Differential effects of sodium selenite in reducing tissue damage caused by three hemoglobin-based oxygen carriers

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Baldwin, Ann L., Elizabeth B. Wiley, and Abdu I. Alayash. Differential effects of sodium selenite in reducing tissue damage caused by three hemoglobin-based oxygen carriers. J Appl Physiol 96: 893–903, 2004. First published October 10, 2003; 10.1152/japplphysiol.00615.2003.—Three “blood substitutes,” a diaspirin cross-linked human hemoglobin (DBBF-Hb), a bovine polymerized hemoglobin (PolyHbBv), and a human polymerized hemoglobin (O-R-PolyHbA0), that have undergone clinical trials are used in this study. Previously, we showed in the rat that coadministration of sodium selenite (Na2SeO3) and DBBF-Hb significantly decreased mesenteric venular leakage and epithelial disruption produced by DBBF-Hb alone but did not reduce mast cell degranulation unless given orally. The purpose of this study was to determine whether Na2SeO3 produced similar beneficial responses when used with PolyHbBv and O-R-PolyHbA0. In anesthetized Sprague-Dawley rats, the mesenteric microvasculature was perfused with PolyHbBv or O-R-PolyHbA0, with and without Na2SeO3 in the perfusate and suffusate, for 10 min, followed by FITC-albumin for 3 min, and then fixed for microscopy. Na2SeO3 did not reduce leak number or area in preparations perfused with PolyHbBv and only reduced leak number (but not significantly) in preparations perfused with O-R-PolyHbA0. Na2SeO3 significantly increased mesenteric mast cell degranulation and impaired epithelial integrity in animals treated with PolyHbBv. In vitro, Na2SeO3 significantly reduced the oxidation rate of DBBF-Hb in the presence of oxidants, had little effect on PolyHbBv, and increased the oxidation rate of O-R-PolyHbA0. These results suggest that Na2SeO3 moderates hemoglobin-induced damage, at least partly, through its redox interactions with the heme sites in the hemoglobin molecules studied and that accessibility of the heme site to Na2SeO3 governs these interactions.

rat-free hemoglobin (Hb)-based oxygen carriers, such as diaspirin cross-linked Hb (DBBF-Hb), PolyHbBv (Oxyglobin), and O-R-PolyHbA0 (Hemolink) have been proposed as blood substitutes for transfusions because of their plasma expansion and oxygen transport capabilities. In addition to their use after accidents or major surgery, such substitutes could also be employed to alleviate anemia in patients with hemotocrits too high to qualify for blood transfusions. In fact, PolyHbBv (Oxyglobin) is currently approved by the Food and Drug Administration for veterinary use in the United States, and its human counterpart has recently been approved for clinical use in humans in South Africa. Hb-based blood substitutes have the added advantages that they can be easily purified, stored for relatively long periods of time, and used in patients of all blood types. However, a number of largely unresolved problems were found during preclinical trials and development of some of these Hb-based substitutes. These include cardiovascular and/or hemodynamic effects, gastrointestinal changes, immune cell activation, coagulation changes, oxidative stress, and decreased host resistance to overwhelming infection (20). A dose-response study performed on dogs by Biopure has shown that Oxyglobin increases arterial oxygen content in the face of normovolemic anemia and produces transient clinical signs (skin discoloration, discolored stools, nausea, vomiting). In addition, histopathology of Oxyglobin administration includes activation of tissue macrophages in multiple organs (Biopure Oxyglobin solution, package insert 2003, Biopure www.biopure.com/products/vetpackageinsert.cfm?cDID=2&cPglD=31#Contraindications). Hemolink has recently been withdrawn from phase III clinical trials in cardiac bypass grafting because it produced adverse cardiac events (www.hemosol.com/companynewsreleases.cfm?newsID =1870&companyID=24).

Previously, our laboratory showed that bolus injection of DBBF-Hb in rats increased venular leakage to bovine serum albumin (BSA) and produced mast cell degranulation in the rat mesentery (6) and also caused detachment of intestinal epithelial cells from each other and from the basement membrane (5, 11). Such changes are characteristic of an inflammatory response (7, 33). This is not surprising because mast cell degranulation, as observed after injection of DBBF-Hb, causes release of histamine and other inflammatory mediators, which then damage cell membranes. It is disadvantageous for a potential blood substitute to cause microvascular leakage because the substitute itself will rapidly leave the circulation and also because alterations in transvascular exchange of plasma proteins will disturb the fluid balance between blood and tissue. Increased microvascular leakage also changes the kinetics of delivery of intravascularly injected drugs and of endogenous enzymes and hormones to various tissues. When transfusions are needed, for example after hemorrhagic shock, it is important that regulation of microvascular exchange is not compromised. In another study, we showed that mesenteric microvascular leakage to BSA, mesenteric mast cell degranulation, and epithelial disruptions were significantly lower in animals treated with PolyHbBv compared with those treated with DBBF-Hb (9). However, even in animals treated with PolyHbBv, the average number of leaks per unit length of venule was still significantly greater than for controls that were perfused with HEPES-buffered saline with 0.5% BSA (HBS-BSA).

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It has been hypothesized that the deleterious effects of intravascularly injected free modified Hbs are caused by their oxidation and subsequent formation of reactive oxygen species (1). In fact, we have shown that the antioxidant sodium selenite (Na₂SeO₃) was effective in significantly reducing the intestinal epithelial damage (8) and microvascular leakage (10) associated with bolus injection of DBBF-Hb in rats. Simoni et al. (28) also demonstrated that treatment with Na₂SeO₃ was very effective in the prevention of oxidative damage induced by Hb. In vitro, Na₂SeO₃ reduced the oxidation rate of DBBF-Hb while in the presence of oxidants (10). Thus it appears that Na₂SeO₃ moderates Hb-induced damage at least partly through its interactions with the Hb. The following study was performed to determine whether coadministration of Na₂SeO₃ would significantly reduce the microvascular damage caused by PolyHbBv and O-R-PolyHbA₀ in the rat mesentery and whether site-specific modifications of Hbs can play a role in the interactions between Hb and selenium (Se). To aid in the interpretation of the results of the animal experiments, the interactions of Na₂SeO₃ with PolyHbBv and O-R-PolyHbA₀ were investigated in vitro and compared with results obtained by using DBBF-Hb and cyanomet-DBBF-Hb (CNmet-DBBF-Hb) (8, 10), a Hb in which the cyanide (CN) groups are tightly bound to the heme such that the molecule is unable to participate in redox reactions. Effects of Na₂SeO₃ on the rates of autoxidation, oxidation, and oxygen affinity of the Hbs were measured. In addition, the effect of Na₂SeO₃ on percent reduction in ferric (met) forms of the modified Hbs were determined.

**MATERIALS AND METHODS**

**Hb Solutions**

Glutaraldehyde-polymerized Hb (PolyHbBv) was purchased from Biopure (Boston, MA). Spectral analysis of the solution just before injection demonstrated the presence of 5–8% of the met form. This percentage of methHb in a modified Hb for clinical use is acceptable according to criteria initiated by the manufacturers of these products. PolyHbBv contains a heterogeneous mixture of polymeric (~95%) and nonpolymeric (~5%) species ranging in size from 32 to 500 kDa (24). Both the oxygen-carrying and redox properties of this Hb have been well documented (2, 16). O-R-PolyHbA₀, a purified human Hb (HbA₀) that is cross-linked with o-raffinose and oligomerized, was obtained from Hemosol Research (Toronto, ON, Canada). The O-R-PolyHbA₀ solution that results from this procedure consists predominantly of polymers of HbA₀ [32 kDa (5%), 64 kDa (33%), 128–600 kDa (63%), ≥600 kDa (3%)] (23). In these experiments both Hbs were diluted to 2 mg/ml with HBS-BSA, pH 7.4, and equilibrated with room air. However, the concentration of Hb used in these experiments was less than one-tenth of that found in blood, and, in addition, oxygen has a low solubility in water. Therefore, the tissues were not exposed to higher concentrations of oxygen than they would experience in vivo. All Hb solutions used in this study were free of red cell enzymes, such as superoxide dismutase or catalase.

**Preexperimental Treatment of Rats**

Animal experiments were conducted with approval from the University of Arizona IACUC. Male Sprague-Dawley rats, weighing 300–350 g, were obtained from Harlan Teklad (Madison, WI). Monthly serology, bacteriology, and parasitology evaluations had been performed on animals from each virus-free barrier at Harlan. The rats were transported to the animal facility at Tucson Veterans Administration Medical Center by truck. The animal facility is small with a low personnel activity, and monthly tests are performed on sentinel rats. On arrival, the animals were housed two per cage in a room (3 m × 4 m) deliberately chosen so as to be remote from noisy air vents, cage washers, and the like. The cages were 45 cm long and 24 cm wide and contained standard Harlan Sanichip bedding. Ten to 20 rats were housed in the room at any given time, and no other rats, apart from those participating in this study, were housed with them. A technician entered both rooms once a day to feed and tend to the rats. The temperature ranged between 72 and 74°F, and the humidity was kept between 55 and 60%. The rats were fed Harlan Tech Lab 485 rat Chow and placed on a light cycle with lights on between 6:00 AM and 6:00 PM.

**Anesthesia**

Sprague-Dawley rats were preanesthetized with 1 mg/kg body wt of the following mixture: ketamine hydrochloride (5 ml of 100 mg/ml), acepromazine maleate (2 ml of 10 mg/50 ml), and xylazine (8 ml of 20 mg/ml). This was followed by an intraperitoneal injection of pentobarbital sodium (30 mg/kg). In each rat, a tracheostomy was performed for artificial ventilation.

**Design of Animal Experiments**

**Venular leakage and mast cell degranulation in mesentery.** Experiments were performed to characterize the mesenteric microvascular leakage to albumin and mast cell degranulation, caused by intravenous injection of PolyHbBv or O-R-PolyHbA₀, in the presence (6 rats per group) and absence (5 and 6 for PolyHbBv and O-R-PolyHbA₀ experiments, respectively) of Na₂SeO₃. The number of animals per group was justified to be sufficient by utilizing a sample-size nomogram in conjunction with estimates of the difference in means that needed to be detected, and the mean standard deviation for each parameter (36). The mesenteric microvasculature was perfused for 10 min with PolyHbBv or O-R-PolyHbA₀, with or without 2 µg/ml Na₂SeO₃. The same concentration of Na₂SeO₃ was also added to the HBS-BSA sulfamate that was applied during the 10-min perfusion. In addition to the animals mentioned, four rats had their mesenteric microvasculature perfused with HBS-BSA instead of a modified Hb, and they served as controls. The results from these experiments were compared with those from a previous experiment to determine the effects of Na₂SeO₃ on microvascular leakage induced by DBBF-Hb (10). To ensure that there was no systematic difference between the two different batches of rats, two of the present rats were perfused with DBBF-Hb and the microvascular leaks were assessed. These animals gave leak numbers and areas that were similar to those of the previous study. For this reason, DBBF-Hb data from the previous study were directly compared with results obtained with PolyHbBv and O-R-PolyHbA₀.

**Epithelial integrity and goblet cell secretion in intestine.** Two groups of rats (5 per group) were used in experiments to determine the effects of PolyHbBv, with and without Na₂SeO₃ (Sigma Chemical, St. Louis, MO), on the epithelial integrity of the intestinal mucosa. One group received 2 µg/ml Na₂SeO₃ in their drinking water. Se has previously been administered to rats in their drinking water at a dose of 0.1 µg/ml and has been shown to be effective in increasing antioxidant enzyme activities (37). In the present experiments, the rats drank an average of 32 ml water per day; therefore they were receiving 64 µg of Se daily. The rats were kept, as described, for 3 wk before experiments were performed on them. The experimental design was as follows: a 5-ml bolus of 10 mg/ml PolyHbBv was injected into the aorta in both groups of rats and allowed to circulate for 30 min before perfusion fixing of the small intestine and preparation of tissue samples for light and electron microscopy. The results from these experiments were compared with those from previous experiments (8) in which DBBF-Hb was used instead of PolyHbBv.
Mesenteric Preparation to Measure Microvascular Leakage and Mast Cell Degranulation

Surgical procedures. The animal procedure was similar to that described previously (31) and is summarized here. After anesthesia, the abdomen was opened and several contiguous well-vascularized mesenteric windows were selected and spread out flat over a Plexiglas platform. A mesenteric window is defined as the tissue extending between two adjacent feeding arterioles in the mesentery. Next, the superior mesenteric artery was cannulated, arterioles and venules surrounding the chosen windows were ligated, and the microvascular network was perfused with HBS-BSA and 1 U/ml heparin at 37°C, followed by 2 mg/ml PolyHbBv or O-R-PolyHbA0 in HBS-BSA, with or without Na2SeO3. The preparation was kept moist during the 10-min perfusion by dripping 20 ml HBS-BSA. The suffusate only contained Na2SeO3 if this was included in the perfusate. When the portal vein was clamped, and veins received adequate perfusion. Next, the pressure was reduced to 40 mmHg, the portal vein was clamped, and fixation was continued at 4°C. After 1 h the mesenteric tissue was carefully excised and each window from the preparation was mounted between two thin glass coverslips by using aqueous mounting medium (Vectashield, Vector Laboratories, Burlingame, CA).

Assessment of venular leakage. An assessment of overall vascular leakage was made by measuring the number and area of regions with extravascular FITC-albumin. Slides were examined by use of a Zeiss Axioplan microscope with a ×10 objective, numerical aperture 0.6, fitted for epifluorescence. The resolution obtained with this system was such that the smallest leak that could be detected was 2 μm2 in area. The light source was a 100-W Hg lamp for epifluorescence and a halogen lamp for transmitted illumination. A video camera (Optronix 750D) was mounted at the camera port of the microscope. Ten images of leaky vessel networks from each mesentery, produced by epifluorescence with the appropriate FITC excitation and emission filters (λ = 488 nm and 515 nm, respectively), were viewed on a black-and-white monitor and also recorded on a video recorder. Each slide was only exposed to the excitation wavelength for 5 s. Recordings were also made of the networks under transillumination. Videotaped images were later analyzed by using an analog-to-digital converter and appropriate software (NIH Image) to measure the length and diameter of each venule, the number of leaks per venule, and the area of each leak. If a leak was positioned at a vascular junction, the leak area was divided by the number of venules involved. Data were pooled within each group and the following values were calculated: 1) average number of leaks per length of venule and 2) average leak area per micrometer of venule.

Assessment of mast cell degranulation. After videotaping of the fluorescent leaks, the tissue was suffused with 1% toluidine blue for 20 s, rinsed with HBS-BSA, and then remounted. Toluidine blue was used to stain the mast cells to determine the numbers that had degranulated. Degranulated mesenteric mast cells, identified by the presence of intracellular granules released into the surrounding tissue, were counted within each circular ×20 microscopic field of view (field area 1.13 mm2). Rows of fields were counted systematically from left to right. Cells located in the periphery of the field were only counted if at least one-half of the cell area was within the field. The error of repeat counting was <2%. From 15 to 30 fields were counted for each mesenteric window.

Data analysis. Each parameter was compared between different groups by using one-way analysis of variance. If a significant difference was found between groups, pairs of groups were compared by using the Student’s t-test with a P value <0.05 to determine statistical significance. All values are presented as means ± SE. The n used in the leakage studies was the number of venules examined per group, and the n used for the mast cell degranulation was the number of mesenteric fields of view examined per group.

Microscopy of Intestinal Mucosa to Assess Goblet Cell Secretion and Epithelial Damage

Surgical procedures. After anesthesia, a midline abdominal incision was made to expose the aorta. The aorta was cannulated just downstream from the superior mesenteric artery in a retrograde direction. The free end of the catheter tubing was connected to a reservoir of HBS-2% BSA, pH 7.4, at 37°C. A loop of intestinal ileum, close to the cecum, was pulled outside the body cavity and arranged on a Plexiglas pillar attached to the plastic stage on which the rat was situated. PolyHbBv (50 mg) in a 5-ml bolus of HBS-2% BSA was injected through a 0.2-μm filter via the aortic cannula and allowed to circulate with the blood for 30 min. With the assumption that the animal’s blood volume in milliliters is equivalent to 8% of the animal’s weight in grams, injection of this amount of blood substitute produced a concentration that was similar to that used in the mesenteric experiments (2 mg/ml). After 30 min, the aorta was clamped proximal to (upstream of) the superior mesenteric artery, and the intestinal circulation was perfused with Karnovsky’s fixative in phosphate buffer, pH 7.4, at 4°C. When perfusion was complete, the inlet pressure was dropped to 40 mmHg and the portal vein was clamped. The animal was killed with an intravenous injection of Beuthanasia. Fixation continued for 60 min, after which the intestinal loop was excised and cut into several segments, each containing a Peyer’s patch; these segments were washed in buffered saline.

Tissue preparation for histology. Tissue squares were immersed in diaminobenzidine (DAB) overnight in the dark to stain specific granules in immune cells and thus make the cells easier to identify. The DAB was prepared as follows (22): DAB (0.1 g) was added to 50 ml 0.1 M monobasic phosphate buffer, and the pH was adjusted to 7.2 very gradually with concentrated NH4OH. The solution became a light tannish-pink color. Next, the tissue squares were rinsed in distilled water. Meanwhile, 25 ml of DAB solution were added to 1.66 ml 3% H2O2 to give final concentration of 0.2%. The tissue was placed in this solution for 60 min and then rinsed three times in 0.15 M sodium cacodylate buffer. Finally, the tissue was dehydrated in increasing concentrations of ethanol and embedded in Spurr’s resin. The pieces of tissue were oriented in the resin so that the blocks could be sectioned perpendicular to the villus plane. Thick sections (2 μm) were cut for light microscopy, mounted on slides, and stained with toluidine blue. Ultrathin sections were cut for electron microscopy (Phillips CM12). Before examination of the sections under electron microscopy, the grids were stained with lead citrate and uranyl acetate.

Data analysis. Thick sections were examined under light microscopy to count the numbers of secreting goblet cells, per villus cross section, in 30–60 villi per animal taken from four different regions of the tissue sample. Only villus sections that contained a central lacteal were included because these villi were centrally sectioned. For collection of data, the slides were coded and the code was not revealed until the data were analyzed, as previously described, with n as the number of villi in each group. Electron microscopy (~3,000) was used to assess the integrity of the epithelium (EI) in each villus cross section examined. Intact epithelium was assigned an EI of 1; epithelium with some cell-cell separation was assigned 2, and epithelial cells detaching from the basement membrane scored 3.
Table 1. Venules containing leaks by group

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Venules with Leaks</th>
<th>Total No. of Venules</th>
<th>Percentage of Venules with Leaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>PolyHbBv</td>
<td>23</td>
<td>298</td>
<td>7.7</td>
</tr>
<tr>
<td>PolyHbBv with Na₂SeO₃</td>
<td>38</td>
<td>395</td>
<td>9.6</td>
</tr>
<tr>
<td>O-R-PolyHbA₀</td>
<td>45</td>
<td>468</td>
<td>9.6</td>
</tr>
<tr>
<td>O-R-PolyHbA₀ with Na₂SeO₃</td>
<td>28</td>
<td>512</td>
<td>5.5</td>
</tr>
<tr>
<td>HBS-BSA only</td>
<td>5</td>
<td>99</td>
<td>5.0</td>
</tr>
<tr>
<td>DBBF-Hb</td>
<td>115</td>
<td>299</td>
<td>38.4</td>
</tr>
<tr>
<td>DBBF-Hb with Na₂SeO₃</td>
<td>10</td>
<td>337</td>
<td>3.1</td>
</tr>
</tbody>
</table>

PolyHbBv, bovine polymerized hemoglobin; O-R-PolyHbA₀, human polymerized hemoglobin; DBBF-Hb, diaspirin cross-linked human hemoglobin; HBS-BSA, HEPES-buffered saline with 0.5% bovine serum albumin.

Effect of Na₂SeO₃ on the Oxidation of PolyHbBv and O-R-PolyHb

Autoxidation experiments. Autoxidation occurs when the ferrous iron (Fe²⁺), the only form of Hb that carries oxygen, undergoes spontaneous oxidation to the ferric (met) form (Fe³⁺). Autoxidation experiments for PolyHbBv and O-R-PolyHbA₀ samples (50 μM) in Hank’s balanced salt solution (HBSS), pH 7.4, 37°C, in the presence of 500 μM of Na₂SeO₃ were carried out in the dark. Absorbance changes in the range of 490–640 nm due to the spontaneous oxidation of PolyHbBv or O-R-PolyHbA₀ were recorded on a Perkin-Elmer Lambda 6 spectrophotometer. Multicomponent analysis was used to calculate the time-dependent changes in concentration of the oxy, met, and deoxy on the basis of known spectra of each species (21). First-order autoxidation rate constants were derived from a fit of the data during 10-h incubation to a single exponential expression by using a nonlinear least squares fitting program (Origin 6.1).

Enzymatic oxidation experiments. Chemically induced oxidation of PolyHbBv and O-R-PolyHbA₀ by low and steady levels of H₂O₂ was achieved in vitro by use of the glucose-glucose oxidase system (GOX) as previously described (17). The reaction mixture was prepared containing 50 μM oxy-PolyHbBv and 10 μM/glucose oxidase in HBSS solution, pH 7.4, which also contained 5.6 mM glucose as substrate at 37°C. Under these conditions, the rate of H₂O₂ production by the GOX system was 1.5 μM H₂O₂/min (17). In another set of experiments, 500 μM Na₂SeO₃ was added to the reaction mixture and spectral changes in the Hb were monitored throughout the experiments.

Se-mediated reduction experiments. In these experiments, the met derivatives of PolyHbBv and O-R-PolyHbA₀ (50 μM) were incubated in HBSS buffer, pH 7.4, 37°C with increasing concentrations of Se (125 μM to 1 mM). The reduction of the ferric iron of the Hb induced by Se was monitored immediately after mixing of Hb solution and Se in a cuvette placed inside a diode-array spectrophotometer.

Effect of Na₂SeO₃ on the Oxygen Affinity of PolyHbBv and O-R-PolyHb

Oxygen equilibrium curves of PolyHbBv and O-R-PolyHbA₀ in the presence of various concentrations of Na₂SeO₃ (250 μM to 1 mM) were obtained by using the Hemox analyzer (TCS Scientific, New Hope, PA). This instrument measures the oxygen tension with a Clark oxygen electrode (model 5331 oxygen probe; Yellow Springs Instruments, Yellow Springs, OH) and simultaneously uses a dual-wavelength spectrophotometer to calculate the Hb oxygenation. Oxygen equilibrium experiments were carried out in 0.1 M phosphate buffer, pH 7.4, and incubation times ranged from 10 to 20 min. The concentration of Hb samples was between 60 and 75 μM (heme) and the temperature was maintained at 37°C. To maintain the ferric (MetHb) content to a minimum level (<2%), 4 μL of the Hayashi enzymatic reduction system, which consists of a number red cell enzymes and cofactors, were included in the final solution (4 mL) (19). In another set of experiments, the Hayashi system was omitted from the Hb solutions to determine the effects of increasing concentrations of Se on methemoglobin build up throughout the experiments and consequently the oxygen affinities of PolyHbBv and O-R-PolyHbA₀. Oxygen equilibrium parameters were derived by fitting the Adair equations to each oxygen equilibrium binding curve by the nonlinear least squares procedure included in the Hemox analyzer software (p50 PLUS, version 1.2) (2).

RESULTS

Mesenteric Preparation to Measure Microvascular Leakage and Mast Cell Degranulation

Distribution of leaks. Table 1 shows the percentage of venules containing leaks for the different groups. For preparations perfused with PolyHbBv without Na₂SeO₃, 7.7% of venules examined contained leaks. Most of the leaks (62/67; 93%) were <100 μm² in area. This compares with a luminal surface area of individual endothelial cells that ranges from 400 to 900 μm² (7). In preparations that were perfused with

![Fig. 1. Mesenteric preparations to measure microvascular leakage. A: light microscopic image of rat mesenteric microvasculature after perfusion with a bovine polymerized hemoglobin (PolyHbBv; 2 mg/ml) followed by FITC-albumin. Several small leakage sites of FITC-albumin from the microvasculature are visible. B: mesenteric preparation after perfusion with PolyHbBv and Na₂SeO₃, with Na₂SeO₃ in the suffusate, followed by perfusion with FITC-albumin. Although more leakage sites can be seen in this micrograph compared with A, the difference overall was not significant. Scale bars = 25 μm.](http://jap.physiology.org/10.1152/jappl.00110.2003)
both PolyHbBv and Na$_2$SeO$_3$, 9.6% of venules examined contained leaks. PolyHbBv preparations, with or without Na$_2$SeO$_3$, resembled controls that were perfused with HBS-BSA when they were viewed by fluorescence microscopy, except that some of the larger venules showed a few small FITC-BSA leaks. Typical mesenteric networks after perfusion with PolyHbBv or with PolyHbBv and Na$_2$SeO$_3$, followed by FITC-BSA, can be seen in Fig. 1, A and B, respectively. For preparations perfused with O-R-PolyHbA$_0$ without Na$_2$SeO$_3$, 9.6% of venules examined contained leaks. Although the majority of the leaks (41/59; 69%) were <100 µm$^2$ in area, this percentage was lower than for PolyHbBv. In preparations that were perfused with O-R-PolyHbA$_0$ and Na$_2$SeO$_3$, 5.5% of venules examined contained leaks. Although the percentage of venules showing leaks after treatment with O-R-PolyHbA$_0$ was relatively low, the individual leaks were, on average, significantly larger than those produced by PolyHbBv [173 ± 42 (n = 59) vs. 23 ± 5 (n = 71)], both without Na$_2$SeO$_3$. Typical mesenteric networks after perfusion with O-R-PolyHbA$_0$ with or O-R-PolyHbA$_0$ and Na$_2$SeO$_3$, followed by FITC-BSA, can be seen in Fig. 2, A and B, respectively.

**Quantification of leaks.** The mean numbers and areas, respectively, of leaks per venule length for networks perfused with PolyHbBv or O-R-PolyHbA$_0$ with and without Na$_2$SeO$_3$, are shown in Fig. 3. Results from previous experiments using diaspirin cross-linked human hemoglobin (DBBF-Hb) or cyanomet (CNmet)-DBBF-Hb are also included for comparison. *Significant difference from corresponding group in which Na$_2$SeO$_3$ was also used.

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**Fig. 2.** Mesenteric preparations to measure microvascular leakage. A: light microscopic image of rat mesenteric microvasculature after perfusion with a human polymerized hemoglobin (O-R-PolyHbA$_0$, 2 mg/ml) followed by FITC-albumin. Several leakage sites of FITC-albumin with a range of sizes are visible in the microvasculature. B: mesenteric preparation after perfusion with O-R-PolyHbA$_0$ and Na$_2$SeO$_3$, with Na$_2$SeO$_3$ in the suffusate, followed by perfusion with FITC-albumin. Scale bars = 25 µm.

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**Fig. 3.** Mesenteric preparations to measure microvascular leakage. A: average number of leaks per venule length (µm$^{-1}$) for networks perfused with PolyHbBv or O-R-PolyHbA$_0$ with and without Na$_2$SeO$_3$. Results from previous experiments using diaspirin cross-linked human hemoglobin (DBBF-Hb) or cyanomet (CNmet)-DBBF-Hb are also included for comparison. *Significant difference from corresponding group in which Na$_2$SeO$_3$ was also used. B: average total leak area per venule length (µm$^2$/µm). Error bars indicate SE. HBS, HEPES-buffered saline; Se, selenium.
which were not significantly different from each other. In preparations perfused with O-R-PolyHbA0, Na2SeO3 somewhat reduced the number of leaks, but the difference was not significant. O-R-PolyHbA0 alone produced an average of 0.59 ± 0.12 × 10⁻³ leaks/μm venule length, and the combination of O-R-PolyHbA0 and Na2SeO3 gave a value of 0.25 ± 0.05 × 10⁻³. Corresponding leak areas per micrometer of venule length were 0.092 ± 0.028 and 0.084 ± 0.032 μm², which were not significantly different from each other.

On the other hand, in a previous study (10) it was shown that in preparations perfused with DBBF-Hb, when Na2SeO3 was included in the perfusate and suffusate, the microvascular leak number was significantly reduced from 2.41 ± 0.33 × 10⁻³ leaks/μm venule length to 0.23 ± 0.09 × 10⁻³ leaks/μm venule length (P < 0.01), and leak area was reduced from 0.10 ± 0.03 μm²/μm to 0.012 ± 0.006 μm² (P < 0.01). In rats perfused with CNmet-DBBF-Hb, without addition of Na2SeO3, the mean leak number per micrometer (0.36 ± 0.14 × 10⁻³) and the mean leak area per micrometer (0.005 ± 0.003 μm²/μm) were significantly higher than after DBBF-Hb perfusion and were similar to controls. Inclusion of Na2SeO3 in the perfusate and suffusate of preparations perfused with DBBF-Hb, when Na2SeO3 was included in the perfusate and suffusate did not significantly alter the already high mast cell degranulation (from 12.88 ± 1.61 to 13.86 ± 1.20). Mesenteric mast cell degranulation in animals whose mesenteric microvasculature was perfused with CNmet-DBBF-Hb (6.85 ± 0.78) was also not affected by inclusion of Na2SeO3 in the perfusate and suffusate (6.06 ± 0.72).

**Microscopy of Intestinal Mucosa to Assess Goblet Cell Secretion and Epithelial Damage**

*Secreting goblet cells.* The extent of goblet cell secretion after injection of PolyHbBv into animals that were not pretreated with Na2SeO3 produced an average of 2.27 ± 0.24 secreting goblet cells per villus near Peyer’s patches. Corresponding values for animals pretreated with Na2SeO3 were 3.10 ± 0.25 and 2.40 ± 0.21, respectively. Thus pretreatment of animals with Na2SeO3 significantly increased goblet cell secretion in both locations. In contrast, in animals injected with DBBF-Hb, pretreatment with Na2SeO3 did not significantly alter goblet cell secretion near Peyer’s patches (from 3.41 ± 0.25 without pretreatment to 3.11 ± 0.25 with pretreatment) or away from Peyer’s patches (from 1.41 ± 0.17 to 1.16 ± 0.12).

*Epithelial damage.* Electron micrographs of intestinal mucosa from rats perfused with PolyHbBv looked very similar to those from another study in which rats were perfused with HBS-BSA instead of with a modified Hb (8). As seen in Fig. 6A, the epithelial intercellular junctions (arrows) were tight and the cells were closely adhering to the basement membrane. In

Fig. 4. Mesenteric preparation to measure mast cell degranulation. Average number of mesenteric degranulated mast cells per microscopic field of view for networks perfused with PolyBvHb or O-R-PolyHbA0, with and without Na2SeO3. Results from previous experiments using DBBF-Hb or CNmet-DBBF-Hb are also included for comparison. *Significant difference from corresponding group in which Na2SeO3 was also used. Error bars indicate SE.
animals that were treated with both PolyHbBv and Na₂SeO₃ (Fig. 6B), the epithelial cells were often partially separated from each other and showed thin, tortuous, cytoplasmic projections along their lateral membranes (arrows). Occasionally, cells were detached from the basement membrane. The average EI indexes for each group are depicted in Fig. 7. Injection of PolyHbBv into animals that were not pretreated with Na₂SeO₃ produced an average EI of 1.08 ± 0.03 near Peyer’s patches and 1.07 ± 0.02 away from Peyer’s patches. Corresponding values for animals pretreated with Na₂SeO₃ were 1.24 ± 0.05 and 1.23 ± 0.05. Treatment with Na₂SeO₃, before injection with PolyHbBv, significantly increased EI and thus reduced epithelial integrity, in both cases. In contrast, treatment with Na₂SeO₃, before injection with DBBF-Hb, had the opposite effect. Near Peyer’s patches, the values for EI without and with Na₂SeO₃ were 1.22 ± 0.03 and 1.03 ± 0.02, respectively, and away from Peyer’s patches the corresponding values were 1.37 ± 0.04 and 1.25 ± 0.04.

**Effect of Na₂SeO₃ on the Oxidation of PolyHbBv and O-R-PolyHbA₀**

We first monitored the effects of inclusion of 500 μM Na₂SeO₃ with PolyHbBv as it undergoes normal spontaneous oxidation under physiological conditions in HBSS buffer, pH 7.4, and 37°C for 6 h. The calculated rates of autoxidation of PolyHbBv with and without Na₂SeO₃ were only very slightly decreased by Na₂SeO₃. We then focused on the effects of Na₂SeO₃ on PolyHbBv oxidation under mild oxidative stress induced by low and steady supplies of H₂O₂, at a rate of 1.5 μM min⁻¹ generated by the GOX system (17). The rate for the exponential decrease in oxy-PolyHbBv under these conditions was calculated to be 0.0454 min⁻¹. However, in the presence of Na₂SeO₃, the rate of oxy-PolyHbBv decay was 0.0355 min⁻¹, ~22% slower than PolyHbBv/GOX medium alone. Similarly, the rate of met-PolyHbBv formation in the presence of Na₂SeO₃ was 0.0445 min⁻¹, ~24% lower than 0.0584 min⁻¹ calculated for PolyHbBv/GOX alone. Na₂SeO₃ had the opposite effect on the oxidation of O-R-PolyHbA₀. Figure 8 shows typical time courses of the oxy-O-R-PolyHbA₀ disappearance and met-O-R-PolyHbA₀ buildup as a function of time in the presence of the GOX system and in the presence or absence of Na₂SeO₃. The rate for the exponential decrease in oxy-O-R-PolyHbA₀ was calculated to be 0.03132 min⁻¹. However, in the presence of Na₂SeO₃, the rate of oxy-O-R-PolyHbA₀ decay was 0.03736 min⁻¹, ~19% faster than O-R-PolyHbA₀/GOX medium alone. Similarly, the rate of met-O-R-PolyHbA₀ formation in the presence of Na₂SeO₃ was 0.05270 min⁻¹, ~11% higher than 0.04736 min⁻¹ calculated for O-R-PolyHbA₀/GOX. Na₂SeO₃ had a more marked effect on suppressing the oxidation of DBBF-Hb under mild oxidative stress than it did with PolyHbBv. For DBBF-Hb, the rate of decrease in oxy-DBBF-Hb was reduced by 50% in the presence of Na₂SeO₃, and the increase in met DBBF-Hb formation was decreased by 70%. To investigate the potential direct redox interactions between modified Hb oxidation forms and Se, we measured the rates of reduction of the ferric iron of the modified Hbs in the presence and absence of Na₂SeO₃. These results were compared with those obtained from incubation of Se with the met form of DBBF (Fig. 9). It is clear that, as the concentration of Se increases, there was a corresponding increase in the reduction in the levels of metHb in PolyHbBv, O-R-PolyHbA₀ and DBBF-Hb solutions, although the effect was most marked for DBBF-Hb and least marked for O-R-PolyHbA₀. The rapid reduction in the met forms of Hbs, even at low Se concentration, is not surprising because Se occupies a higher position in the pecking order of free radicals than iron in terms of standard reduction potential and is therefore capable of donating electrons to the ferric iron of Hb (13). However, similar concentrations of Se showed no effects on the levels of metHb trapped by cyanide, confirming the need...
for an unliganded binding site for the redox communication to occur between Se and the ferric iron of PolyHbBv, O-R-PolyHbAO, and DBBF-Hb.

Effect of Na₂SeO₃ on the Oxygen Affinity of DBBF-Hb

Increasing concentrations of Na₂SeO₃ did not appear to affect the oxygen affinity of PolyHbBv, as demonstrated by the constancy of values of P₅₀ and the cooperativity (Hill number) in the presence or absence of reducing enzymes. In the presence of enzymes, an average P₅₀ value of 30.8 ± 0.4 Torr was obtained for PolyHbBv regardless of the Se concentrations used. This value is close to that calculated for Hb solutions without Se (control) (P₅₀ = 31.21 Torr). We have previously obtained similar results using DBBF-Hb (10) and more recently for O-R-PolyHbAO; the average P₅₀ value for DBBF was 29.4 ± 0.9 Torr in the presence of 125 μM to 1 mM Na₂SeO₃ and 29.0 Torr in the absence of Na₂SeO₃. For O-R-PolyHbAO the average P₅₀ value was 36.5 ± 1.9 Torr in the presence of 125 μM to 1 mM Na₂SeO₃ and 29.0 Torr in the absence of Na₂SeO₃.

DISCUSSION

This study has shown that the modified Hbs, DBBF-Hb and O-R-PolyHbAO, both produce microvascular leakage to FITC-BSA in the rat mesentery to a similar degree, as judged by total leak area per unit length of venule, that is significantly greater than for preparations perfused with HBS-BSA. On the other hand, PolyHbBv does not produce significant microvascular leakage. There is a growing experimental evidence to support the idea that modified Hbs, injected in vivo, can cause tissue damage and organ dysfunction by producing highly reactive oxygen species (8, 20, 28). Modified Hbs spontaneously autooxidize to produce metHb and superoxide anions (O₂⁻).

Superoxide is also produced from the respiratory burst of phagocytes that are activated by Hb. Superoxide undergoes the dismutation reaction, in the presence of superoxide dismutase, which is contained within the cells, with consequent production of H₂O₂. The H₂O₂ is normally decomposed by its reaction with catalase or with glutathione peroxidase. However, these enzymes are inhibited by superoxide (12). It has been postulated that the excess H₂O₂ then reacts with metHb in vivo to initiate further oxidation cycles resulting in the formation of highly reactive ferryl Hb, in which the iron center is at higher oxidation state (Fe⁴⁺) (28). The interaction of ferryl Hb with H₂O₂ results in the formation of rhombic heme, which is considered to be one of the best measures of the toxicity of a blood substitute (23). The rhombic heme in which the geometry of the iron is distorted due in some instances to the
chemical modification of the protein then initiates a cascade of oxidative side reactions resulting in the formation of free iron. Iron in turn catalyzes the production of the highly reactive hydroxyl radical. Interestingly, measured values for formation of rhombic heme were more than tenfold greater for DBBF-Hb and O-R-PolyHbA0 than for PolyHbBv (23), consistent with the relative degrees of microvascular injury produced by these substances as reported in the present study.

This study has demonstrated that coinjection of Na2SeO3 with modified Hbs in the microvasculature may significantly reduce, have no effect on, or somewhat enhance the tissue damage mediated by the Hb and that the result is dependent on the interaction of the Na2SeO3 with the particular modified Hb. Dietary supplemented Se, an antioxidant, has been shown to reduce Hb-mediated lipid peroxidation in the liver (28). Se is thought to act as an antioxidant in the body because it is a component of the enzyme glutathione peroxidase, which catalyzes removal of H2O2 (30). However, Simoni et al. (28) only found slightly increased concentrations of glutathione peroxidase in the livers of Se-supplemented rats, even though the tissue was resistant to Hb-induced damage. They hypothesized that the Se had been incorporated into other, nonglutathione peroxidase antioxidant cellular systems, such as seleno-amino acids (18, 35). As another possibility, a selenoprotein from the heart and muscle of lambs has been isolated that may be a Se-containing cytochrome with a possible participation in oxidation-reduction reactions (32). However, Simoni et al. concluded that the exact mechanism by which Se exerts its protective role against Hb-induced oxidative injury could not be proposed but that the concomitant injection of Se with Hb solutions seemed to be the most practical in the case of emergency situations.
In a more recent study, Chen and Lin (15) gave rats SeO2 daily for 14 days and observed increases in oxyHb in which iron is in its ferrous form and decreases in metHb in the blood. Because they also saw increased concentrations of glutathione peroxidase activity in the blood of rats fed SeO2, they concluded that this enzyme must be responsible for change in the oxy-met ratio of the Hb because it reduces the presence of H2O2. However, the results of the present study demonstrate that there is no need for glutathione peroxidase to be involved in this process; merely combining Na2SeO3 with DBBF-Hb, or to a lesser extent with PolyHbBv, in vitro, produced a similar result. Thus Se can directly alter the redox state of modified Hbs, such that the oxy-met ratio is increased, within a very short time period.

The results of the in vivo experiments described in the present study demonstrate that there is no need to pretreat rats previous to injection with modified Hbs to produce the beneficial effects of Se on the microvasculature. These results are consistent with the idea indicated by the in vitro experiments that Se exerts its beneficial effects by reacting directly with the Hb rather than by becoming incorporated into an enzyme that scavenges reactive oxygen species. This study has also shown, in three different modified Hbs, that the interaction of Se does not alter the oxygen-carrying capacities of the Hbs and thus does not compromise their function as Hb-based oxygen carriers. However, although Na2SeO3 significantly reduced the tissue damage produced by DBBF-Hb, it had no effect on damage induced by O-R-PolyHbA0, and its effect with PolyHbBv was deleterious. These differing effects in vivo are consistent with the results of the in vitro experiments showing that Na2SeO3 does not affect the redox properties of PolyHbBv and O-R-PolyHbA0 as much as those of DDBBF-Hb. For example, Na2SeO3 was not so effective at decreasing the oxidation of PolyHbBv and O-R-PolyHbA0, compared with DBBF-Hb, under mild oxidative stress or of increasing the rates of reduction of the met forms of PolyHbBv and O-R-PolyHbA0. It is possible that Na2SeO3 may alter the extent of formation of heme degradation products, such as rhemic heme, but further experiments are required to test this hypothesis.

The in vivo experiments demonstrated that, for PolyHbBv and O-R-PolyHbA0, Na2SeO3 actually increased mesenteric mast cell degranulation. For PolyHbBv, further experiments showed that goblet cell secretion and epithelial disruption were also increased with coadministration of Na2SeO3. These results are consistent with the evidence that the Na2SeO3 did not interact with the PolyHbBv and O-R-PolyHbA0 as effectively as it did with DBBF-Hb, and thus some Se was free to react with other components in the body. Our previous experiments in which Na2SeO3 was perfused in the microvasculature with phosphate-buffered saline, rather than with a modified Hb, or administered as a dietary supplement to animals that were not intravascularly injected with a modified Hb showed similar results (10). A possible explanation is that the Se activated neutrophils and macrophages to produce oxidants (3, 4). In addition, Na2SeO3 is metabolized by interaction with reduced glutathione, and reactive oxygen species are by-products of this reaction (27, 34). Reduced glutathione is present in erythrocytes and also in neutrophils (14) and macrophages (26). Reaction of the Se with immune cells, or with reduced glutathione, could be responsible for the effect of Na2SeO3 on preparations perfused with PolyHbBv or O-R-PolyHbA0. In cases in which DBBF-Hb is present, Na2SeO3 interacts with the DBBF-Hb, as shown by our in vitro results and thus may not be free to stimulate immune cells. Thus the interaction is synergistic and the prooxidant properties of both molecules are reduced. These experiments indicate that Se compounds will only be useful adjuncts to Hb-based blood substitutes for which it can be shown, in advance, that the oxidation state is significantly reduced by interaction with the Se.

The relatively small degree of microvascular leakage and mast cell degranulation accompanying perfusion with CNmet-DDBBF-Hb, vs. DBBF-Hb, is consistent with the hypothesis that the tissue damage induced by Hbs is caused by their oxidation and accompanying formation of reactive oxygen species. As mentioned previously, CNmet-DDBBF-Hb is a Hb in which the CN groups are tightly bound to the heme such that the molecule is unable to participate in redox reactions. The fact that Na2SeO3 did not significantly affect the microvascular leakage and mast cell degranulation that accompanied perfusion with CNmet-DDBBF-Hb, whereas it increased these responses after Hb-BSA perfusion, suggests that the Na2SeO3 interacted with the CNmet-DDBBF-Hb and thus was not free to stimulate immune cells.

Both the in vivo and the in vitro experiments in this study are consistent with a hypothesis called the Se link hypothesis that was introduced by Nyberg-Swenson (24). In this model, Se constitutes a protective system that prevents the direct transfer of electrons and perhaps also prevents contact between the substrate being oxidized and oxygen, thus impeding the formation of oxygen compounds. If this hypothesis is applied to autooxidation of oxyHb, this would mean that the Se would be placed in such a position such that the transfer of one electron from the HbFe2+/H2O2 to the attached O2 would be impeded, and so the metHb, or HbFe3+, and superoxide would not form. However, in the present study, Na2SeO3 had little effect on the autooxidation of the modified Hbs, and thus it is unlikely that Se formed such a link. In contrast, Na2SeO3 reduced the oxidation of DDBBF-Hb and, to a lesser extent, PolyHbBv in the presence of H2O2. When HbFe2+ reacts with H2O2, it donates two electrons to the H2O2 to form ferryl Hb (HbFe4+) and OH-. The ferryl Hb then accepts one electron from another HbFe2+ molecule to form two molecules of metHb or HbFe3+ (16). Alternatively, a second H2O2 molecule reduces the ferryl Hb to metHb, and a superoxide radical is formed in the heme pocket, which then attacks the heme. If the Se link hypothesis were applied to the reaction to form ferryl Hb, this would mean that the Se would be positioned such that the Fe2+ would be separated from the H2O2 and hence unable to donate two electrons. The effect of Na2SeO3 in decreasing the rate of oxidation of oxyHb to metHb, demonstrated in the presence of H2O2, is consistent with the Se link hypothesis.

To summarize, the in vivo and in vitro experiments performed in this study demonstrate that Se can reduce the oxidative damage produced by some modified Hbs, but only if it interacts sufficiently with the Hb molecule to reduce its rate of oxidation. The fact that Se compounds can reduce oxidative damage by directly interacting with the modified Hb, and without changing its oxygen-carrying capacity, makes them ideal for use in clinical situations. Because the effect is not dependent on incorporation of the Se into an enzyme system, there is no need to pretreat the patient; the Se compound can be coinfected with the modified Hb. This technique could also be
used to improve the storage properties of blood substitutes. However, the differing efficacy of Na2SeO3 in reducing the tissue damage caused by the three Hb-based oxygen carriers used in the present study highlights the importance of knowing the redox chemistry of the blood substitute in question.

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GRANTS

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