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Short communication

Reduced nitric oxide reactivity of a new recombinant human hemoglobin attenuates gastric dysmotility

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Abstract

The objective of this investigation was to compare the gastric motility effects of two recombinant hemoglobin variants, rHb1.1, and rHb3011, the latter having decreased nitric oxide reactivity via mutagenic alteration. Variants were administered to rats at 750 and 1500 mg/kg, i.v., prior to feeding a meal. The percentage of meal emptied was determined 45 min after feeding. rHb1.1 reduced gastric emptying significantly (48% and 71%), whereas rHb3011 was significant (42%) only at the higher dose ($p < 0.05$). The results suggest that rHb1.1 inhibits gastric emptying by scavenging NO, and this effect is significantly reduced by rHb3011, which has decreased NO reactivity.

Keywords: Nitric oxide (NO); Hemoglobin; Gastrointestinal motility

1. Introduction

As investigation continues in search of a safe and effective hemoglobin-based oxygen therapeutic, several chemically and genetically altered hemoglobin molecules are under development (Tsuchida, 1995) including a recombinant human hemoglobin, rHb 1.1 (Looker et al., 1992). Compared to human banked blood, these molecules have multiple advantages including increased storage stability and biocompatibility with all blood groups. As clinical trials of these molecules progress, reports of patient gastrointestinal discomfort have arisen (Tsuchida, 1995; Viele et al., 1997). At least part of this observed effect on gastrointestinal dysmotility has been ascribed to the nitric oxide (NO) binding activity of these molecules (O'Kelly et al., 1993; Conklin et al., 1995; Conover et al., 1996).

A recent advance in the genetic and protein engineering of recombinant hemoglobins (rHbs) has yielded a series of new molecules having reduced reactivity with NO (Doherty et al., 1998). One such new rHb, rHb3011, was produced by site-directed mutagenesis at the distal heme pockets, which results in a NO reaction rate constant thirty-fold slower than rHb1.1. In this investigation, two different rHbs, one having high (rHb1.1) and one having low (rHb3011) NO reactivity, were tested for their respective effects on gastric emptying in the rat.

2. Materials and methods

All procedures in this investigation were performed in compliance with the Declaration of Helsinki, the Animal Welfare Act Regulations, 9 CFR parts 1, 2 and 3 and with the *Guide for the Care and Use of Laboratory Animals*, DHEW Publication (NIH) 85–23, 1985. The protocol was approved by the company's Animal Care and Use Committee.

2.1. Animal preparation

Male Sprague–Dawley rats (Charles River, Raleigh, NC) weighing 250–350 g were deprived of food for at least 17 h before experimentation with water available ad libitum. The animals were group housed in cages with wire–mesh bottoms to attenuate coprophagy. Water was removed just prior to experimentation. All rats were studied under conscious, resting conditions.

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2.2. Experimental protocol and surgery

Each rat was given an i.v. injection of a test or control article by the tail vein and returned to a cage. Treatment groups included the proteins human serum albumin (10% HSA, control solution), and two different recombinant hemoglobin solutions (rHbs, [10%] in formulation buffer) rHb1.1 and rHb3011, which were intravenously administered at doses of 350 mg/kg (rHb1.1 only), 750 mg/kg and 1.5 g/kg. A formulation buffer control group was given a vehicle volume equivalent to all proteins dosed at 750 mg/kg. Each treatment group consisted of 10–12 rats. Positive control test article, N^G-nitro-L-arginine methyl ester (L-NAME), was given i.v. at doses of 0 (saline control), 1, 3, and 10 mg/kg (n = 11,7,10, and 10, respectively), to three additional treatment groups. Forty-five minutes after test article administration (sixty minutes after administration for animals that received L-NAME), each rat was orally gavaged with 3 g of a nutrient meal and again returned to a cage. Forty-five minutes after feeding, rats were euthanized by CO_2 asphyxiation, and laparotomized to expose the stomach. Upon exposure, the pylorus and cardia were quickly ligated to intragastrically isolate the residual meal. The stomach was removed, weighed, cleared of contents, and the weight (Wt.) was determined again.

Percent emptying of the stomach was expressed as:

$$\%\,\text{emptied} = \left[\left(\text{Wt. meal given} - \left(\text{Wt. full stomach} - \text{Wt. empty stomach}\right)\right)/\text{Wt. meal given}\right] \times 100.$$

2.3. Preparation of nutrient meal

The nutrient meal was composed of methylcellulose dispersed in ice water to which was added casein, cornstarch, powdered confectioner's sugar, and beef bouillon (Droppleman et al., 1980). This mixture was thoroughly blended after the addition of each ingredient to insure proper dispersion and homogeneity. The meal was divided into aliquots and then refrigerated for at least 24 h prior to use to allow trapped air to escape. Aliquots were frozen until needed. The day prior to experimentation, meal aliquots were allowed to thaw in a refrigerator overnight. Just prior to use, the meal was warmed to approximately 28°C and thoroughly mixed. Each animal received 3 g (approximately 3 ml) of the meal via an intragastric tube.

2.4. Agents

L-NAME, methylcellulose, and casein were purchased from Sigma (St. Louis, MO). The HSA was supplied by Alpha Therapeutics (Los Angeles, CA). Saline was purchased from Baxter Healthcare (Deerfield, IL).

Formulation buffer was composed of 150 mM NaCl, 5 mM sodium phosphate, and 4 μM EDTA at ~pH 7.2.

Briefly, the rHbs were synthesized using the following genetic and biochemical methods. rHb1.1 or rHb3011 was expressed from a single plasmid cloned into *Escherichia coli* (Looker et al., 1992; Doherty et al., 1998). Both molecules contained the tandem fusion of the alpha genes, which upon expression formed a stabilized hemoglobin containing a dialpha subunit and two beta subunits. Nitric oxide reactivity was altered by site-directed, PCR mutagenesis of the specific regions coding for the heme pocket amino acids (Eich et al., 1996).

E. coli expressing rHbs were grown in small fermentors, and after bacterial lysing, purifications were performed using Fast Flow Chelating Sepharose (Pharmacia) followed by anion-exchange chromatography using Fast Flow Q Sepharose (Pharmacia) (Looker et al., 1994). Purified protein was concentrated and mixed with formulation buffer via diafiltration. Final hemoglobin concentration was adjusted to 10% with phosphate-buffered saline.

The in vitro properties of both rHbs and HSA are listed in Table 1. P_{50} values were determined from oxyhemoglobin dissociation curves produced by a Hemoxanalyzer (TCS, Southampton, PA). Hemoglobin concentrations were determined by the Drabkins test, and endotoxin levels were assayed by the limulus amoebocyte lysate gel clot test. Percentages of total hemoglobin converted to methemoglobin were determined by a spectrophotometric method (Evelyn and Malloy, 1938). The rate constants for the reaction of NO with oxyhemoglobins were measured spectrophotometrically via a method described previously (Eich et al., 1996).

2.5. Statistical analysis

In the present investigation, 174 rats were used to produce 125 successful experiments. Forty-nine rats were discarded either because of the extra-vascular presence of injected test article, evidence of intra-gastric feces (coprophagia), or reflux of gastric contents subsequent to gavage.

Table 1
In vitro properties of two recombinant hemoglobins (rHbs) and HSA

	rHb1.1	rHb3011	HSA
P_{50} (mm Hg)	32	46	–
$k'_{NO,ox}$ ($\mu M^{-1}/s$)	60	2	–
Protein (mg/ml)	100	100	100
Endotoxin (Eu/ml)	0.2	0.3	–
% met-rHb	4.5	5.9	–

$k'_{NO,ox}$: rate constant for the reaction of oxyhemoglobin with nitric oxide.
% met-rHb: percent methemoglobin of total hemoglobin.

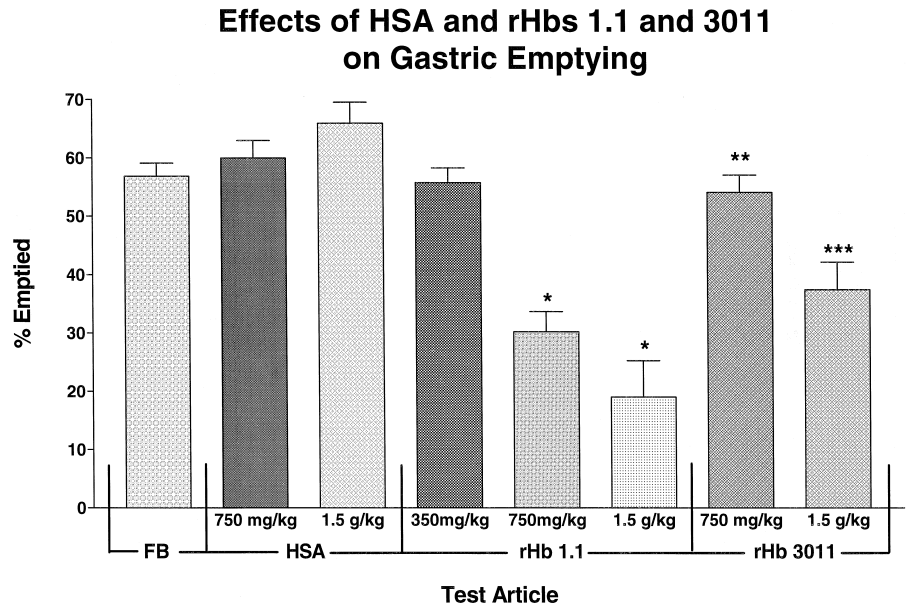

Fig. 1. Effects of formulation buffer vehicle control (FB), two doses of HSA and rHb 3011, and three doses of rHb1.1 (*x*-axis), on rat gastric emptying (*y*-axis). Data are mean ± S.E.M. * Significantly different ($p < 0.05$) from corresponding HSA doses, ** ($p < 0.05$) different from same rHb1.1 dose but not HSA, *** ($p < 0.05$) different from both same HSA and rHb1.1 doses.

All results are presented as mean ± S.E.M. Comparisons of gastric emptying between treatment groups were analyzed by one-way ANOVA. Treatment means were compared for equality via Tukey's multiple comparison test. Means were considered significantly different at $p < 0.05$.

3. Results

Positive control groups treated with L-NAME (1, 3, and 10 mg/kg, i.v.) were performed to verify that this rat model of gastric dysmotility was sensitive to reductions in endogenous NO by inhibition of nitric oxide synthase. L-NAME produced a dose-dependent reduction in absolute percent gastric emptying to 55 ± 4, $43 \pm 4*$ and $33 \pm 5*$, respectively. Emptying was significantly decreased by the two highest doses ($* = p < 0.05$) as compared to 55 ± 5 for the saline vehicle control.

Fig. 1 compares the gastric emptying effects of rHb1.1 and rHb3011 to formulation buffer vehicle (FB) and HSA negative controls. Percent gastric emptying for these controls were 57 ± 2 (FB), 60 ± 3 (HSA low dose), and 65 ± 4 (HSA high dose), respectively. The two highest doses of rHb1.1 significantly reduced percent gastric emptying (30 ± 4 and 19 ± 7), which were 48% and 71% below corresponding HSA controls (100%), respectively. Only the highest dose of rHb3011 decreased percent emptying (38 ± 5) below HSA control (a decrease of 42%), and the 750 mg/kg dose of this molecule was not significantly different (54 ± 3) from HSA. Gastric emptying was greater with both doses of rHb3011 than with corresponding doses of rHb1.1.

4. Discussion

This investigation examined the hypothesis that a recombinant hemoglobin having reduced NO reactivity (rHb3011) would attenuate gastric motility less than a variant having a higher reactivity (rHb1.1). The results indicate that rHb3011 interfered with gastric emptying significantly less than rHb1.1.

The participation of NO in the coordinated propulsion of a meal through the digestive tract has been well documented (Guslandi, 1994). Both generalized gastrointestinal smooth muscle as well as the specialized sphincter musculature are normally controlled by multiple mechanisms which include non-adrenergic, non-cholinergic neurons, some of which release NO (Sanders and Ward, 1992). Although NO is involved in non-propulsive digestive activities, the experimental (Conklin et al., 1995; Conover et al., 1996) and clinical manifestations (Viele et al., 1997) of hemoglobin solutions suggest that NO depletion by these proteins primarily affects gastrointestinal smooth muscle. Genetic alterations of heme pocket structure may lead to diminished NO scavenging (Doherty et al., 1998), and thereby, to reduced gastrointestinal and other pharmacologic effects.

The rat gastric emptying preparation utilized in the present investigation was designed to determine the significance of lowered NO reactivity on gastrointestinal motil-

ity. Although rHbs may exert dysmotility effects at multiple regions along the gastrointestinal tract (Conover et al., 1996; Viele et al., 1997), this rat model focuses only on gastric and pyloric function. The present rat model does demonstrate reproducible gastric emptying upon administration of several negative control agents (saline, formulation buffer, HSA). Positive control experiments with L-NAME showed that inhibiting endogenous NO synthesis attenuated gastric emptying in a dose-related manner, similar to results reported previously (Plourde et al., 1994). rHb3011 had a significantly decreased effect on gastric motility than did rHb1.1, indicating a correlation between reduced action on gastric emptying and reduced NO reactivity. The results are consistent with the hypothesis that inhibition of gastric emptying by hemoglobins is primarily due to scavenging of NO.

Alternatively, there could have been mechanisms by which rHb3011 produced the more favorable gastric action unrelated to regional NO effects. rHb1.1 produces a transient systemic vasoconstriction producing hypertension in rats, whereas rHb3011 does not (Doyle et al., 1998). This circulatory action is most likely related to differential rHb reactivity with vascular endothelial NO. In the present investigation, a secondary effect related to hemodynamic changes could have produced an alteration in gastric perfusion to affect motility of the stomach. rHb3011 could also have been cleared faster than rHb1.1, thereby reducing the effective concentration of rHb3011 in the relevant tissues over the course of the experiment. However, these two rHbs have been recently demonstrated to have similar half-lives in the rat, rendering this explanation unlikely (results not shown). Also, little is known about the fluid shifts and resulting motility alterations that could occur as a result of administering a hemoglobin protein load. However, the two rHbs used in this study have identical colloid oncotic pressures (both 16 mm Hg), suggesting that in vivo differences in fluid balance should be minimal. In addition, multiple endogenous receptor-mediated mechanisms related to gastrin, cholecystokinin, acetylcholine and opioids are known to participate in the control of gastric motility. The effect of free hemoglobin on these effector systems is unknown.

Although recombinant hemoglobins may impact other mechanisms controlling gastrointestinal propulsion as mentioned above, the present results suggest that reducing NO scavenging by distal heme pocket mutagenesis can lessen the impact of extracellular hemoglobin on gastric motility. This strategy creates the potential for synthesis of recombinant hemoglobins having reduced gastrointestinal dysmotility effects while preserving oxygen delivery capability.

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