Second-generation recombinant hemoglobin molecules do not stimulate sphincter of Oddi, gallbladder or duodenal motility in the Australian brush-tailed possum

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BACKGROUND: Several studies have investigated the effects of hemoglobin-based oxygen carriers on gastrointestinal motility. Diaspirin cross-linked hemoglobin reduces sphincter of Oddi trans-sphincteric flow and increases duodenal motility in the Australian brush-tailed possum, effects attributed to nitric oxide (NO) scavenging. Recently, second- generation recombinant hemoglobin molecules with reduced NO scavenging ability have been developed.

AIM: To determine the effects of two second-generation recombinant hemoglobin solutions and the prototype recombinant hemoglobin with high NO binding, on duodenal and biliary motility in the Australian brush-tailed possum.

METHOD: Blood pressure, duodenal, sphincter of Oddi and gallbladder motility; and trans-sphincteric flow were recorded. The effects of recombinant hemoglobin or human serum albumin (control) solutions on these parameters were investigated. Each solution was infused intravenously at 1 mL/kg/min to deliver 250 mg/kg or 500 mg/kg.

RESULTS: Duodenal contraction frequency was stimulated by the high dose of prototype recombinant hemoglobin, but not by a comparable dose of second-generation recombinant hemoglobin. The induced duodenal activity occurred in the later phase of the experimental period. In contrast, biliary motility and trans-sphincteric flow were not altered by any hemoglobin solution. The high dose of all the hemoglobin solutions elevated blood pressure, whereas the low dose solutions did not alter any parameter measured.

CONCLUSION: At the doses studied, the second-generation recombinant hemoglobin with reduced NO binding capacity did not significantly alter duodenal and biliary motility, supporting the need for further studies to evaluate their potential usefulness as blood substitutes.

Key Words: Biliary motility; Diaspirin cross-linked hemoglobin; Duodenal motility; Hemoglobin-based oxygen carrier; Nitric oxide; Recombinant hemoglobin
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The shortage of donated blood and the increasing risk of infections have led to the development of blood substitutes as oxygen carriers. Various types of hemoglobin-based oxygen carriers (HBOC) have been produced (1,2). Chemically or genetically modified hemoglobin solutions have several advantages including compatibility with all blood types and superior storage stability compared with donated human blood (3,4).

A number of studies have reported physiological effects of HBOCs mediated by nitric oxide (NO) scavenging, mainly in the cardiovascular system (5,6). In the gastrointestinal tract, abdominal discomfort, pain, nausea and vomiting have been reported following the application of various HBOC solutions (7-10) and effects on esophageal (11,12) and sphincter of Oddi (SO) motor function (13,14) and gastric emptying (15) have been reported.

Recombinant human hemoglobin (rHb) molecules that display similar oxygen delivery to human hemoglobin have been developed, and are free of infectious agents (16). The prototype rHb molecule (rHb1.1) displayed extensive NO binding capacity (17), but recently developed second-generation rHb molecules, such as monomer rHb and rHb2.0, have markedly reduced NO scavenging ability (18). For example, rHb2.0 has approximately 20- to 30-fold lower NO scavenging compared with that of rHb1.1 (19). These second-generation molecules display reduced gastric motility and hemodynamic effects (15,18,19).

The effects of the second-generation rHb on duodenal and biliary motility have not been reported. Therefore, the aims of the present investigation were to compare the effects of three rHbs with various rates of NO scavenging on duodenal and biliary motility in anesthetized Australian brush-tailed possums.

METHODS

Ethical approval for these studies was granted by the Flinders University Animal Welfare Committee, Australia.

Animal preparation

Adult Australian brush-tailed possums (n=30) of either sex (1.6 kg to 3.2 kg) were used in the present study. The animals were fasted for 18 h and anesthesia was induced with intramuscular xylazine (Rompun; 5 mg/kg, Bayer Australia Ltd, Australia) and ketamine (Ketalar, 20 mg/kg, Parke-Davis Pty Ltd, Australia) injections. The left femoral vein was cannulated and a continuous infusion of pentobarbital sodium (Nembutal, 15 mg/kg/h to 45 mg/kg/h, Rhone Merieux Pty Ltd, Australia) was used to maintain anesthesia throughout the experimental period. The animals were intubated through a tracheotomy and mechanically ventilated using a small animal respirator (Phipps and Bird Inc, USA). A constant infusion of saline (2 mL/kg/h to 4 mL/kg/h) was delivered via the left femoral vein. Blood pressure was measured via a catheter in the left femoral artery connected to a pressure transducer (Transpac IV, Abbott Critical Care Systems, Ireland). Animal body temperature was maintained at 37°C with a homeothermic blanket (Harvard Apparatus Ltd, United Kingdom).

Measurements of duodenal and biliary motility

The basic experimental techniques for measuring duodenal and biliary motility have been described previously (14). Intraperitoneal access was gained by a midabdominal incision. An incision was then made in the common bile duct 5 mm to 10 mm distal to the cystic duct through which three catheters were inserted (Figure 1). One end of a bile diversion tube, a polyvinyl chloride single lumen catheter (1.52 mm outside diameter [OD], 0.86 mm inside diameter [ID], length 25 cm), was inserted 2 mm toward the liver and secured with a ligature. The other end of this catheter was located in the lumen of the distal duodenum, thereby maintaining the enteroxirculation of bile salts. The two other catheters, to measure trans-sphincteric flow and SO manometry, were inserted towards the duodenum and secured in position with a ligature.

The trans-sphincteric flow catheter (polyvinyl chloride, 1.52 mm OD, 0.86 mm ID, length 40 cm), with an end hole, was positioned 2 mm proximal to the SO. The other end of this catheter was connected to a reservoir containing 20 mL saline and elevated 10 cm above the common bile duct, corresponding to physiological bile duct pressure. The reservoir was attached to an isometric force transducer and trans-sphincteric flow was measured gravimetrically as follows. The weight of the reservoir was continuously recorded via the force transducer, which acted as an electromagnetic balance. As trans-sphincteric flow occurred, the weight of the reservoir decreased and the rate of change (slope) represented trans-sphincteric flow. Aliquots of saline (1 mL) were delivered into the reservoir at regular intervals to maintain a relatively constant inflow (20).

The single lumen manometry catheter (polyethylene, 0.60 mm OD, 0.20 mm ID, length 20 cm), with a single side hole 1 mm from the tip, was positioned in the distal segment of the SO. The manometry catheter was connected in series with a low compliance pneumatic hydraulic capillary infusion system (Armador Medical Specialities, USA) and a pressure transducer (Transpac IV). The manometry catheter was perfused with bubble-free saline at a rate of 0.12 mL/min. A saline-filled balloon catheter was placed into the gallbladder through a small incision made at the fundus and connected to a pressure transducer.

To measure duodenal contractions, a strain gauge transducer (KFG-I-120-C3-11, Kyowa Electronic Instruments Co Ltd, Japan) embedded in silicon, was attached to the anterior duodenal serosal surface (circumferential orientation) at 1 cm oral to the
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Figure 2) Representative recordings illustrating the effect of 500 mg/kg infusion of the recombinant human hemoglobin (rHb) and human serum albumin (HSA) solutions. In each figure, the left panels represent the entire experimental period and the right panels represent an expanded time scale showing the 5 min period immediately before and the 15 min after infusion. In each panel, four recordings are presented in the following order: HSA (1st), rHb1.1 (2nd), monomer rHb (3rd) and rHb2.0 (4th). A Arterial blood pressure: rHb infusion of any of the 3 rHbs caused a rapid increase in blood pressure. In contrast, rHb infusion did not significantly alter sphincter of Oddi (SO) motility (B) or trans-sphincteric flow (C). Infusion of the rHb solutions produced inconsistent effects on gallbladder motility (D). No obvious change was observed with rHb1.1, whereas infusion of monomer rHb caused a transient, small increase in motility. In rHb2.0 infusion, increased motility was seen at 1.5 h post-infusion. Infusion of rHb1.1 produced a prolonged increase in duodenal contraction frequency, whereas infusion of monomer rHb or rHb2.0 resulted in smaller transient increases. Infusion of the HSA solutions did not significantly alter duodenal contraction frequency (E)
SO using n-butyl-cyanoacrylate (Vetbond, 3M Animal Care Products, USA).

Fluctuations in gastric and duodenal pressure can influence SO motility. To avoid these sources of variability, the pylorus was ligated and a silastic rubber catheter (3.5 mm OD, 2.8 mm ID, length 15 cm) with several side holes was inserted into the antrum and fixed in position with a ligature to drain the gastric contents and maintain a constant gastric pressure (Figure 1). Similarly, another silastic rubber catheter was inserted in the duodenum, 4 cm distal to the SO-duodenal junction, towards the SO, via a small incision and secured with a ligature. This catheter served to drain the contents of the resultant pyloric-duodenal segment and maintain a constant pressure.

Data acquisition
Arterial blood pressure, SO, gallbladder and duodenal motility and trans-sphincteric flow were continuously recorded using a MacLab recording system and Chart 3.5 software (ADInstruments Pty Ltd, Australia).

Experimental protocol
All preparations displayed spontaneous biliary and duodenal motility. After a 30 min equilibration period, a test dose of 200 ng/kg cholecystokinin octapeptide (CCK-8, Auspep Pty Ltd, Australia) was administered intravenously to confirm that SO, gallbladder and duodenal recordings were satisfactory. The responses to CCK-8 were short-lived with all parameters returning to baseline within 15 min of administration. An additional 60 min re-equilibration period was allowed before rHbs or human serum albumin (HSA) solution, iso-oncotic to rHb2.0 (Baxter Healthcare Corporation, USA), administration.

One of three rHb solutions (rHb1.1, monomer rHb or rHb2.0, Baxter Healthcare Corporation), 10% v/v or HSA was infused at 1 mL/kg/min for 2.5 min to deliver 250 mg/kg or for 5 min to deliver 500 mg/kg clinically relevant doses recommended by the supplier (n=5 animals/dose/rHb solution). These doses were selected based on the results of preliminary studies. The rHb molecules display a range of NO binding: high for rHb1.1, low for rHb2.0, and a molecule did not influence SO, gallbladder or duodenal motility. Our data indicated that intravenous administration of this molecule did not influence SO, gallbladder or duodenal motility.

RESULTS
Administration of second-generation rHb molecules enhanced blood pressure with little to no effect on biliary and duodenal motility. Figure 2 illustrates typical recordings of each parameter following infusion of 500 mg/kg of HSA, rHb1.1, monomer rHb or rHb2.0 solutions. Group data representing each parameter for all solutions are shown in Figure 3 (500 mg/kg) and Figure 4 (250 mg/kg).

Mean arterial blood pressure
Infusion of 500 mg/kg of rHb solutions (first- and second-generation molecules) resulted in an elevated mean arterial blood pressure; however, the peak change induced by the first generation molecules were higher than that of the second generation molecules. This increase in blood pressure persisted for at least 1 h and had an onset within 1 min after the infusion commenced, whereas infusion of HSA did not significantly change blood pressure (Figure 3A). Of the rHb solutions infused, rHb1.1 produced the greatest peak increase in blood pressure and maintained an elevated blood pressure for most of the experimental period compared with the HSA infusion. Monomer rHb infusion produced a significant increase in blood pressure for only 30 min to 45 min after infusion began, whereas infusion of rHb2.0 resulted in a prolonged elevation for the entire experimental period, with peak elevation intermediate between that produced by rHb1.1 and monomer rHb, compared with HSA solution (Figure 3A). Infusion of the low dose (250 mg/kg) rHb1.1, monomer rHb or rHb2.0 solutions failed to significantly alter blood pressure, compared with the HSA infusion (Figure 4A).

Duodenal motility
Infusion of the high dose (500 mg/kg) rHb1.1 solution stimulated duodenal contractile activity which was evident in the later phase of the experimental period (210 min to 300 min postinfusion) compared with HSA infusion (Figure 3B). In contrast, monomer rHb and rHb2.0 failed to significantly alter duodenal contraction frequency. Similarly, infusion of the low dose (250 mg/kg) rHb solutions did not significantly influence duodenal motility compared with the HSA infusion (Figure 4B).

SO motility, trans-sphincter flow and gallbladder motility
Infusion of the high and low doses of each rHb solution failed to significantly alter SO or gallbladder motility compared with HSA infusion (Figure 3C-F, Figure 4C-F).

DISCUSSION
The present study demonstrates for the first time that infusion of second-generation rHb molecules with reduced NO binding capacity, in contrast to first generation rHb molecules, have no significant effects on SO, gallbladder or duodenal motility and trans-sphincteric flow when compared with HSA infusion.

The first generation HBOCs had a number of undesirable side effects. These included renal toxicity, activation of inflammatory responses, systemic vasoconstrictions and increased esophageal, duodenal and SO motility (8,10,12,14,21). Several side effects were attributed to the NO binding effect of these molecules. The recently developed second-generation HBOC, rHb2.0, had NO binding comparable to that of hemoglobin. Our data indicated that intravenous administration of this molecule did not influence SO, gallbladder or duodenal motility.
Figure 3) Group data (mean ± SEM; n=5 per group) illustrating the responses to intravenous administration of recombinant human hemoglobin (rHb) (500 mg/kg) compared with human serum albumin (HSA) infusion. In each panel, data are presented in the following order: top, rHb1.1 (open circle) vs HSA (closed circle); middle, monomer (open triangle) vs HSA (closed circle); and bottom, rHb2.0 (open diamond) vs HSA (closed circle). Infusion of rHb1.1 or rHb2.0 produced a significant increase in mean arterial pressure for most of the experimental period. A Infusion of the monomer rHb increased blood pressure 30 min to 60 min after infusion. B Infusion of rHb1.1, but not monomer rHb or rHb2.0, significantly increased duodenal (DU) contraction frequency between 4 h and 6 h following infusion. rHb infusion did not significantly change sphincter of Oddi (SO) basal pressure (C), SO area under the curve (AUC) (D), trans-sphincteric flow (E) and gallbladder (GB) AUC (F), compared with HSA.

*P<0.05 compared with HSA group; #P<0.05 compared with rHb1.1 group.
A human study (9) with the prototype rHb (rHb1.1) reported that 68% of the volunteers experienced gastrointestinal symptoms including nausea, vomiting, diarrhea, dysphagia and abdominal pain. We also demonstrated that the first generation HBOC molecule, diaspirin cross-linked hemoglobin (DCLHb), had a stimulatory effect on possum duodenal motility over 5 h (14). In the present series, rHb1.1 also caused prolonged excitation of duodenal contraction. This action may explain the side effects experienced by human subjects given rHb1.1. The second-generation rHbs with lower NO binding ability...
produced no significant effects on the duodenum. These findings are consistent with the role of NO as an inhibitory neurotransmitter in the gut (22).

No rHbs investigated in the present study significantly altered possum SO or gallbladder motility. This contrasts with a previous study (13) in the American opossum where administration of rHb1.1 increased SO motility; this may reflect a species difference effect. Our results are important in that we have previously shown that hyperstimulation of the SO leads to pancreatitis in the Australian brush-tailed possum (23). Hyperamylasemia or mild acute pancreatitis has been reported in humans following the administration of DCLHb or rHb1.1 (24-26). SO stimulation may be a possible mechanism for the induction of pancreatitis in these studies.

With regard to gallbladder motility, we noted variable changes following the administration of rHb and HSA solutions. Similar variability was observed in our previous study with DCLHb (14). Several patterns of change in gallbladder motility, including acute transient excitation, late sustained excitation, inhibition and no change, were recorded. For rHb1.1, these variable results may be derived from complex interactions relating to NO binding and/or the possible production of endothelins in the gallbladder, due to handling of the organ. Other factors, however, may be involved because rHb2.0-treated animals displayed an increased gallbladder activity, particularly from 3 h to 6 h postinfusion, although there was considerable variability within this group (Figure 3F).

Endothelins have been shown to induce gallbladder contraction (27-28) and have been implicated in DCLHb's cardiovascular effects (5,29-31). An alternative explanation may be the variable action of NO on the gastrointestinal tract. Both inhibitory and excitatory effects have been attributed to NO in the gastrointestinal tract (32,33). For example, Alkon et al (34) reported NO had excitatory or inhibitory effects on the guinea pig gallbladder depending on the status of oxidative stress. Furthermore, because of the long duration of our experiments, direct and indirect effects may influence the parameters measured. Thus, these various mechanisms may interact and their relative contribution could dictate the final motor response observed. Because of the complex interactions that can potentially occur in vivo the precise mechanisms underlying our findings are unclear.

Infusion of rHb2.0 solution induced a rapid elevation in blood pressure which was sustained for at least 6 h. The duration of this response was greater than that observed with the other rHbs tested and may reflect a longer biological half-life of this molecule. The reason for sustained elevation with rHb2.0 is still unclear, however, it may be species-specific. In other species, infusion of rHb2.0 has been noted to increase cardiac output (M Doyle, personal communication). This finding suggests that, at least in the Australian brush-tailed possum, the mechanism(s) responsible for elevated blood pressure may not be related to NO scavenging or that the low NO binding capacity of the second-generation rHb molecules is still sufficient to influence the cardiovascular system.

CONCLUSIONS

rHbs with reduced NO binding capacity increase blood pressure without duodenal and biliary motility effects. These findings suggest that the second-generation rHbs do not display significant gastrointestinal or biliary side effects; however, further studies are required to evaluate their potential usefulness as blood substitutes.

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