Control	4.7 ± 0.2	7.4 ± 0.3*	7.6 ± 0.3*	8.1 ± 0.2*	8.8 ± 0.3*	9.8 ± 0.3*
	HBOC-201	4.3 ± 0.2	6.4 ± 0.2†*	6.7 ± 0.2†*	7.2 ± 0.2†*	8.2 ± 0.2*	9.0 ± 0.3*
SVR (l/min.mmHg^{-1})	Control	19 ± 1	11 ± 0*	11 ± 0*	10 ± 0*	10 ± 0*	9.0 ± 0*
	HBOC-201	27 ± 1†	17 ± 1†*	16 ± 1†*	15 ± 1†*	13 ± 1†*	12 ± 0†*
Pulmonary hemodynamics							
MPAP (mmHg)	Control	14 ± 1	21 ± 2*	21 ± 1*	23 ± 1*	28 ± 1*	31 ± 1*
	HBOC-201	26 ± 2†	32 ± 2†	34 ± 3†*	35 ± 2†*	39 ± 2†*	42 ± 2†*
LAP (mmHg)	Control	1.5 ± 1	2.4 ± 1	3.3 ± 0.6	4.9 ± 0.6*	7.7 ± 1*	8.3 ± 1*
	HBOC-201	9.8 ± 1.7†	7.7 ± 1.1†	7.0 ± 1†	8.1 ± 1.1†	9.0 ± 0.7	9.6 ± 1
PVR (l/min.mmHg^{-1})	Control	2.7 ± 0.2	2.5 ± 0.2	2.4 ± 0.2	2.3 ± 0.2	2.3 ± 0.2	2.4 ± 0.2
	HBOC-201	3.8 ± 0.4†	4.1 ± 0.4†	4.3 ± 0.5†	4.1 ± 0.4†	4.0 ± 0.4†	3.8 ± 0.4†

Data are means ± SE. HR, Heart rate; MAP, mean arterial pressure; SV, stroke volume; CO, cardiac output; SVR, systemic vascular resistance; MPAP, mean pulmonary artery pressure; LAP, left atrial pressure; PVR, pulmonary vascular resistance; HBOC-201, hemoglobin-based oxygen carrier 201. *P ≤ 0.05 vs. $Rest_{Lying}$, † P ≤ 0.05 vs. Control.

Figure 1. Systemic and pulmonary hemodynamics at rest and during exercise following administration of hemoglobin-based oxygen carrier (HBOC)-201 alone (n = 3 pigs, left), demonstrating reproducibility of 3 separate administrations of HBOC-201, and in combination with infusion of adenosine (ADO) (n = 6 pigs in full crossover study design, right). * $P \leq 0.05$, ** $P \leq 0.10$, compared with control; † $P \leq 0.05$, compared with HBOC-201 alone; ‡ $P \leq 0.05$, effect of HBOC+ Ado different from HBOC-201 alone.

The pressor response to HBOC-201 was maintained during exercise (Figure 1). In the systemic circulation, both MAP and SVR remained elevated throughout the exercise protocol as compared to control, although the elevation of SVR tended to wane with incremental levels of exercise. Similarly, in the pulmonary circulation both PAP and PVR remained elevated at all exercise intensities.

Reversal of pressor effect of HBOC-201 by adenosine in the systemic and pulmonary vasculature.

Coinfusion of ADO was carefully titrated to maintain MAP at a level similar to MAP before HBOC-201 infusion (Figure 1). Dosages required to stabilize MAP fluctuated throughout the experiment, but they were on average 0.17±0.01 mg/kg/min (range between 0.08 and 0.38 mg/kg/min). Although the HBOC-201-induced changes in SVR and PVR were abolished by ADO (Figure 1), PAP tended to remain slightly higher (P=0.08) due to a slight increase in left atrial pressure (not shown). MAP increased by ~15 mmHg upon cessation of the ADO (not shown).

Role of NO

The exogenous administration of NO, by coinfusion of the NO-donor NTG, was also titrated to counteract systemic pressor responses to HBOC-201. The dose of NTG required to stabilize MAP increased from 0.11±0.01 mg/kg/min at 20 min of HBOC-201 infusion to 0.22±0.06 mg/kg/min upon completion of HBOC-201 infusion (P=0.05) and remained essentially unchanged during the exercise protocol, being 0.16 mg/kg/min at maximal exercise (range from 0.06 to 0.49 mg/kg/min). This dose of NTG negated the HBOC-201-induced increase in SVR as well as PVR and, thereby, the elevated pressures in these vascular beds (Figure 2). Similar to ADO, MAP increased upon cessation of the NTG-infusion (not shown).

Endothelial NOS (eNOS) blockade with L-NNA resulted in peripheral vasoconstriction, as evidenced by a significant increase in SVR and an increase in MAP. The increase in MAP was accompanied by increases in LV systolic pressure, as well as left atrial pressure, and probably, by a baroreflex-mediated decrease in HR and CO, as stroke volume was not altered (Figure 2). However, subsequent infusion of HBOC-201 did not result in a further increase in MAP or SVR. In contrast to the findings in the systemic vasculature, HBOC-201 induced an increase in mean PAP and PVR even in the presence of L-NNA (Figure 2). Thus, HBOC-201 induced further pulmonary vasoconstriction following the vasoconstriction induced by L-NNA, both at rest and during exercise, suggesting that, in addition to scavenging of NO, HBCO-201 exerts its vasoconstrictor effect through another pathway in pulmonary vasculature. Of note, when HBOC-201 and L-NNA were coinfused, systemic pressor responses appeared to be increased as compared to the effect of HBOC-201 alone (Figure 2), indicating that not all NO is scavenged by HBOC-201.

Figure 2. Systemic and pulmonary hemodynamics at rest and during exercise following administration of the nitric oxide (NO)-donor nitroglycerin (NTG; left) or the eNOS inhibitor N^{ω}-nitro-L-arginine (L-NNA; right) in combination with HBOC-201. * P ≤ 0.05, compared with control; † P ≤ 0.05, compared with HBOC-201 alone; ‡ P ≤ 0.05, effect of HBOC+ NTG different from HBOC-201 alone; § P ≤ 0.05, §§ P ≤ 0.1 compared with L-NNA alone; n = 6 pigs (NTG) or 5 pigs (L-NNA) in a full cross-over study design. For the sake of clarity, statistics comparing HBOC-201 with control are not shown, but they are identical to Figure 1.

Other vasoactive pathways

Administration of the mixed ET_A and ET_B receptor antagonist tezosentan reduced MAP by 10±4 mmHg ($P<0.05$), and negated the systemic hypertension caused by subsequent HBOC-201 by preventing the increase in SVR (Figure 3). Also, in the pulmonary vasculature, HBOC-201 had no effect on either pulmonary pressure or PVR, in the presence of tezosentan (Figure 3). These data suggest that activation of the endothelin system is an important contributor in the vasoconstrictor response to HBOC-201.

ROS scavenging in itself had no significant effect on MAP (ΔMAP 12±8 mmHg, $P=0.21$). Coinfusion of ROS scavengers with HBOC-201 slightly reduced the effect of HBOC-201 on mean arterial pressure but did not significantly affect SVR (Figure 3). Similarly, in the pulmonary vascular bed, no reduction of pressor effects could be detected at rest, and the effects of HBOC-201 tended to be exacerbated during exercise following administration of the ROS scavenger cocktail (Figure 3).

DISCUSSION

In the present study we report, in accordance with previous publications[9,36-40], that intravenous administration of HBOC-201 resulted in systemic and pulmonary hypertension as a result of vasoconstriction, which was maintained during exercise. Pressor responses could be prevented by coinfusion of NTG or ADO both at rest and during exercise; however, this required careful titration of the dosage of these vasodilators. eNOS inhibition prevented HBOC-201-induced increase in systemic vasoconstriction, and it reduced but did not abolish HBOC-201-induced pulmonary vasoconstriction. ET_A/ET_B blockade with tezosentan prevented the HBOC-201–induced pressor responses in the systemic and pulmonary vasculature, while ROS-scavenging tended to blunt the pressor response in the systemic but not pulmonary vasculature.

Abolishing pressor effects

The main hemodynamic effects of HBOC-201 occurred during the first 10 min of its administration and were maintained throughout the entire infusion and after infusion. Repeated administration of HBOC-201, following complete washout, induced virtually identical effects, corroborating results from ECMO priming with HBOC-201 in piglets[41,42] and indicating that no immune reaction occurred in response to the protein and that repeated administration is safe. Also, plasma clearance of HBOC-201, which has been shown to follow first-order pharmacokinetics with an elimination half time of 20 h, for either single or multiple dosage regimens[1], was comparable with previous studies.[1,43] As anticipated, HBOC-201 produced an increase in arterial Hb, metHb and plasma metHb (Table 2); however, the level of metHb in this study was well below toxic levels[44], and coinfusion of NTG with HBOC did not elevate metHb levels further (not shown).

Figure 3. Systemic and pulmonary hemodynamics at rest and during exercise following administration of the endothelin ET_A/ET_B- receptor blocker Tezosentan (n = 4 pigs in full cross-over study design; left) or a reactive oxygen species (ROS) scavenger cocktail comprised of N-acetylcysteine (NAC), Tempol, and mercaptopropionyl glycine (MPG; n = 5 pigs in full cross-over study design; right) in combination with HBOC-201. * $P \leq 0.05$, compared with control; † $P \leq 0.05$, compared with HBOC-201 alone; ‡ $P \leq 0.05$, effect of HBOC+ Tezo or ROS different from HBOC-201 alone. For the sake of clarity, statistics comparing HBOC-201 with control are not shown, but they are identical to Figure 1.

Table 2. Effects of HBOC-201 on blood gas values at rest and during exercise

		Rest	Exercise level (km/h)				
	Treatment	Lying	1	2	3	4	5
Arterial							
Hemoglobin (g%)	Control	8.4 ± 0.2	8.7 ± 0.2	8.8 ± 0.2	8.9 ± 0.2	9.0 ± 0.2	9.3 ± 0.2*
	HBOC-201	9 ± 0.2	9.3 ± 0.1†	9.5 ± 0.2	9.6 ± 0.2†*	10.1 ± 0.2†*	10.4 ± 0.2†*
Met-hemoglobin (%)	Control	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0	0.3 ± 0	0.3 ± 0.1	0.3 ± 0.1
	HBOC-201	1.1 ± 0.1†	1.2 ± 0.1†	1.2 ± 0.1†	1.2 ± 0.1†	1.2 ± 0.1†*	1.0 ± 0.1†
Plasma hemoglobin (g%)	Control	0.02 ± 0	0.01 ± 0	0.02 ± 0	0.02 ± 0	0.02 ± 0	0.02 ± 0
	HBOC-201	2.06 ± 0.05†	2.08 ± 0.04†	2.04 ± 0.05†	2.03 ± 0.05†	2.03 ± 0.05	2.05 ± 0.04†
SaO_2 (%)	Control	97 ± 0.3	95 ± 1	98 ± 1	98 ± 1	96 ± 1	94 ± 1*
	HBOC-201	91 ± 0.4	90 ± 1†	90 ± 1†	90 ± 1†	90 ± 1†	90 ± 1†
O_2 Hb (%)	Control	96 ± 0.3	94 ± 1	95 ± 0.5	95 ± 1	95 ± 1	94 ± 1*
	HBOC-201	89 ± 0.4†	88 ± 1†	89 ± 1†	89 ± 1†	89 ± 1†	88 ± 1†
pO_2 (mmHg)	Control	102 ± 2	94 ± 3*	97 ± 2	96 ± 3	94 ± 2	90 ± 3*
	HBOC-201	102 ± 2	94 ± 3*	98 ± 3	94 ± 3	92 ± 3*	90 ± 3*
pCO_2 (mmHg)	Control	42 ± 1	41 ± 1	40 ± 1	40 ± 1	39 ± 1	38 ± 1*
	HBOC-201	43 ± 1	41 ± 1	41 ± 1	43 ± 3	39 ± 1*	38 ± 1*
pH	Control	7.44 ± 0.04	7.46 ± 0.01	7.47 ± 0*	7.47 ± 0.01*	7.48 ± 0.01*	7.48 ± 0.01*
	HBOC-201	7.45 ± 0.01	7.46 ± 0.01	7.46 ± 0	7.46 ± 0.01	7.47 ± 0.01*	7.47 ± 0.01
Lactate (mmol/L)	Control	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	1.9 ± 0.2*
	HBOC-201	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	2.2 ± 0.4*
Mixed venous							
SaO_2 (%)	Control	50 ± 1	39 ± 1*	37 ± 1*	37 ± 1*	33 ± 1*	26 ± 2*
	HBOC-201	39 ± 2†	30 ± 2†*	29 ± 2†*	29 ± 2†*	25 ± 2†*	33 ± 4
pO_2 (mmHg)	Control	42 ± 1	37 ± 1	36 ± 1*	36 ± 1*	33 ± 0.5	31 ± 1*
	HBOC-201	38 ± 1†	33 ± 1†	33 ± 1†*	33 ± 1†*	30 ± 1†	35 ± 2
pCO_2 (mmHg)	Control	51 ± 1	50 ± 2	52 ± 1	51 ± 1	51 ± 1	51 ± 1
	HBOC-201	53 ± 1	54 ± 1	54 ± 1	52 ± 1	53 ± 2	52 ± 2
pH	Control	7.36 ± 0.01	7.36 ± 0.01	7.38 ± 0.01	7.37 ± 0	7.38 ± 0.01	7.35 ± 0
	HBOC-201	7.35 ± 0.01	7.35 ± 0.01	7.35 ± 0.01	7.35 ± 0.01	7.35 ± 0.01	7.34 ± 0.01
Lactate (mmol/L)	Control	1.0 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1.6 ± 0.2*
	HBOC-201	0.9 ± 0	0.9 ± 0	0.9 ± 0.1	0.8 ± 0	1.1 ± 0.1	1.9 ± 0.4*

Data are means ± SE; n = 11. SaO_2, oxygen saturation; O_2 Hb, fraction of oxyhemoglobin in total hemoglobin; Po_2, O_2 tension; Pco_2, CO_2 tension. *P ≤ 0.05 vs. $Rest_{lying}$; † P ≤ 0.05 vs. control.

The pressor effect of HBOC has been ascribed to scavenging NO, primarily by plasma ferrous heme, thereby lowering NO concentration.[38] Our results support previous findings that NTG is capable of negating HBOC-201-induced vasoconstriction and the accompanying increases in systemic and pulmonary blood pressures.[45] NTG reduces vascular resistance in small and large vessels through endothelium-independent, but NO-mediated, vasodilation[46] However, earlier studies were skeptical using NTG coadministration as a therapeutic option to negate the pressor effect of HBOC due to its short half-life, requiring continuous infusion. Moreover, profound systemic vasodilation and hypotension might occur in response to NTG, potentially jeopardizing resuscitation from hemorrhagic shock.[45,47] In the present study, we avoided NTG-induced hypotension through careful NTG titration to maintain MAP within physiological limits. Importantly, the NTG dosage required to normalize systemic pressures was also capable of normalizing pulmonary pressures.

NTG was compared to ADO, a purine nucleoside and NO-independent vasodilator.[24,48] At ADO infusion rates that eliminated HBOC-induced systemic hypertension, the pulmonary pressor effect was not fully eliminated, despite restoration of normal PVR. The persistence of pressor effect was likely due to elevated left atrial pressure, secondary to adenosine-induced negative cardiac inotropy.[49]

Indirect oxidation of hemoglobin involves a process of cooxidation in which the methemoglobin-forming agent is cooxidized with heme iron by hemoglobin-bound oxygen (HbO_2).[50] $O_2^{\cdot-}$ and H_2O_2 are produced when HbO_2 accepts electrons from ferrous heme and the methemoglobin-forming agent. However, ROS scavenging only marginally influence the pressor response to HBOC-201 in the systemic vasculature, while the pulmonary pressor response was unaffected, suggesting that ROS do not play a major role in the pressor effect of HBOC-201. Alternatively, it is possible that oxidative stress indirectly modulates vascular tone. Indeed, it has been shown that oxidative stress enhances ET production through stabilization of prepro-endothelin mRNA.[51-53] In the present study, the ET-receptor blocker tezosentan was capable of negating the pressor responses of HBOC-201 in both the systemic and pulmonary vasculature. Therefore, it is plausible that pressor effects of HBOC-201 may result from disinhibition of endothelin synthesis and release.[54,55] ET is a potent and long-lasting vasoconstrictor and ET-receptor blockade could, therefore, potentially provide another strategy to oppose HBOC-201-induced vasoconstriction. Although tezosentan is a relatively short-acting receptor blocker with a half-life of 3 h[56], longer-acting ET-receptor blockers are available. ET-receptor blockade would require less patient monitoring and have a lower risk of inducing hypotension.

Effects of HBOC-201 during exercise

To our knowledge, this is the first study to analyze the effect of a cell-free oxygen carrier on exercise hyperemia. In the normal healthy vasculature, exercise-induced vasodilation is regulated via an intricate interplay of vasoactive molecules, including NO, ROS, and endo-

thelin.[19,20,57] As outlined above, HBOC-201 could potentially scavenge NO and enhance production of ROS and ET and could, therefore, interfere with exercise-induced vasodilation. However, although the pressor responses of HBOC-201 were essentially unaffected by exercise, SVR did decrease during exercise following administration of HBOC-201, indicating that exercise-induced vasodilation is essentially intact.

To assess the role of NO in HBOC-201 pressor effects at rest and during exercise, HBOC administration was repeated following eNOS-inhibition. If indeed, scavenging of NO is the main contributor to the pressor effect of HBOC-201, eNOS inhibition would be expected to block the pressor effect by HBOC-201. Indeed, in accordance with previous studies[39,58], following inhibition of eNOS and the consequent vasoconstriction, no additional vasoconstriction was induced by HBOC-201 infusion in the systemic vasculature. In contrast, HBOC-201 did result in a further increase in PVR. It is not clear why the pulmonary and systemic vasculature responded differently to the combination of L-NNA and HBOC-201. However, it is possible that L-NNA did not completely inhibit eNOS in the pulmonary circulation, although this is unlikely given the high dose (20 mg/kg iv) of L-NNA administered. An alternative explanation for the divergent effects in the systemic and pulmonary vasculature is that it has been shown that the nature of the chemical interaction between NO and Hb is dependent on the amount of oxygen present. Formation of $Fe^{II}NO$Hb occurs principally when Hb is deoxygenated (T-state) in peripheral tissue. NO bound to the heme-group can be transferred to a specific cystein residue (β93Cys) upon reoxygenation of Hb in the lung, resulting in formation of SNO-Hb.[59,60] This SNO-Hb formation can also occur directly but only when Hb is oxygenated (R-state). From SNO-Hb, NO can be either released or transferred to another thiol group, thereby preserving part of NO signalling.[61,62] Thus, S-nitrosylation of Hb is governed, in part, by the state of the Hb molecule undergoing an allosteric shift from R to T shift during passage in the circulatory system.[63] These varying degrees of Hb S-nitrosylation at different molecular states (R and T) may explain, at least in part, the different hemodynamic responses to HBOC-201 in the systemic and pulmonary vasculature following NOS inhibition by L-NNA. In peripheral micro vessels, because of low oxygen tension, SNO-Hb levels are low and NO released from SNO-Hb may be consumed by biological targets, such as those mediated by GSNO reductase.[62] Consequently, the availability of bioactive NO may be closely coupled to de novo synthesis by NOS that is inhabitable by L-NNA, leaving little residual NO for scavenging by HBOC-201. By contrast, bioactive NO in the form of SNO-Hb is abundant in lungs and well protected inside erythrocytes but, upon release, is susceptible to scavenging by free Hb, manifesting as a further increase in PVR following eNOS inhibition.

A third explanation may be that the vasoconstrictor effect of HBOC-201 is not solely mediated through scavenging of NO. A ROS scavenging cocktail failed to appreciably alter hemodynamic responses to HBOC-201 and, unlike either NTG or ADO, failed to restore SVR or PVR to control levels. As glutaraldehyde-polymerized HBOC in itself was shown

to exhibit catalase-like properties, it is possible that the increase in free radicals induced by administration of this HBOC was negated by HBOC itself.[64] Indeed, lipid peroxidation as measured by thiobarbituric acid reactive substances was not significantly elevated by glutaraldehyde-polymerized HBOC. Similar to our study, these observations suggest that HBOC-201 fails to significantly stimulate ROS formation or that any HBOC-induced increase in ROS is adequately scavenged by endogenous antioxidants. However, it is possible that in certain disease states generally characterized by elevated oxidative stress[26], such as reperfusion following myocardial infarction or in the presence of severe endothelial dysfunction, HBOC may exacerbate oxidative stress, either directly or through scavenging NO. In vitro studies have suggested that HBOCs may amplify ROS formation that could, in turn, react with NO to generate nitroxide radicals[15] and/or uncouple NO synthase secondary to insufficient cofactors tetrahydrobiopterin (BH4) and NADPH required to convert L-arginine to L-citrulline and NO.[65]

The pattern of SVR and PVR during exercise with HBOC-201 very much resembles the pattern found with ET antagonism, as we previously showed that the vasodilator effect of ET-receptor blockade waned with increasing exercise intensity in the systemic circulation while it increased in the pulmonary vasculature.[35] Moreover, reduced bioavailability of NO and/or oxidative stress could contribute to overexpression of ET.[16] Although we did not measure plasma ET-levels in the present study, an increase in ET-mediated vasoconstriction as a cause of the pressor effects of HBOC-201 is consistent with the ability of tezosentan to negate these pressor effects both at rest and during exercise. Importantly, dosing of the ET-receptor blocker tezosentan did not need to be altered during exercise, and endothelin-antagonists by themselves have only very modest effects on hemodynamics, making endothelin-antagonists clinically attractive antagonists of the pressor effect of HBOC-201.

Finally, several clinical trials have shown that other HBOC products with other compositions than HBOC-201 may increase the risk of myocardial infarction and death.[66] However, clinical studies conducted with HBOC-201 in patients with documented cardiovascular disease showed both intravenous and intracoronary infusion of HBOC-201 to be safe and well tolerated.[2]

Methodological Considerations

Although plasma ET-1 measurements could possibly strengthen the conclusion that HBOC-201 induced vasoconstriction is ET-mediated, a number of both physiological and methodological issues complicate interpretation of such measurements. First, ET is released for more than 80% into the abluminal side, while less than 20% is secreted into the lumen side. Hence circulating ET-1 does not reflect the local concentration of ET-1 in the vessel wall. Second, an ET-mediated pressor effect may be caused by changes in ET-receptor sensitivity through altered nitrosylation of the ET-receptors. Finally, we have previously found that L-NNA and indomethacin yield a false-positive result in plasma ET-1 measure-

ments. Similarly, an interaction with HBOC-201 cannot be excluded, making it difficult to interpret the results. Therefore, blocking ET receptors with tezosentan is the best way to assess the interaction of HBOC-201 with the ET-system.

Conclusion and future directions

HBOC-201 can disrupt hemodynamic homeostasis, mimicking some aspects of endothelial dysfunction, resulting in elevated systemic and pulmonary blood pressures. HBOC-201 induced vasoconstriction is mediated by scavenging NO and likely by upregulating ET production. Pressor effects can be restored by NO donors or direct vasodilators, such as nitroglycerin or ADO, but dosages must be carefully monitored to avoid hypotension. However, hemodynamic normalization was more easily achieved via administration of an ET receptor blocker. Future studies should focus on coadministration of long-acting ET_A receptor antagonists (e.g., ambrisentan or sitaxentan) and, although oxygen-derived free radicals do not appear to play a significant role in HBOC-201-induced pressor responses of healthy subjects, the possible role of ROS in HBOC-induced vasoconstriction in subjects with documented preexisting endothelial dysfunction would be of interest.

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GRANTS

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