Modified hemoglobins produce venular interendothelial gaps and albumin leakage in the rat mesentery

ANN L. BALDWIN
Department of Physiology, College of Medicine, University of Arizona, Tucson, Arizona 85724-5051

Baldwin, Ann L. Modified hemoglobins produce venular interendothelial gaps and albumin leakage in the rat mesentery. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H650–H659, 1999.—Cross-linked hemoglobin (αα-Hb) and polyethylene glycol (PEG)-conjugated Hb have both been considered as possible "blood substitutes." Previously, we showed that PEG-Hb extravasates rapidly in the intestinal mucosa and causes transient epithelial sloughing, resulting in temporary opening of the intestinal epithelial barrier. In the present study, the rat mesenteric preparation was used to quantify the effects of the two Hbs on microvascular leakage to albumin and to investigate possible changes in the integrity of the interendothelial cell junctions and the endothelial actin cytoskeleton. In anesthetized Sprague-Dawley rats, the microvasculature of a mesenteric window was perfused with HEPES-buffered saline (HBS) containing 0.5 mg/ml BSA and 2 mg/ml αα-Hb (n = 16) or PEG-Hb (n = 5) for 2 or 10 min. Controls (n = 4) just received HBS-BSA. In some experiments (n = 9 for αα-Hb; n = 5 for PEG-Hb), the perfusate was then replaced by FITC-albumin in HBS-BSA for the next 3 min. The vasculature was then perfusion fixed, stained for filamentous actin and for mast cells, and viewed microscopically. In the remaining experiments, the mesenteric microvasculature was stained with silver nitrate to determine the number of endothelial junctional gaps per length of venules. Both Hbs increased the number and area of leaks per micrometer of venular length compared with control, but αα-Hb increased to a greater extent than PEG-Hb. Formation of leaks was accompanied by changes in the endothelial actin cytoskeleton and by an increased number of endothelial gaps. Mast cell degranulation was significantly greater (P < 0.05) in Hb-treated preparations compared with controls, but there was no direct correlation between sites of degranulation and albumin leakage. These Hbs appear to induce venular leakage in the mesentery by mechanisms similar to those previously observed after treatment with histamine or nitric oxide synthase inhibitors.

blood substitutes; actin cytoskeleton; silver nitrate; microscopy

HEMOGLOBIN (Hb)-based oxygen carriers, such as cross-linked Hb (αα-Hb) and polyethylene glycol (PEG)-Hb, have been proposed as blood substitutes for transfusions due to their plasma expansion and oxygen transport capabilities. The development of such substances is motivated by the dangers of blood incompatibility reactions or infectious diseases in homologous blood transfusions. In addition, Hb-based oxygen carriers have the advantage that they can be sterilized and stored for prolonged periods of time. However, little is known about the microvascular effects of blood replacement solutions and, in particular, their effects on microvascular permeability. It is important to determine whether potential blood substitutes cause microvascular leakage, because alterations in transvascular exchange of plasma proteins disturb the fluid balance between blood and tissue. In addition, increased microvascular leakage changes the kinetics of delivery of intravascularly injected drugs, and of endogenous enzymes and hormones, to various tissues. At times during which transfusions are needed, for example, after hemorrhagic shock, it is important that regulation of microvascular exchange is not compromised.

In previous publications (2, 5), using electron microscopy, we showed that PEG-Hb extravasates from intestinal mucosal capillaries via widened endothelial junctions and causes various ultrastructural changes in the intestinal mucosa and accompanying capillaries. Apart from epithelial detachment, these changes include mast cell degranulation, goblet cell secretion, and tissue edema. Mast cell degranulation can result in release of inflammatory mediators, such as histamine, leukotrienes, prostaglandin D2, and cytokines, which increase microvascular permeability, causing edema, and recruit and activate inflammatory cells, such as eosinophils and neutrophils (10).

In the present study, we investigated the effects of αα-Hb and PEG-Hb on extravasation of BSA in the rat mesentery. The mesentery is an excellent preparation for quantifying the degree of vascular leakage invoked by particular mediators because the tissue is very thin and the microvascular networks are almost two dimensional, thus facilitating image acquisition and analysis. We have previously used the mesentery to detect venular leaks to albumin after administration of histamine (3, 29) or nitric oxide synthase (NOS) inhibitors (4). In those experiments, we showed that the formation of leaks is accompanied by changes in the endothelial actin cytoskeleton and by increases in the number of endothelial junctional gaps per surface area of venule. In the present study, we compare our results with αα-Hb and PEG-Hb with those obtained using other mediators.

MATERIALS AND METHODS

αα-Hb. The αα-Hb was obtained from the United States Army (kind gift of Dr. Alan Rudolph, Naval Research Center, Washington, DC). It is stabilized by intramolecularly cross-linking the α-subunits within the Hb tetramer and contains 4.74% methemoglobin and 0.125 EU/ml endotoxin. This product has a half-life of 3–24 h in the circulation, depending on the species and dose (10–12).

PEG-conjugated Hb. The PEG-Hb was formulated in 5 mmol/l NaHCO3, 4 mmol/l Na2HPO4, and 150 mmol/l NaCl (methemoglobin <5%, endotoxin <0.5 EU/ml) at a concentra-
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tion of 60 mg/ml. This product has a longer half-life in the circulation of dogs (58.3 h; see Ref. 8), when the solution is exchange transfused 30%, than does Hb (4.0 h; see Ref. 22). Other details regarding the PEG-Hb have been described elsewhere (22).

Both Hbs were stored in 1-ml aliquots at −20°C in microcentrifuge tubes. Immediately before use, an aliquot was thawed at 4–8°C, diluted to 2 mg/ml with HEPES-buffered saline (HBS), and warmed to 37°C by placing the container in a water bath, and the pH was measured to ensure that it had not drifted from 7.4.

Cannulation and perfusion of rat mesentery. The animal procedure was similar to that described previously (3) and is summarized here. Twenty-seven male Sprague-Dawley rats (350–400 g) were anesthetized with an intramuscular injection of pentobarbital sodium (6 mg/100 g). The abdomen was opened, and several contiguous well-vascularized mesenteric windows were selected and spread out flat over a Plexiglas platform. The superior mesenteric artery was cannulated near the selected mesenteric windows, and the appropriate arteries and venules bordering the windows were ligated to allow perfusion only of the chosen windows.

The mesenteric windows were flushed clear of blood with HBS (pH 7.4) containing 0.5% BSA and 1 U/ml heparin at 37°C and were perfused at an inlet pressure of 100 mmHg, with this solution alone (controls, 4 animals), HBS-BSA plus 2 mg/ml α-Hb (3 animals for 2 min; 12 animals for 10 min), or HBS-BSA plus 2 mg/ml PEG-Hb for 10 min (5 animals). In nine of the α-Hb and in all the PEG-Hb experiments, the perfusate was then replaced by FITC-albumin (Sigma, St. Louis, MO) in HBS-BSA for the next 3 min. As soon as the vasculature of the windows was filled with FITC-albumin, as judged by the color, the pressure was adjusted to 40 mmHg, and the portal vein, which acted as the outflow outlet, was clamped. After 3 min, the clamp was removed, and 3 ml of fixative (3% formaldehyde in HBS) were perfused via the cannula at a pressure of 100 mmHg. Next, the pressure was reduced to 40 mmHg, the portal vein was clamped, and fixation was continued for 30 min. The vasculature of the mesenteric windows was then perfused, via the cannula, with a cocktail of 3% formaldehyde, 0.1% Triton X-100, and rhodamine phalloidin (10 U/ml; Molecular Probes, Eugene, OR) in HBS for 30 min at 4°C to promote staining of endothelial actin fibers. After staining, the vasculature was perfusion fixed for 30 min with 3% formaldehyde and then flushed with HBS. The mesenteric tissue was carefully excised, and two windows from each preparation were mounted between two thin glass coverslips using aqueous mounting medium (Vectashield; Vector Laboratories, Burlingame, CA). The remaining mesenteric windows from each experiment were spread flat on microscope slides and suffused with 1% toluidine blue for 15 min before mounting. Toluidine blue was used to stain the mast cells to determine the numbers that had degranulated. The PEG-Hb preparations were not included in the mast cell study due to shortage of material. Degranulated mesenteric mast cells, identified by the presence of intracellular granules released into the surrounding tissue, were counted within each circular ×20 field of view of a Zeiss light microscope (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%. About 30 fields were counted for each mesenteric window.

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 using a Zeiss Axiosplan microscope with a ×10 objective and numerical aperture (NA) 0.6, fitted for epifluorescence. The light source was a 100-W mercury lamp for epifluorescence and a halogen lamp for transmitted illumination. A video camera (Optronix V1470) was mounted at the camera port of the microscope. Five images of leaky vessel networks from each mesentery, produced by epifluorescence with the appropriate FITC excitation and emission filters (λex = 488 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 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 (i.e., α-Hb for 10 min, and the following values were calculated: 1) average number of leaks per length of venule, 2) average leak area per micrometer of venule, and 3) size distribution of leaks.

Actin cytoskeleton. Epifluorescence microscopy was used to examine the structure and degree of integrity of the endothelial actin cytoskeleton of mesenteric venules from all groups. Photographs of venules were taken, in pairs, using the FITC filter and rhodamine filter in turn, through a ×63 NA 1.25 oil immersion objective. The photographs were taken to record representative examples of leaky and nonleaky venules, and the only criterion used for selecting a vessel for photography was its clarity under epifluorescence microscopy.

Silver staining. The preparations that were not perfused with FITC-albumin were stained with silver nitrate to delineate the endothelial cell junctions and demonstrate the presence of junctional gaps (7 controls and 6 α-Hb for 10 min). The procedure used was a modified version of that developed by McDonald (16). Briefly, the preparation was perfusion fixed with sodium cadoxylate-buffered 1% paraformaldehyde and 0.5% gluteraldehyde for 5 min, using a handheld syringe. To obtain the desired results, it was essential to flush a relatively large volume of fixative (25 ml) through the system. Next, 0.9% NaCl was flushed through for 2 min, followed by 5% glucose for 10 s, 0.2% silver nitrate for 15 s, and glucose for 10 s. Finally, fixative was flushed through for 1 min. The tissue was quickly excised, spread out in cadoxylate buffer, and exposed to bright light for 8–15 min. When the vascular tree became visible through a dissecting microscope, the tissue was removed from the light, dehydrated, cleared in toluene, and mounted for microscopic observation. Junctional gaps were easily discernible in mesenteric venules as focal deposits of silver along the cell borders. Between 5 and 15 venules were chosen from each preparation in which to count the number of gaps. Venules were chosen according to whether they had stained with sufficient intensity. In each case, the venule length was measured, using a microscopic eyepiece calibrated scale, so that the number of gaps per length of venule could be calculated. Finally, the diameters of 40–130 venular silver deposits for each treatment group were measured using photomicrographs. All deposits appearing in the images were included in the counts.

Statistical analysis. For parameters, such as mast cell degranulation and numbers of junctional gaps, the slides were coded and later analyzed. Each parameter was compared between different groups using one-way ANOVA. If a significant difference was found between groups, pairs of groups were compared using the Student's t-test with a P-
value <0.05 to determine statistical significance. All values are presented as means ± SE. The number of venules per group.

RESULTS

The results from this study have been compared with those from three of our previous studies, one using histamine as a mediator (3), one using N(G)-monomethyl-L-arginine (L-NMMA; see Ref. 4), and the third using histamine or L-NMMA followed by silver staining to identify junctional gaps (1). Control values from the histamine study for FITC-BSA venular leakage have also been used in the comparison.

Distribution of leaks. Microscopic examination of control mesenteric preparations by epifluorescence revealed very few leaky sites after perfusion with HBS-BSA (6 experiments), but preparations treated with \( \alpha \alpha \)-Hb or PEG-Hb showed many leaky sites. The leakage occurred in venules but not in arterioles or capillaries. The total number of venules examined for \( \alpha \alpha \)-Hb (2 min), \( \alpha \alpha \)-Hb (10 min), and PEG-Hb (10 min) were 41, 128, and 299, respectively. The percentage of venules demonstrating leaks for \( \alpha \alpha \)-Hb (2 min), \( \alpha \alpha \)-Hb (10 min), and PEG-Hb (10 min) were 39, 84, and 41%, respectively. Corresponding values for L-NMMA (3 min) and histamine (3 min) were 71 and 46%. After 2 min of \( \alpha \alpha \)-Hb treatment, most leaks were <500 \( \mu \text{m}^2 \) in area. This compares with a luminal surface area of endothelial cells that ranges from 400 to 900 \( \mu \text{m}^2 \) (3). Perfusion with \( \alpha \alpha \)-Hb or PEG-Hb for 10 min resulted in the appearance of some larger leaks. In many cases, a few of the leaks were >5,000 \( \mu \text{m}^2 \) in area.

Quantification of leaks. The number of leaks per venule length, as a function of type and duration of treatment, is shown in Fig. 1A. Values for 2-min \( \alpha \alpha \)-Hb and 10-min PEG-Hb were very similar to each other, significantly greater than for controls, and significantly smaller than for histamine, 10-min \( \alpha \alpha \)-Hb, and 3-min L-NMMA. With regard to average leak area per venule length (Fig. 1B), all mediators gave values that were significantly greater than for control. The value for 10-min \( \alpha \alpha \)-Hb was significantly greater than for all other cases. Thus \( \alpha \alpha \)-Hb causes leaks that increase in number and in area with time from 2 to 10 min. PEG-Hb for 10 min produced leak numbers per venule length and leak area per venule length similar to those of 2-min \( \alpha \alpha \)-Hb, but the size distribution of leaks was wider. There were fewer very small leaks and several large leaks in PEG-Hb specimens.

It has been suggested that FITC-BSA can increase the microvascular leakage produced by NOS inhibitors (27). To determine whether this effect significantly affected the results, two experiments were performed in which mesenteric windows were perfused for 10 min with \( \alpha \alpha \)-Hb, followed by tetramethyl rhodamine isothiocyanate-labeled BSA for 3 min. In these experiments, although the area of leaks per venule length was markedly reduced compared with FITC-BSA, the numbers of leaks were very similar (41.9 and 43.5 \( \times \) 10\(^{-4} \) vs. a mean value of 53.0 \( \times \) 10\(^{-4} \) for FITC-BSA). Therefore, it is unlikely that stimulation of the FITC-BSA was responsible for the formation of leaks after perfusion of \( \alpha \alpha \)-Hb.

Appearance of venular leaks and accompanying changes in endothelial actin cytoskeleton. In control preparations, as noted previously (4), there were very few venular leaks to FITC-BSA, and the endothelial cell actin cytoskeleton consisted of peripheral fibers at the cell-cell junctions. After 10 min of treatment with \( \alpha \alpha \)-Hb, many leaks could be seen (Figs. 2A and 3A), some small and others more extensive. In Fig. 2A, FITC-BSA appears to be leaving the venule in the form of streams. This phenomenon occurred occasionally and was also observed in preparations treated with L-NMMA (4). The endothelial cell actin cytoskeletons of vessels depicted in Figs. 2A and 3A are demonstrated in Figs. 2B and 3B (taken using filters for FITC-albumin and rhodamine-phalloidin, respectively). Comparison of Fig. 2, A and B, reveals that a cluster of small leaks are coincident with the endothelial cell junction, as outlined by rhodamine phalloidin staining of the peripheral actin rim (PAR). The precise positions of these small leaks coincide with distinct gaps in the PAR. We have observed this phenomenon previously in prepara-
tions treated with histamine (3, 29) or with \( \text{L-NMMA} \) (4). This type of disruption was rarely seen in control preparations. In controls, the PARs formed fairly continuous boundaries around each endothelial cell (3, 4).

In regions of venules in which the leaks were more extensive (i.e., Fig. 3A), the PARs were very disrupted, and, in addition, distinct actin fibers, largely aligned with the longitudinal axis of the vessel, were evident in the central region of the cell. As shown in Fig. 3B, the central actin fibers were often absent within the cell most closely associated with the large leak but were present within surrounding cells. This observation is consistent with our hypothesis (3) that the central fibers provide extra support for the cells so that they may retain their original shape in the face of the action of various mediators.

Mast cell degranulation. In the preparations that were perfused with \( \text{\( \alpha \alpha \)-Hb} \) for 2 or 10 min, toluidine blue staining revealed the presence of degranulated mast cells (Fig. 4). Figure 5 shows the number of degranulated mast cells per microscopic field of view for \( \text{\( \alpha \alpha \)-Hb} \) preparations, compared with previously published results for histamine and \( \text{L-NMMA} \) (1). The \( \text{\( \alpha \alpha \)-Hb} \)-treated preparations show significantly more mast cell degranulation than those undergoing the other treatments. However, the \( \text{\( \alpha \alpha \)-Hb} \)-treated and control preparations, unlike the other groups shown in Fig. 5, were not pretreated with the mast cell stabilizer chromolyn. If the other groups (histamine and \( \text{L-NMMA} \) had not been pretreated with chromolyn, the degree of mast cell degranulation observed in those cases would have been higher. The reason for not using a mast cell stabilizer was that \( \text{\( \alpha \alpha \)-Hb} \) has been used extensively in clinical trials without stabilizers, and we wished to determine the effects of the Hb in the form in which it is currently being administered.
Because mast cells, on degranulation, release substances such as histamine and prostaglandins that cause venular leakage (7), we determined whether there was any correlation between extent of venular leakage and degree of mast cell degranulation for each preparation treated with $\alpha$-Hb. The mean number of degranulated mast cells was plotted against the mean number of leaks per venule length, or the mean leak area per venule length, for each experiment (plots not shown). There was no correlation between degree of mast cell degranulation and mean number of leaks per venule length ($r^2 = 0.01$) or between mast cell degranulation and mean leak area per venule length ($r^2 = 0.02$).

Silver-stained endothelial junctional gaps. The appearance of mesenteric microvessels from 10-min $\alpha$-Hb-treated preparations, after staining with silver nitrate, is shown in Fig. 6. Figure 6A shows an arteriole in which the endothelial cell boundaries are very clearly visible as continuous silver lines, with no silver deposits (gaps). Gaps were never seen in arterioles. Note the characteristic long, thin, spindly shape of the arteriolar endothelial cells. Figure 6, B and C, shows silver-stained small venules. The staining is lighter, and silver deposits (gaps) along the cell borders are clearly visible. The endothelial cells are less spindly than those observed in arterioles. Larger venules showed similar silver deposits, but due to the difficulty of locating reasonable lengths of large venules in a single plane of focus, photographs are not presented. Figure 6C demonstrates a cluster of silver deposits that we noticed in vessels treated with $\alpha$-Hb. One endothelial cell is partially separated from its neighbors by seven gaps. The mean number of gaps per venule length is shown in Fig. 7 for control and 10-min $\alpha$-Hb-treated preparations. Previously published results for 3-min histamine and 3-min L-NMMA (1) are also included for compari-
son. The results for αα-Hb and L-NMMA are similar to each other and significantly greater than for histamine, which is significantly greater than for controls. However, control preparations were not devoid of gaps. All controls showed some gaps scattered throughout the venular endothelium.

Unfortunately, we were not able to show fluorescent leaks and silver-stained gaps together in the same preparation. The processes involved in the silver staining removed all traces of fluorescence. Therefore, we were unable to show a direct correlation between leak sites and gap positions. However, a plot of mean leak area per venule length versus mean number of gaps per venule length obtained from different treatments demonstrated a very high correlation ($r^2 = 0.99$ for a second-degree polynomial fit; Fig. 8A). Each data point represents the mean value for a given treatment (i.e., αα-Hb, histamine, etc.). This finding is consistent with a previous observation (30) that the number of venular gaps, as determined by electron microscopy, was corre-

Fig. 4. Light micrograph showing mesentery stained with toluidine blue to reveal mast cells. Intact mast cells (arrows) and degranulated mast cells (arrowheads) can be seen. Scale bar = 50 µm.

Fig. 5. Average number of degranulated mast cells (dmc) per microscopic field of view for different treatments. Significant differences: * greater than control; ** greater than for all other treatments.

Fig. 6. Light micrographs of mesenteric microvessels from preparations treated with αα-Hb for 10 min, followed by the silver nitrate staining procedure. Black lines mark the endothelial cell junctions. A: arteriole; B and C: venules with junctional gaps, identified by circular silver deposits (arrowheads). Note the clustering of silver deposits in C. Scale bars = 10 µm.
lated with the amount of extravasation of $\alpha$-lactalbumin from rat mesenteric venules exposed to histamine. A plot of the mean number of leaks per venule length versus mean number of gaps per venule length did not show a strong correlation (Fig. 8B). Preparations treated with L-NMMA for 3 min showed a greater number of leaks per venule length, but fewer gaps per venule length, than did preparations treated with $\alpha\alpha$-Hb for 10 min.

The mean diameters of venular silver deposits (means ± SE) from mesenteries treated with HBS-BSA, L-NMMA, histamine, and $\alpha\alpha$-Hb (10 min; 4 rats in each case) were $0.94 \pm 0.06 \mu m$ ($n = 41$), $1.30 \pm 0.04 \mu m$ ($n = 50$), $1.42 \pm 0.04 \mu m$ ($n = 51$), and $1.78 \pm 0.04 \mu m$ ($n = 131$), respectively. All three mediators gave silver deposit diameters that were significantly larger than control values, and the $\alpha\alpha$-Hb mean value was significantly larger than values for L-NMMA and histamine.

**DISCUSSION**

This study has demonstrated that $\alpha\alpha$-Hb and PEG-Hb, both of which have been considered as possible blood substitutes, cause venular leakage in the rat mesentery, similar to that produced by histamine and NOS inhibitors, such as L-NMMA. This finding is contrary to the results reported by the only other group that has used epifluorescence to determine the effects of Hb-based oxygen carriers on the permeability of microvascular networks (23). Those authors concluded that $\alpha\alpha$-Hb does not enhance permeability for FITC-dextran (mol wt 150,000) in the microcirculation of striated skin muscle in conscious hamsters. There are several ways to explain this apparent discrepancy. First, the $\alpha\alpha$-Hb came from different sources; the $\alpha\alpha$-Hb used by the other authors came from Baxter Healthcare, whereas that used in these experiments came from the United States Army. Even so, it is unlikely that this difference is the reason for the discrepancy, because the two products contain the same chemical modifications. However, as stated by D’Agnillo and Alayash (9), a strict comparison between the two products has not been made in the literature. Second, the tracer used by Nolte et al. (23) was considerably larger than BSA, which has a molecular weight of 69,000 and a Stokes-Einstein radius of 37 Å, and many studies have shown that larger molecules do not extravasate from microvessels as easily as smaller molecules (i.e., Ref. 26). Third, the fluorescent tracer was injected into the circulating blood and thus was present within the vasculature when the observations were made. This means that the leaks could only be detected in cases in which the FITC-dextran had diffused away from the vessels into the tissue. In the mesentery, as shown in Fig. 2C, small leaks may be confined to the vessel wall and difficult to visualize if the microvasculature has not been flushed free of tracer before the observations are made. Occasionally, some vessels show streams of FITC-BSA entering the tissue (Fig. 2A). A reason for the lack of extensive diffusion from some microvascular beds was suggested by Majno and Palade in 1961 (15). They observed, using electron microscopy, that particles of mercuric sulfide (HgS), ranging in diameter from 70 to 350 Å, leaked through venular endothelium of striated muscle after histamine treatment but were then retained by the underlying basement membrane. Thus the tracers were restricted to a compartment close to the venule. They concluded, “The average diameter of the blood proteins is close to 40 Å, and if the basement membrane is capable of retaining almost all the particles of HgS down to a diameter of 70 Å, it is clear that the filtration of protein molecules should encounter some difficulty.” McDonald (16) made a similar observation. He reported that once the tracer, monastral blue,
had leaked through junctional gaps, it tended to cross the endothelial basal lamina but not the basal lamina of the underlying pericytes. If the 150,000 molecular weight dextran was being impeded in a similar way in its passage from microvessels of striated muscle, it is unlikely that Nolte et al. (23) could have detected such local, constrained leaks. They assessed leakage by densitometric quantification of the mean fluorescence intensity in the perivascular tissue. Thus they did not measure individual leaks. No information is given regarding the exact location of the perivascular tissue regions with respect to the vessels. If the regions were too close to the vessels, the leaks would have been masked by the intravascular tracer, and if the regions were too distant, the leaks would have been missed. Fourth, it is possible that the microvasculature of the hamster striated skin muscle is just less permeable than that of the rat mesentery.

In this study, we have presented evidence that suggests that FITC-BSA leaks through localized endothelial junctional gaps similar to those produced by histamine and inhibition of NOS (6, 13). Because local disruption of the endothelial actin cytoskeleton occurs at leak sites, it is probable that alterations in the arrangement of the cytoskeleton play a role in the formation of the junctional gaps. The findings of this study will now be discussed in detail.

Venular leakage. Common to the response of the mesenteric microvasculature to L-NMMA (4), there was a slight widening of the leak size distribution after 10 min of αα-Hb treatment time compared with 2 min. Increased treatment time from 2 to 10 min increased the number of leaks per venule length 2.4-fold and the leak area per venule length 4.8-fold. These results suggest that, during treatment with αα-Hb, the mean size of individual leaks increases.

The fact that perfusion with PEG-Hb for 10 min produced significantly less leakage than perfusion with αα-Hb for the same time period is noteworthy. One possible explanation for the difference could be that, since PEG-Hb is a larger molecule than αα-Hb (Stokes-Einstein radius of 250 Å vs. 56 Å), it does not accumulate so easily in the interendothelial cell clefs. Thus the amount of iron released from the Hb derivative per unit volume of fluid in the cleft may be smaller. Free iron is known to catalyze production of the hydroxyl radical that causes tissue damage. Any direct effects that the PEG-Hb might have on junctional integrity would thus tend to be reduced relative to those of αα-Hb. Alternatively, PEG-Hb may be more stable and may not release its iron so easily. However, this latter option is unlikely, considering the rate at which it extravasates from intestinal mucosal capillaries (2).

Changes in endothelial actin cytoskeleton. Changes in the endothelial actin cytoskeleton produced by αα-Hb were only present at leaky sites and were very similar to those previously noted after treatment with L-NMMA (4). These include focal breaks in the PAR, extensive disruption of the actin cytoskeleton accompanying larger leaks, and the appearance of actin fibers in the central region of the cell that were oriented, to some degree, with the longitudinal axis of the vessel. It is possible that the focal breaks in the PARs could have led to the formation of the endothelial gaps, identified by the presence of silver deposits. Assuming that the PAR acts as a scaffold to maintain endothelial cell shape, a break in the PAR would tend to reduce cell membrane tension in that region, causing the cell to passively retract from its neighboring cell in that specific region, thus forming a gap. The fact that the focal breaks in the PAR caused by Hb were similar in size (~1.8 μm) to the silver deposits marking the gaps supports this hypothesis.

Mast cell degranulation. We previously reported that perfusion of mesenteric networks with L-NMMA caused some mast cell degranulation, even in the presence of chromomycin (4). We hypothesized that, since mast cells can be degranulated by superoxide and nitric oxide (NO) acts as a scavenger for superoxide, the L-NMMA was causing mast cell degranulation by depleting the tissue of NO. Because αα-Hb also scavenges NO, it could be causing mast cell degranulation by a similar mechanism. We previously suggested (4) that, because mast cell potency and strategic location, we could not rule out mast cell secretory products as contributors to at least part of the leak production and endothelial cell cytoskeletal rearrangement caused by L-NMMA. In fact, in the case of αα-Hb, when comparing 2- and 10-min values, the mean number of degranulated mast cells and the mean number of leaks per venule length increase by factors of 1.9 and 2.4, respectively. Thus, at first sight, it appears that mast cell degranulation plays a major role in αα-Hb-induced leak formation. However, when the analysis is separated into individual experiments and correlation coefficients are calculated, the correlation no longer holds. Therefore, it appears that, although mast cell degranulation definitely accompanies leak formation, it does not directly produce the leaks.

Silver-stained endothelial junctional gaps. The average number of gaps per venule length in control preparations [100 ± 40 (SE)/μm × 10⁻³] was considerably greater than the average number of leaks per venule length [3.6 ± 0.2 (SE)/μm × 10⁻³]. Therefore, either a large proportion of the apparent gaps are not open and/or several gaps contribute to a single leak. The existence of gaps that do not allow leakage of large tracers has been reported previously. For example, McDonald (16) observed that, 10 min after application of substance P, venules in the rat trachea showed silver deposits but no leakage to monastral blue. At shorter time points after application of substance P, leaks were observed. The disparity between the number of gaps versus number of leaks is also seen after treatment with αα-Hb, histamine, or L-NMMA, but its magnitude is reduced.

Our study demonstrated that the leak area, rather than the leak number, per venule length was closely correlated with the number of gaps per venule length. This finding is consistent with the possibility that several gaps can result in one large leak, rather than in a few small, discrete leaks. However, the correlation...
between leak area and number of gaps was not linear when effects of different mediators were compared (i.e., $\alpha\alpha$-Hb caused an average leak area per venule length 5.3 times larger than that produced by histamine, but the number of gaps produced was only 1.6 times higher). Because the FITC-BSA was contained in the microvasculature at the same pressure in all cases, this finding suggests that the gaps caused by a given mediator are not identical to those produced by another mediator. In fact, we showed that the average size of the silver deposits observed after treatment with $\alpha\alpha$-Hb was significantly larger than that of deposits observed after histamine or l-NMMA. This size difference could account, at least partially, for the increased leak area observed after perfusion with $\alpha\alpha$-Hb. Differences in the average sizes of different populations of silver deposits have been reported previously. Hirata et al. (13) reported that, in the rat tracheal mucosa after treatment with substance P, deposits in postcapillary venules were larger than those in collecting venules. Our control value for mean diameter of silver deposits closely approximates the value obtained by Baluk et al. (6), 1.16 ± 0.02 µm, in postcapillary venules of the rat trachea 10 min after application of substance P, at which time venular leaks had vanished. Our histamine value is very similar to the value obtained by the same authors 1 min after application of substance P when venular leaks were apparent. Apart from the larger diameter of silver deposits produced by $\alpha\alpha$-Hb, the clustering of gaps that we observed in $\alpha\alpha$-Hb-treated preparations could account for the larger leaks compared with histamine, because several neighboring gaps could contribute to the same leak.

Possible mechanisms for leak formation by Hbs. One property of Hb that lends itself to the disruption of endothelial junctions is its strong affinity for NO. As we have stated previously (4), inhibition of NO results in a reduction of intracellular cGMP (20), which, in cultured endothelial cells, leads to contraction of actomyosin filaments and possible cell contraction to produce a widening of the intercellular defts (24). Our previous experiments, in which we demonstrated that perfusion of the mesenteric microcirculation with NOS inhibitors nitro-l-arginine methyl ester and l-NMMA also caused venular leakage (4), support this hypothesis. Second, levels of superoxide anions, produced by mitochondrial function, may increase if there is NO available to scavenge the superoxide (14). Excess quantities of superoxide anions may lead to tissue damage. In addition, $\alpha\alpha$-Hb and PEG-Hb may produce reactive oxygen species (ROS) independent of their ability to deplete the tissue of NO. For example, oxyhemoglobin can convert to methemoglobin and release superoxide as a by-product. Also, levels of superoxide anions in the tissue increase when blood is diluted with solutions such as HBS-BSA, because, unlike plasma, these solutions do not contain ROS scavengers, such as catalase and superoxide dismutase (19). The excess ROS then react with Hb and cause it to release some free iron from the heme group (25). The free iron catalyzes production of the hydroxyl radical (OH·) by the Fenton reaction (18). The hydroxyl radical is a far more potent mediator of tissue damage than is superoxide, and it propagates lipid peroxidation (21). Thus the effects of various Hb-based blood substitutes on venular leakage may depend on the affinity of the product for NO and/or on the propensity of the product to release free iron.

The effects of $\alpha\alpha$-Hb and PEG-Hb on venular leak formation have important implications regarding their use as blood substitutes. In a previous study (2), we showed that PEG-Hb rapidly extravasates from the intestinal mucosal microcirculation. Therefore, the effect on microvascular leakage is not limited to the mesentery. If $\alpha\alpha$-Hb and PEG-Hb increase transport of plasma proteins from the blood stream into the tissue, this means that transport of nutrients and drugs between blood and tissue will also be compromised. Thus it is important to develop Hb-based blood substitutes that do not cause excess microvascular leakage.

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Address for reprint requests and other correspondence: A. L. Baldwin, Department of Physiology, College of Medicine, University of Arizona, Tucson, AZ 85724-5051 (E-mail: abaldwin@u.arizona.edu). Received 8 October 1998; accepted in final form 28 March 1999.

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