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Effects of *S*-Nitrosation on Hemoglobin-Induced Microvascular Damage

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ABSTRACT

Blood substitutes, such as diaspirin cross-linked hemoglobin (Hb), cause microvascular leakiness to macromolecules. Because of the potentially stabilizing effects of nitric acid (NO) on endothelium, experiments were performed to determine whether *S*-nitrosohemoglobin (SNO-Hb), a potential NO-donor Hb-based blood substitute, would not cause microvascular damage. Release of NO, or its metabolites, from the SNO-Hb was facilitated by addition of glutathione, which aids in the decomposition of *S*-nitrosothiols. In anesthetized rats, the mesenteric microvasculature was perfused with SNO-Hb with glutathione (six rats), SNO-Hb alone (six rats), or saline (eight rats) for 10 min, followed by fluorescein isothiocyanate (FITC)-albumin for 1 min, and finally fixed for epifluorescence microscopic examination. When comparing the SNO-Hb group with saline, both the numbers and areas of leaks were significantly increased [0.019 ± 0.003 (SEM) μm vs. 0.0030 ± 0.0004 and 7.36 ± 1.50 vs. 0.156 ± 0.035 ($p < 0.005$)]. With the addition of glutathione, leakage was still high (0.005 ± 0.00005 μm and 5.086 ± 0.064 μm) but decreased compared with SNO-Hb alone ($p < 0.005$). In conclusion, NO, or a related vasodilator, when released from SNO-Hb, significantly reduces but does not eliminate microvascular damage. Further improvements may result by *S*-nitrosating a more stable form of modified hemoglobin. *Antioxid. Redox Signal.* 8: 1093–1101.

INTRODUCTION

The necessity for blood substitutes is underscored by current concerns over shortages in donated blood supplies, contamination with infectious agents, and limitations associated with immune responses elicited by differing blood-group antigens between donor and recipient. Furthermore, a blood substitute would ideally also have a longer shelf-life than would donated blood. Hemoglobin-based oxygen carriers, or blood substitutes, have received much attention in this regard, but a variety of biologic properties associated with them have hindered their development. It is now well appreciated that cell-free hemoglobin disrupts a variety of biologic processes, leading to toxicity and observed failure of such compounds in clinical trials. These include effects on the cardiovascular system, disruption of vascular homeostasis mechanisms, changes in gastrointestinal metabolism, immune/inflammatory cell activation, induction of coagulation cascades, oxidative stress, and decreased host resistance to overwhelming infections (16).

We previously showed that one mechanism through which hemoglobin-based blood substitutes induce vascular toxicity is stimulating microvascular permeability. Specifically, diaspirin cross-linked hemoglobin (DBBF-Hb), polyethylene glycol (PEG)-conjugated Hb, bovine polymerized hemoglobin (PolyHbBv), and a human polymerized hemoglobin (O-R-PolyHbAo) induced macromolecular leakage in the rat mesenteric microvasculature (4, 5). The underlying mechanism for this effect has been postulated to include scavenging of endothelium-derived nitric oxide (NO; which also results

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in hypertensive responses) and formation of reactive oxygen species (ROS) via either metal release or heme redox cycling or both (1, 8). Interestingly, we also showed that NO donors protect mesenteric venules against histamine-induced leaks (2). However, little is known about the interplay between therapeutic delivery of NO and hemoglobin-induced microvascular leakage.

S-Nitrosohemoglobin (SNO-Hb) represents a derivative of hemoglobin in which the β93cysteine residue is *S*-nitrosated. SNO-Hb has been extensively discussed in the context of hypoxic blood flow, with studies providing conclusions that range from a key role to little or no role for SNO-Hb in modulating vascular tone under physiologic conditions (9, 13, 25). More-recent studies implicate a role for this derivative in contributing to dysfunctional hemodynamics associated with sepsis (12). Despite the contrasting views on the biologic function of SNO-Hb, a consistent observation has been that *S*-nitrosation of Hb imparts NO-donor–like properties on this protein. In the presence of thiols, cell-free SNO-Hb stimulates vasodilatation of isolated vessels, inhibits platelet aggregation consistent with NO-donor functions (8, 14, 19, 29), and recently has been shown to modulate tumor blood flow *in vivo* (26). The precise mechanisms and identity of NO-dependent species released from SNO-Hb/thiol interactions that mediate NO-dependent responses in the vascular wall remain unclear but could involve formation of other NO-derivatives including *S*-nitrosothiols (via transnitrosation reactions) or nitroxyl (via reduction reactions) (27). Nevertheless, such properties of SNO-Hb have led to the hypothesis that *S*-nitrosation of cell-free hemoglobin may ameliorate toxic effects of cell-free hemoglobin, providing a novel therapeutic strategy in the development of blood substitutes (22, 25). Consistent with this concept, administration of an *S*-nitrosated polyethylene glycol–modified hemoglobin to rats did not induce hypertensive effects (20).

In this study, we hypothesized that because of NO delivery, *S*-nitrosation of hemoglobin would prevent increases in microvascular permeability.

METHODS

Hemoglobin solutions

SNO-Hb, *N*-ethylmaleimide (NEM)-Hb, and OxyHb were prepared as previously described by using human oxyhemoglobin (17, 21). In brief, SNO-Hb was prepared by transnitrosation by using *S*-nitroso-*N*-acetylpenicillamine (10:1 ratio heme) in borate buffer + 100 μ*M* diethylenetriaminepentaacetate (DTPA), pH 8, at 20°C for 30 min, and subsequent purification by gel-filtration chromatography with phosphate-buffered saline (PBS) + 100 μ*M* DTPA, pH 7.4. NEM-modified hemoglobin (to block β93cysteine and thus act as a control for SNO-Hb) was prepared similarly by using a final ratio of NEM to heme (10:1). Hemoglobin solutions were stored at 4°C and used within 3–5 days of preparation. Immediately before experiments, hemoglobin solutions were characterized by visible spectroscopy (samples with >0.5% metHb were excluded) and Saville reaction to quantify *S*-nitroso concentration and were between 1 and 1.5 SNO/ tetramer. DBBF-Hb was a generous gift from the Walter Reed Army Institute of Research (Washington, DC) and represents a hemoglobin cross-linked by bis(3,5-dibromosalicyl) fumerate.

Pre-experimental treatment of rats

The research described in the study adheres to the American Physiological Society's "Guiding Principles of the Care and Use of Animals," and the protocol was approved by the Institutional Animal Care and Use Committee. Sprague–Dawley rats weighing between 300 and 400 g were used. The rats were housed two per cage, in enriched cages (16 x 12 × 12 in, with ramp leading to 6 × 4-in shelf and 8-in long PVC tube). Noise from personnel was minimized in the facility. Monthly tests are preformed on sentinel rats.

Anesthesia

Fifty-seven Sprague–Dawley rats were anesthetized with 1 ml/kg body weight of the following mixture: ketamine hydrochloride (5 ml of 100 mg/ml), acepromazine maleate (2 ml of 0.2 mg/ ml), and xylazine (8 ml of 20 mg/ml).

Mesenteric preparation to measure microvascular leakage

Surgical procedure. Experiments were performed to characterize the mesenteric microvascular leakage to albumin caused by intravenous injection of either SNO-Hb, SNO-Hb with glutathione, NEM-Hb, NEM-Hb with glutathione, OxyHb, OxyHb with glutathione, DBBF-Hb, glutathione alone, or HEPES-buffered saline with 0.5% bovine serum albumin, pH 7.4 (HBS-BSA) as control. The mesenteric microvasculature was perfused for 10 min with 5 ml of one of these solutions. All of the blood products were diluted to 2 mg protein/ml. When glutathione was used, the concentration was 0.03 g/1,000 ml in the perfusate.

The surgical procedure was similar to that described previously (2, 4, 5). In brief, after anesthesia, the abdomen was opened along the midline, and mesenteric windows that were well vascularized were exposed. A mesenteric window is defined as the tissue extending between two adjacent feeding arterioles in the mesentery (4). The superior mesenteric artery was cannulated and used to perfuse with HBS-BSA and 1 U/ml heparin at 37°C, followed by one of the solutions described earlier. The preparation was kept moist with HBS-BSA. A portal clamp was used to prevent backflow of blood. After 10 min, the clamp was removed, and the windows were perfused with 5 ml BSA labeled with fluoroscein isothiocyanate (FITC) (Sigma Chemical), a fluorescent tracer. The system was then perfused with 5 ml of fixative (3% formaldehyde in HBS), and the animal killed with 1 ml of phenytoin and pentobarbital (Beuthanasia) into the heart. Gauze pads covering the mesenteric windows were soaked with the fixative, left for 60 min, and the windows nonquenching mounting medium (Vectashield; Vector Laboratories; Burlingame, CA).

Assessment of venular leakage. Venular leakage was assessed by measuring the numbers and areas of regions along venules that showed extravascular FITC-albumin. Each

slide was examined by using a Zeiss Axioplan microscope with ×10 objective, numeric aperture 0.6, fitted for epifluorescence. The light source was a 100-W mercury lamp for epifluorescence and a halogen lamp for transmitted illumination (5). A video camera (Olympus DP70) was mounted at the camera port of the microscope. Microvascular networks in each mesenteric window were scanned systematically by using epifluorescence with the appropriate excitation and emission filters (λ = 488 and 515 nm, respectively) and then videorecorded. The images (~10 per window) were analyzed by using an analog-to-digital converter and appropriate software (NIH-Image) to measure the length and diameter of each vessel, the number of leaks per venule, and the area of each leak. The data for each group were pooled and calculated to yield the average number of leaks per unit length of venule and the average leak area per unit length of venule.

Statistical analysis

Both the numbers of leaks and areas of leaks were compared between groups by using the Mann–Whitney Rank Sum Test with a p value <0.05.

RESULTS

Quantification of leaks

Microscopic examination of venular leaks by epifluorescence microscopy showed that administration of cell-free Hbs increased FITC-albumin leakage compared with control. The total numbers of venules examined for each preparation were as follows: Oxy-Hb (n = 164), Oxy-Hb with glutathione (n = 205), SNO-Hb (n = 246), SNO-Hb with glutathione (n = 371), NEM-Hb (n = 214), NEM-Hb with glutathione (n = 371), DBBF-Hb) (n = 209), glutathione (n = 341), control 1 (n = 334), control 2 (n = 128). Interestingly, the severity of leakage, as judged by leak area per unit length of venule, was significantly greater in preparations perfused with SNO-Hb relative to control, Oxy-Hb, or NEM-Hb (Figs. 1 and 4B). Coadministration of glutathione with SNO-Hb preparation significantly decreased the number of leaks and the area of leaks relative to SNO-Hb alone (Figs. 2 and 4). Although SNO-Hb produced the greatest leak area per unit length of venule, the number of leaks was significantly lower than for Oxy-Hb, Oxy-Hb with glutathione, NEM-Hb, and NEM-Hb with glutathione (see Figs. 3 and 4).

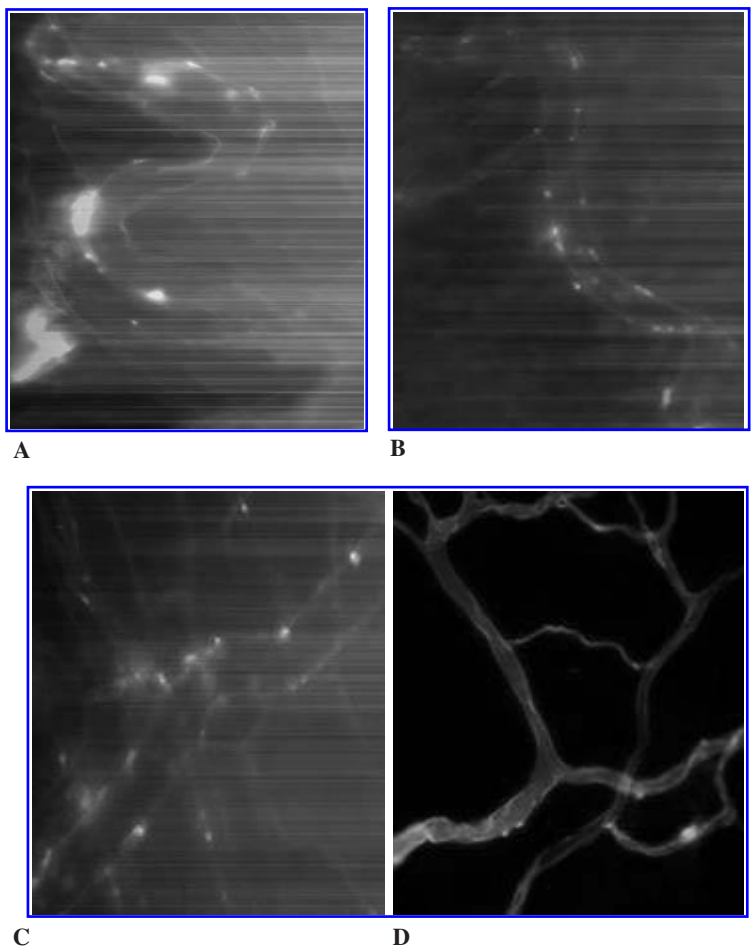

FIG. 1. Light micrographs of FITC-albumin leakage after perfusion of the following: (A) SNO-Hb with large leaks; (B) Oxy-Hb with small leaks; (C) NEM-Hb with many small leaks; and (D) HBS-BSA with no leaks.

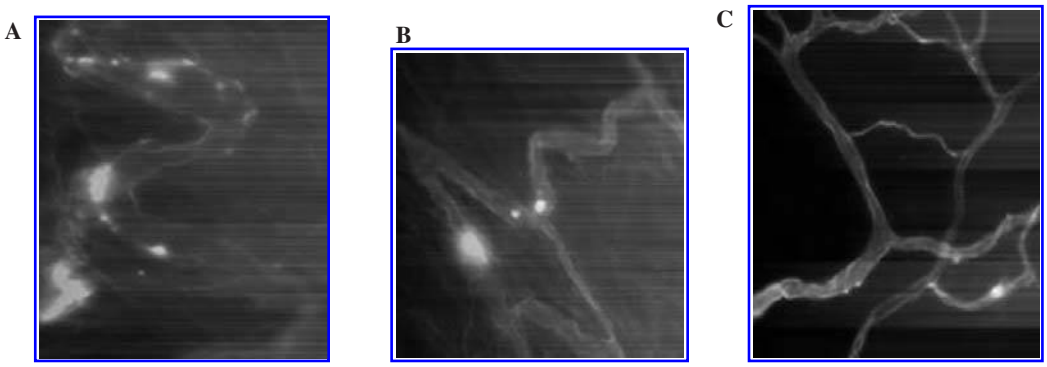

FIG. 2. Light micrographs of FITC-albumin leakage after perfusion of (A) SNO-Hb, where many large leaks are visible; and (B) SNO-Hb with glutathione, where the leakage is decreased but not to the point of (C) control.

The mean number of leaks per micrometer of venule length for each treatment were as follows (Fig. 4A): OxyHb (0.030 ± 0.002), OxyHb with glutathione (0.036 ± 0.002), SNO-Hb (0.019 ± 0.003), SNO-Hb with glutathione (0.010 ± 0.001), NEM-Hb (0.025 ± 0.002), NEM-Hb with glutathione (0.036 ± 0.002), DBBF-Hb (0.014 ± 0.001), glutathione (0.006 ± 0.001), Control 1 (0.0030 ± 0.0004), and Control 2 (0.008 ± 0.001). Figure 4B illustrates the mean leak area per micrometer of venule length. The mean leak areas per micrometer of venule length were as follows: OxyHb (2.647 ± 0.306),

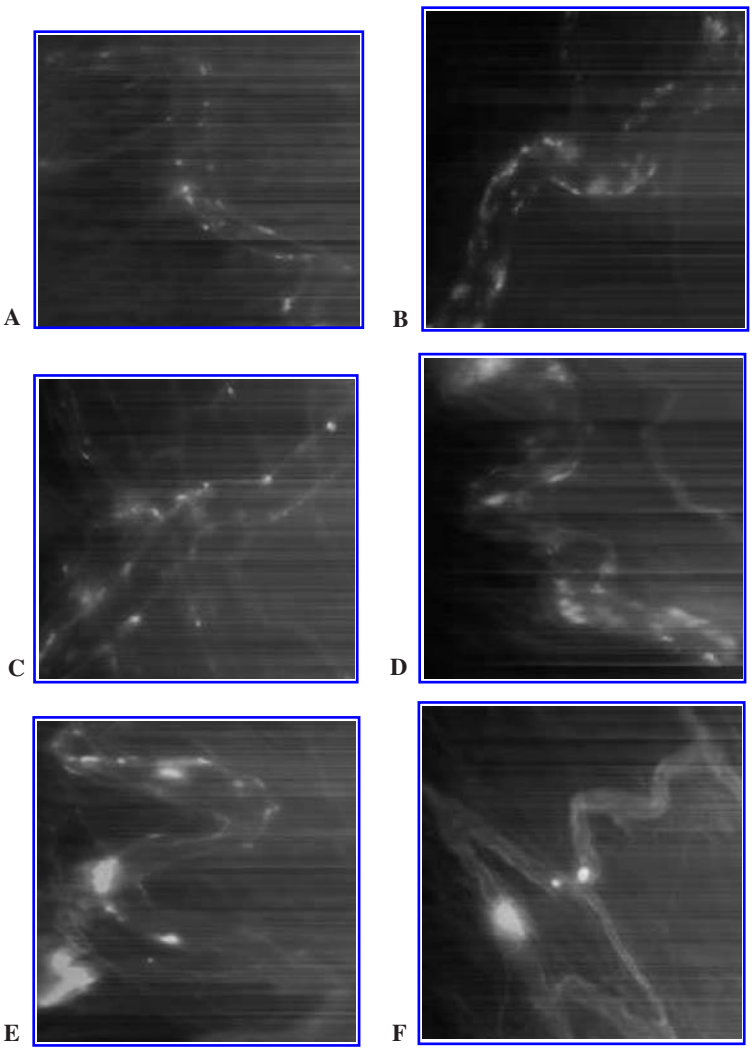

FIG. 3. Light micrographs of FITC-albumin leakage after perfusion of: Oxy-Hb (A), Oxy-Hb with glutathione (B), NEM-Hb (C), NEM-Hb with glutathione (D), SNO-Hb (E), and SNO-Hb with glutathione (F). Greater numbers of smaller leaks were observed after perfusion with OxyHb and NEM-Hb, with and without glutathione, compared with perfusion with SNO-Hb, with and without glutathione.

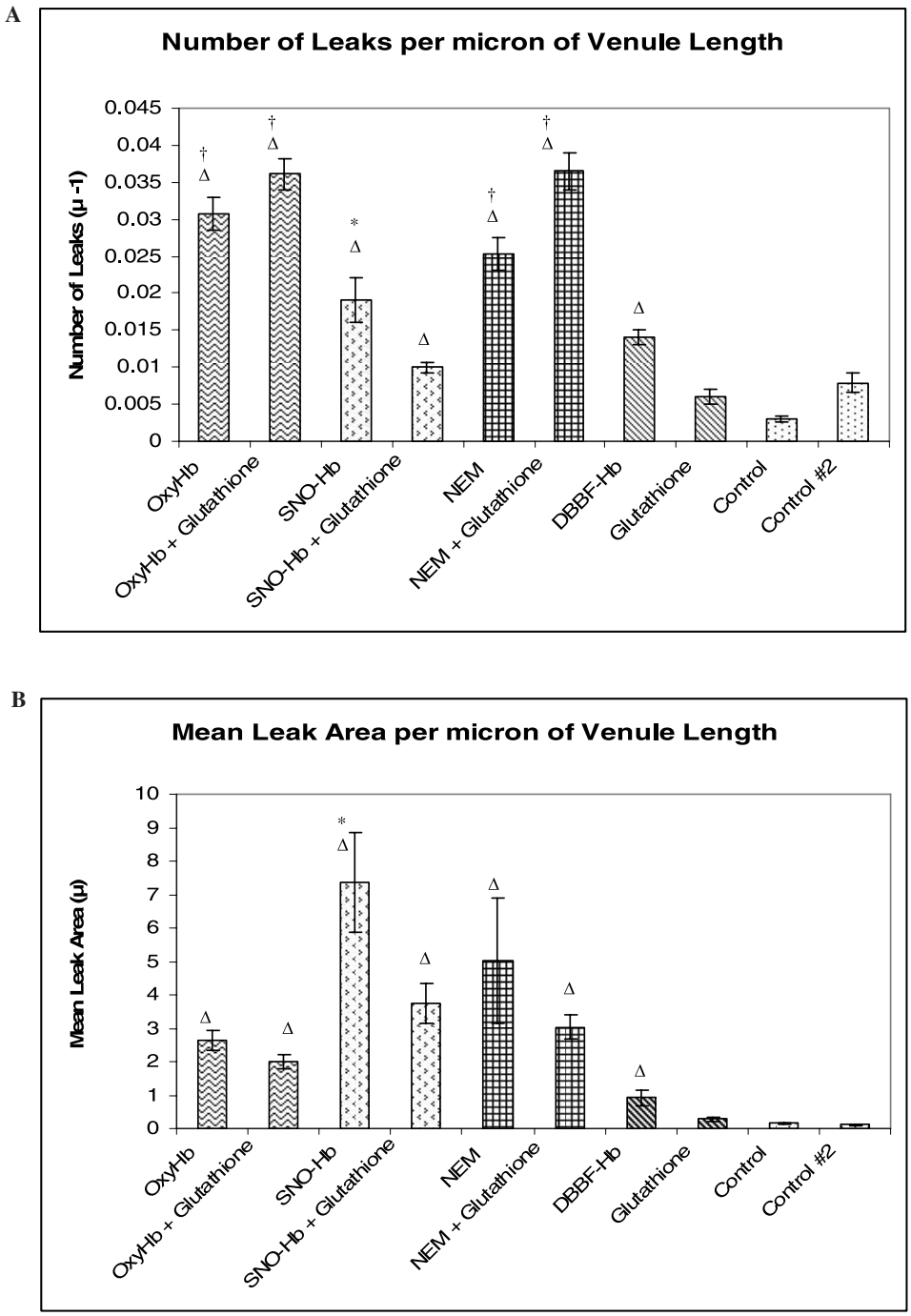

FIG. 4. (A) Average number of mesenteric leaks per venule length (μm) for different treatments. (**B)** Average total leak area per venule length ($\mu m^2/\mu m$). *Significantly greater than SNO-Hb with glutathione; †significantly greater than SNO-Hb; Δsignificantly greater than control.

OxyHb with glutathione (2.008 ± 0.210, SNO-Hb (7.360 ± 1.500), SNO-Hb with glutathione (3.740 ± 0.590), NEM-Hb (5.035 ± 1.872), NEM-Hb with glutathione (3.040 ± 0.370), DBBF-Hb (0.930 ± 0.230), glutathione (0.283 ± 0.056), Control 1 (0.156 ± 0.030), and Control 2 (0.110 ± 0.035). The control 1 data were taken at the beginning of the experiment, whereas the control 2 data were taken at the end to verify no change over the period of time. Because no significant difference was found between the two values, the control data were combined for statistical comparison with the other groups.

To test whether modification of the β93 cysteine and loss of this reactive thiol were mediating the increased area of

leaks caused by SNO-Hb, this residue was alkylated by NEM. Similar to SNO-Hb, NEM-Hb significantly increased the area of leaks compared with Oxy-Hb (Fig. 4B). Interestingly, the addition of glutathione to SNO-Hb significantly decreased the number and area of leaks but had no effect on leakage when used with Oxy-Hb, suggesting that direct antioxidant effects of glutathione were not affecting changes in permeability, but interactions with SNO-Hb were required. Although addition of glutathione to NEM-Hb significantly reduced leak area, the number of leaks was increased, indicating that the effects of glutathione are dependent on the type of hemoglobin (*S*-nitrosated vs. alkylated) and also that this low-molecular-weight thiol may inhibit expansion and fusion of small leaks.

The size distribution of leaks varied considerably within preparations, ranging from <5 μm^2 to 150,000 μm^2 in the case of SNO-Hb and to 12,000 for NEM-Hb and Oxy-Hb. It should be noted that these areas were derived from measurements of diffused fluorescence, and so the structural gaps between the endothelial cells were far smaller.

Because glutathione reduced leak number only when used with SNO-Hb but not with the other Hb products, it was hypothesized that a specific interaction between glutathione and SNO-Hb was preferentially inhibiting formation of small leaks. To test this hypothesis, the leak data (Fig. 4) were modified by omitting the smallest leaks (with an area of ≤ 5 μm^2) (Fig. 5). Figure 5A represents the number of leaks per micrometer of venule length, whereas Fig. 5B represents the mean area per micrometer of venule length. This evaluation was performed only for OxyHb, SNO-Hb, and NEM-Hb, with and without glutathione. As illustrated for all six groups, when the small leaks were omitted from data analysis, the numbers of leaks per unit length of venule were decreased, whereas the mean leak area did not change significantly. The percentage decrease in leak number was 18%, 19%, 39%, 28%, 17%, and 15% for OxyHb, OxyHb with glutathione, SNO-Hb, SNO-Hb with glutathione, NEM-Hb, and NEM-Hb with glutathione, respectively. Thus, the glutathione reduced the percentage of small leaks only when used in conjunction with the SNO-Hb, as hypothesized. In addition, a greater proportion of the leaks formed by SNO-Hb, with or without glutathione, were small (≤ 5 μm^2) compared with those induced by OxyHb or NEM-Hb. This finding may seem inconsistent with the fact that SNO-Hb produced the largest mean leak area per square micrometer but can be explained by the presence of some much larger leaks formed by SNO-Hb compared with OxyHb and NEM-Hb.

DISCUSSION

S-Nitrosation of hemoglobin has been proposed as a potential strategy to ameliorate hypertensive and other effects that limit the development of cell-free hemoglobins as blood substitutes (22, 25). Many studies have documented that SNO-Hb can stimulate vascular cell signaling typically associated with NO bioactivity, and as proof of concept, *S*-nitrosation of PEG-Hb prevented vasoconstriction when administered to rats (20). This study was undertaken to determine the effects of hemoglobin *S*-nitrosation on another important element in maintaining vascular homeostasis, microvascular permeability.

S-nitrosation of cell-free hemoglobin decreased the number of leaks compared with OxyHb. However, the ultimate functional impact of increased microvascular permeability is a determinant not only of the number of leaks, but also of the size (*i.e.*, mean area) of leaks. Surprisingly, SNO-Hb–induced leaks were 2 to 3 times greater in total area than those formed by Oxy-Hb, even though the number of leaks was significantly lower. To explain these findings, we hypothesize that small leaks form first and then increase in number with severity of damage, so that eventually the small leaks coalesce to form large leaks, so that the actual number of leaks may be reduced. Evidence for this hypothesis is derived from a study in which we perfused the mesenteric microcirculation with DBBF-Hb and then stained the endothelial cell junctions with silver nitrate that deposited in the junctional gaps formed by the DBBF-Hb (4). In these preparations, the gaps were arranged in clusters along the venules, and the number of gaps was more closely correlated with the area of fluorescent leaks than was the number of leaks, suggesting that neighboring gaps may contribute to the same leak. A few large leaks would be more detrimental to tissues than would many small leaks because a greater range of molecular species would be able to enter the tissue from the bloodstream. For that reason, even though SNO-Hb produced significantly fewer leaks than oxyHb, we conclude that SNO-Hb caused the most damage because it produced the largest mean leak area.

Similar effects were observed with NEM-Hb, suggesting that modification of the β93cys residue rather than *S*-nitrosation *per se* was responsible for the increased mean leak area. One possibility relates to the effect of β93cys modification on the structural stability of the hemoglobin tetramer. *S*-nitrosation or alkylation (by NEM) of Hb increases the oxygen affinity, and by inference, the "R"-state conformation of Hb (6, 18, 21). Relative to the "T"-state, "R"-state Hb has a higher propensity to dissociate into dimers that may account for the increases in permeability observed. Indeed, cross-linking Hb to stabilize the tetrameric structure inhibits the increased permeability, a result confirmed with the use of DBBF- Hb in this study. Conversely, Boykins *et al*. (7) showed that if cys93 is blocked, as is the case with *O*-raffinose polyHbAo (Hemolink), the Hb becomes locked in the T-state, damaging the heme, which can then damage the surrounding tissue and increase microvascular permeability. Another possible explanation for the high-leak area caused by SNO-Hb is that modification of the β93cys residue results in a net loss of a thiol that could otherwise participate in scavenging and limiting vasculotoxicity caused by reactive species. Indeed, previous studies have shown that the β93cys is readily oxidized on exposure of hemoglobin to oxidants and thus has a protective function (3). Moreover, cell-free hemoglobin-induced increases in microvascular permeability occur via heme redox cycling and secondary generation of reactive species. Our data suggest that the β93cys may provide an important source of reducing equivalents endogenous to hemoglobin that limit heme-induced oxidative damage.

We also tested the effects of coadministration of glutathione, because this low-molecular-weight thiol is re-

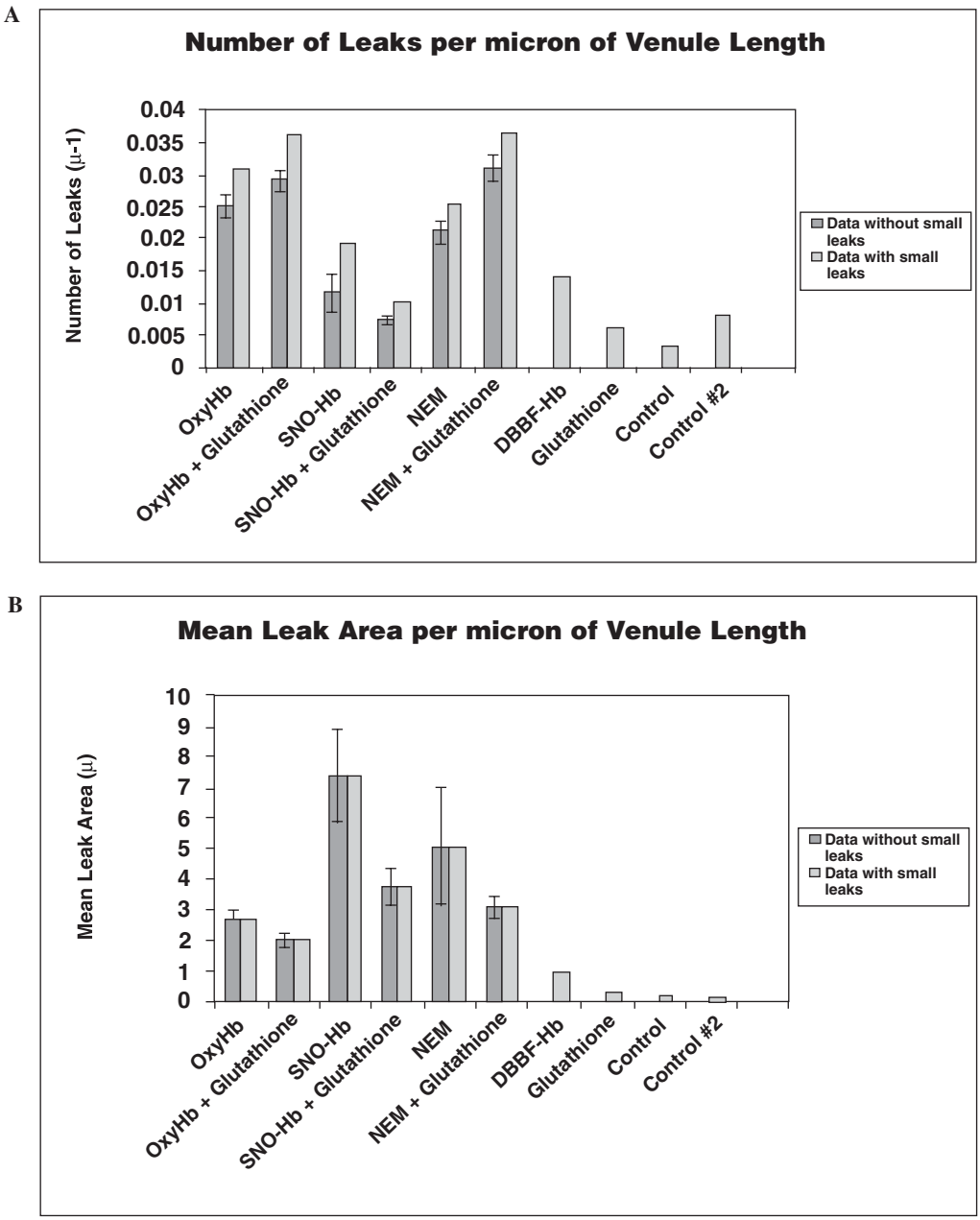

FIG. 5. (A) Average number of mesenteric leaks per venule length (μm) for different treatments with leaks <5 μm² in area omitted. (B) Average total leak area per venule length (μm²/μm) with leaks <5 μm² in area omitted.

quired to stimulate SNO-Hb–induced vasodilation of isolated vessels. Glutathione alone had no effect, nor did it modulate Oxy-Hb–induced permeability. However, glutathione did reduce the number and size of leaks stimulated by SNO-Hb. The lack of similar effects of glutathione when administered alone, with OxyHb or with NEM-Hb, suggests that specific interactions between glutathione and SNO-Hb result in the decreased microvascular permeability, and that direct antioxidant effects of glutathione, *per se*, are unlikely to play a role in limiting the number of leaks caused by cell-free Hb. Based on previous observations that NO donors protect microvascular integrity (2) and that glutathione is required for SNO-Hb–dependent vasodilation, we suggest that similar mechanisms can operate to limit SNO-Hb–dependent vasotoxicity. The precise mediator produced from SNO-Hb/thiol interactions that result in the observed protection remains unclear, but it is consistent other NO-dependent effects, as reported earlier. Potential mechanisms by which NO can protect against microvascular permeability include prevention of heme-redox cycling and secondary generation of ROS or inhibition of endothelial permeability or both.

PERSPECTIVE

In summary, *S*-nitrosation of hemoglobin increases permeability that is reduced by coadministration with glutathione. These data suggest that if future therapeutic strategies using *S*-nitrosated hemoglobins are developed, their possible effects on microvascular permeability should be considered, emphasizing the potential of thiols to maintain endothelial integrity. Finally, we propose that *S*-nitrosation of cross-linked hemoglobins, with enhanced structural stabilities, will be less effective at stimulating microvascular permeability while preserving vascular NO signaling and so will offer a novel strategy in blood-substitute development. Endothelial-derived NO activates soluble guanylate cyclase and generates guanosine 3',5'–cyclic monophosphate (cGMP), which stabilizes endothelial cell junctions and maintains their barrier function (2).

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ABBREVIATIONS

DBBF-Hb, diaspirin cross-linked hemoglobin; DTPA, diethylenetriamine pentaacetic acid; FITC, fluoroscein isothiocyanate; HBS-BSA, HEPES-buffered saline containing bovine serum albumin; NEM-Hb, *N*-ethylmaleimide; NO, nitric oxide; *O*-*R*-PolyHbAo, human polymerized hemoglobin; OxyHb, oxygenated hemoglobin; PBS, phosphate-buffered saline; PEG-Hb, polyethylene glycol–conjugated hemoglobin; PolyHbBv, bovine polymerized hemoglobin; ROS, reactive oxygen species; SNO-Hb, *S*-nitrosohemoglobin.

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