Disodium cromoglycate stabilizes mast cell degranulation while reducing the number of hemoglobin-induced microvascular leaks in rat mesentery

Maxwell I. Ginsburg and Ann L. Baldwin

Department of Physiology, College of Medicine, University of Arizona, Tucson, Arizona 85724-5051

Submitted 26 June 2003; accepted in final form 23 December 2003

Ginsburg, Maxwell I., and Ann L. Baldwin. Disodium cromoglycate stabilizes mast cell degranulation while reducing the number of hemoglobin-induced microvascular leaks in rat mesentery. Am J Physiol Heart Circ Physiol 286: H1750–H1756, 2004. First published January 2, 2003; 10.1152/ajpheart.00605.2003.—Blood substitutes, such as diaspirin cross-linked Hb (DBBF-Hb), have been considered for use during blood transfusions. Unfortunately, bolus injection of modified Hb has been shown to rapidly increase the leakage of microvessels to plasma albumin. This effect may result from production of excess reactive oxygen species (ROS) and could be linked to the observed increase in degranulated mast cells (DMC). Disodium cromoglycate (cromolyn) stabilizes mast cells and therefore might minimize the venular permeability in the rat mesentery. In 10 anesthetized Sprague-Dawley rats, the mesenteric preparation was continuously suffused with cromolyn while the microvasculature was filled with DBBF-Hb solution (10 mg/ml) for 10 min. Six animals received cromolyn pretreatment [two intravascular injections over 30 min (experiment A)] and four animals received pretreatment with 2% HEPES-buffered saline (HBS)-BSA (experiment B). Two more animals were pretreated with HBS-BSA without DBBF-Hb infusion but with cromolyn suffusion (experiment C). Another set of experiments was performed on five animals without cromolyn suffusion or any pretreatment but with DBBF-Hb infusion (experiment D). All groups then received a 1-min perfusion of FITC-albumin, fixation for 60 min, and microscopic examination. Experiments A and B demonstrated a significant reduction in the number of venular leaks and DMC compared with experiment D, but not in the area of venular leaks. These results suggest mast cell degranulation is not a major contributor to microvascular leakage induced by DBBF-Hb.

blood substitutes; cromolyn; venular leaks; diaspirin cross-linked hemoglobin

In the last decade, the desire for a more efficient and convenient blood substitute for use in transfusions and major surgery has resulted in the development of synthetic oxygen transporters, such as diaspirin cross-linked hemoglobin (DBBF-Hb). Previous studies have indicated that DBBF-Hb alone is not a safe and effective blood substitute because it increases microvascular permeability and mast cell degranulation (5). Autoxidation of Hb is the primary oxidative reaction that initiates the oxidative cascade and is an important concern when considering the use of chemically modified blood substitutes (3, 15, 16, 20). D’Agnillo and Alayash (14) described how DBBF-Hb has been shown to autoxidize faster than native Hb. When autoxidized, Hb produces superoxide, which in turn reacts with Hb, oxidizing oxyhemoglobin to methemoglobin or reducing methemoglobin to oxyhemoglobin (27). The superoxide can also be spontaneously converted to hydrogen peroxide via superoxide dismutase (19). The hydrogen peroxide can then react with DBBF-Hb to produce ferryl hemoglobin (HbFe4+). HbFe4+ is a highly reactive oxygen species (ROS) that can induce lipid peroxidation and oxidize other biological molecules (1, 13). These physiological adverse effects are detrimental for several reasons. For example, ROS activate mast cell degranulation (21), which contributes to the vascular permeability by increasing the amount of released histamine (16, 28, 29). Mast cells are potent mediators that cause most of the symptoms observed during hypersensitivity reactions (22). With an increase in venular leakage, the blood substitute itself can leave the circulation and the patient may be deficient regarding oxygen transport. Also, the increase in leakage will alter the kinetics of delivered hormones and enzymes to appropriate tissues, all of which are critical, especially during hemorrhagic shock when a transfusion would be necessary (2, 9, 18). In other words, there is sufficient evidence to indicate that modified Hb can be unstable in vivo.

Previously, we have shown that injection of the mesenteric microcirculation with DBBF-Hb causes venular leakage to FITC-albumin and also produces mast cell degranulation. However, it is not clear whether leak formation is exacerbated by the mast cell degranulation or whether these two responses are independent of each other. This study addresses the following question: if the mast cells are stabilized, do the leaks still form by some mast cell-independent mechanism? No experiments have previously been performed to determine the role that mast cell degranulation plays in Hb-induced microvascular leakage.

It is well documented that disodium cromoglycate (cromolyn) works well as a mast cell stabilizer. Cromolyn has been widely used as a treatment for asthma, most likely because of its ability to prevent the release of the mediators of anaphylaxis from mast cells (24) and because no cromolyn-induced toxicity has been observed in studies using the prescribed therapeutic routes (10). Little information is known about the mechanism by which cromolyn works; nevertheless, previous studies, as well as our own, indicate that cromolyn does in fact reduce the number of degranulated mast cells (DMC) (10, 22–24, 29). Crossman et al. (12) demonstrated that cromolyn may function by modifying tachykinin actions, thus reducing the effects of substance P. Substance P is a neuropeptide that can activate mast cells (25). This mechanism is further supported by the findings of Yamawaki et al. (29), who determined that cromolyn inhibits the response of microvascular leakage to substance P aerosols. Previous studies suggest that cromolyn acts as a tachykinin antagonist in the airways and reduces vascular permeability (25, 29).
For this reason, cromolyn was used in experiments on the rat mesentery to determine whether mast cell stabilization reduces the microvascular leakage produced by DBBF-Hb. As Baldwin et al. (9) and Valeski and Baldwin (25) have previously determined, the rat mesentery provides an excellent preparation to quantify 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 (5–7, 9, 26).

**MATERIALS AND METHODS**

*Preexperimental treatment of rats.* The research described in this study adheres to the American Physiological Society’s “Guiding Principles in the Care and Use of Animals,” and the protocol has been approved by Institutional Animal Care and Use Committee. The subjects of the experiment were male Sprague-Dawley rats from Harlan Teklad (Madison, WI) weighing between 440 and 485 g. Extreme care was taken in the ways in which the rats were housed and tended, as explained in Baldwin et al. (9).

Hb solution. DBBF-Hb was obtained from the Walter Reed Army Institute of Research (Washington, DC) as a generous gift. It is human Hb cross-linked by bis(3,5-dibromosalicyl)fumerate (DBBF-Hb). Its half-life is ~3–24 h in the circulation.

*Cannulation and injection of rat mesenteric circulation.* Fourteen 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). After the preanesthetic, an intraperitoneal injection of pentobarbital sodium (30 mg/kg) was given before the first incision. To maintain respiration, a tracheostomy was performed. The ventilator was set for 100 strokes/min. The abdomen was then opened, and the mesenteric windows were carefully wrapped in gauze. Rats in experiments A–C (see Table 1, cromolyn drip) received a suffusion of cromolyn, and therefore the gauze was presoaked in warm (37°C) cromolyn solution [5 g cromolyn in 1 liter HEPES-buffered saline (HBS-BSA)]. The cromolyn-soaked wrap was then placed on a platform of wet gauze level with the abdomen. The warm cromolyn-HBS-BSA solution was continuously dripped on the gauze-wrapped mesentery throughout the experiment to ensure adequate cromolyn suffusion. Rats in experiment D (Table 1) did not receive the cromolyn drip and were continuously suffused with HBS solution.

Once the portal vein was exposed, rats in experiment A received a warm cromolyn-HBS-BSA injection (0.1 ml/100 g body wt) in the portal vein in the direction of blood flow using a 28.5-gauge needle. This procedure was repeated 30 min later. Cromolyn was injected before the mesentery was manipulated because mast cells are exquisitely sensitive to the physical disturbances that are likely to occur during surgery. Rats in experiments B and C were administered two warm 2% HBS-BSA injections (0.1 ml/100 g body wt) instead of the cromolyn injection into the portal vein.

To expose the superior mesenteric artery, surgical forcesps were used for blunt tissue dissection. Once the artery was free of adipose and connective tissue, two 4-0 surgical sutures were tightly secured around the artery with sufficient space between each to allow perfusion of ~6–10 mesenteric windows. We define a mesenteric window as the mesenteric tissue lying between two adjacent pairs of feeding arteries/collecting venules. These ligatures allow for perfusion of only one select number of windows and ensure proper clearance and pressure of fluid within the feeding arteries and venules. In Fig. 1, only one mesenteric window is shown for clarity. The cannula (catheter that would later be placed into the mesenteric artery for the injection of appropriate reagents) was positioned just upstream of the downstream ligature, and a clamp was placed just upstream of the cannula. This clamp helped to prevent backflow of blood into the cannula, or catheter, that would later be placed into the mesenteric artery for the perfusion of appropriate reagents. Another 4-0 suture was loosely tied around the site of cannulation to help secure the cannula when in place.

After the cannula was inserted retrograde into the artery and secured in place with the suture, the clamp was removed. A small incision was made in the portal vein to allow the microvasculature to be flushed clear of blood. First, the blood was cleared by injecting 5 ml of warm heparin flush (22 mg heparin/4 ml deionized water at pH 7.4) through the cannula. In all cases, the heparin flush could be seen entering the feeding arteriole, followed by the microvascular network, and finally flowing through the collecting venule into the mesenteric vein. Next, in 15 of the animals (groups A, B, and D), 5 ml of warm DBBF-Hb solution (2 mg/ml in HBS-BSA) was injected. The portal vein was then clamped, and the DBBF-Hb solution was allowed to incubate within the mesenteric microvasculature over a 10-min interval. During the 10-min interval, another 1 ml of DBBF-Hb solution was injected every 3 min, removing the portal vein clamp for each extra 1 ml added. Therefore, a total of 8 ml of DBBF-Hb solution was injected into the rat mesentery over 10 min. Two of the animals that received the HBS-BSA injections did not receive the DBBF-Hb infusion (group C). Thus the effects of HBS-BSA injections and cromolyn suffusion alone on microvascular leakage could be determined.

The portal vein clamp was removed once more and DBBF-Hb was replaced by warm FITC-albumin solution (5 mg FITC in 10 ml HBS-BSA). The portal vein was re-clamped, and the FITC-albumin was allowed to incubate in the microvasculature for 1 min. Next, the clamp was removed, 3 ml of cold (4°C) fixative (3% formaldehyde in HBS) were perfused via the cannula, and the portal vein was then re-clamped. An extra 2 ml of fixative were added while the vein was still clamped to maintain pressure inside the mesenteric microcirculation (~40 mmHg). At this time, the animal was promptly terminated with 1 ml of Beuthanasia into the heart. The cromolyn drip (or HBS drip) was removed, and the gauze was soaked in the fixative and covered in ice. The fixation was allowed to continue for 60 min at 4°C. Every 10–15 min, the portal vein clamp was removed, an extra 1 ml of fixative was added, and the clamp was replaced.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Treatment</th>
<th>No. of Leaks $\times 10^{-3}$</th>
<th>Leak Area</th>
<th>No. of Rats</th>
<th>No. of Venules</th>
<th>Average No. of DMC</th>
<th>No. of Fields of View</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>DBBF-Hb with Crom injection and drip</td>
<td>0.500$\pm$0.140</td>
<td>0.100$\pm$0.040</td>
<td>6</td>
<td>490</td>
<td>1.07$\pm$0.12</td>
<td>458</td>
</tr>
<tr>
<td>B</td>
<td>DBBF-Hb with HBS-BSA injection and Crom drip</td>
<td>0.670$\pm$0.150</td>
<td>0.114$\pm$0.032</td>
<td>4</td>
<td>330</td>
<td>1.68$\pm$0.23</td>
<td>212</td>
</tr>
<tr>
<td>C</td>
<td>HBS-BSA injection with Crom drip</td>
<td>0.090$\pm$0.060</td>
<td>0.008$\pm$0.006</td>
<td>2</td>
<td>156</td>
<td>0.17$\pm$0.08</td>
<td>90</td>
</tr>
<tr>
<td>D</td>
<td>DBBF-Hb only</td>
<td>1.517$\pm$0.359</td>
<td>0.119$\pm$0.037</td>
<td>5</td>
<td>221</td>
<td>4.40$\pm$0.40</td>
<td>340</td>
</tr>
<tr>
<td></td>
<td>HBS-BSA only</td>
<td>0.120$\pm$0.080</td>
<td>0.020$\pm$0.020</td>
<td>7</td>
<td>374</td>
<td>1.28$\pm$0.20</td>
<td>152</td>
</tr>
</tbody>
</table>

Values are means $\pm$ SE. DBBF-Hb, diaspirin cross-linked hemoglobin; Crom, cromolyn; HBS-BSA, HEPES-buffered saline-bovine serum albumin; DMC, degranulated mast cells.

**Table 1. Data recorded for each treatment**

*www.ajpheart.org*
The mesenteric tissue was carefully excised, placed in a small vial containing fixative, and refrigerated for at least 1 h. The mesenteric windows were then carefully separated and individually mounted between a glass slide and a glass coverslip using aqueous mounting medium (Vectorshield, Vector Laboratories; Burlingame, CA). Baldwin and Thurston (6), we altered the procedure. The low leak numbers and areas obtained in these two animals were sufficient to prove that the injection process, per se, was not increasing microvascular leakage. An additional group from a previous study (9) was used in this analysis for further comparisons. This group entailed HBS-BSA perfusion without any pretreatment or cromolyn suffusion. If a significant difference was found between groups, pairs of groups were compared using Student’s t-test with a P value < 0.05 indicating statistical significance. All values are presented as means ± SE; n used in these studies is the number of venules per group and that used for mast cell degranulation was the number of mesenteric fields of view examined per group, both of these quantities being the units of variation.

RESULTS

Distribution of leaks. A previous study (9) showed that microscopic examination of the HBS-BSA perfusion experiments (n = 374) by epifluorescence revealed very few leaky sites. In the present study, the HBS-BSA injection pretreatment with cromolyn suffusion (n = 156, experiment C) produced similar results. However, the DBBF-Hb perfusion treatment revealed an abundance of microvascular leaks, which occurred mainly in the venules (n = 221, experiment D) as opposed to the arterioles and capillaries. This result was similar to that demonstrated in a previous study (9). Significantly fewer leaks were seen in venules of preparations that were suffused with cromolyn and pretreated with either cromolyn (n = 490, experiment A) or HBS-BSA injections (n = 330, experiment B).

Quantification of leaks. Figure 2A illustrates the average number of leaks per micrometer of venule that resulted from each treatment. The mean number of leaks per micrometer of venule length for each treatment was as follows: experiment A, 0.50 ± 0.14 × 10⁻³ leaks/μm venule; experiment B, 0.67 ± 0.15 × 10⁻³ leaks/μm venule; experiment C, 0.09 ± 0.06 × 10⁻³ leaks/μm venule; and experiment D, 1.52 ± 0.37 × 10⁻³ leaks/μm venule.
leaks/μm venule. Suffusion with cromlyn, with or without cromolyn injection pretreatment (groups A and B), significantly reduced the number of leaks per micrometer of venule produced by DBBF-Hb (group D).

As illustrated in Fig. 2B, the mean area of leaks per micrometer of venule length for each treatment was as follows:

- **Experiment A**: 0.100 ± 0.040 μm²/μm venule; **Experiment B**: 0.114 ± 0.032 μm²/μm venule; **Experiment C**: 0.008 ± 0.006 μm²/μm venule; and **Experiment D**: 0.119 ± 0.034 μm²/μm venule. The value for experiment A did not differ significantly from that found in experiments B or D. On the other hand, experiment C produced very low leak areas (no statistics, n = 2 for group C). Thus although cromolyn suffusion (with or without cromolyn injection) significantly reduced the number of leaks per micrometer of venule produced by DBBF-Hb, it did not reduce the average leak area per micrometer of venule. This observation suggests that the individual leaks must be larger on average with the cromolyn suffusion than with DBBF-Hb injection alone. Therefore, either cromolyn inhibits the formation of the smaller leaks or the leaks are aggregating into larger leaks, thus reducing the number of leaks without changing the total area. The average individual leak areas for the treatments including DBBF-Hb were as follows (means ± SD): experiment A, 160 ± 238 μm² (percentage of leaks <50 μm² = 45%); experiment B, 212 ± 307 μm² (percentage of leaks <50 μm² = 35%); and experiment D, 70 ± 75 μm² (percentage of leaks <50 μm² = 70%). Thus treatment with cromolyn (groups A and B) significantly increased the individual leak area of preparations perfused with DBBF-Hb (group D).

**Mast cell degranulation.** DMC were easily distinguished from intact mast cells because of the fashion in which the mast cell granules are expelled into the surrounding tissue, as demonstrated by toulidine blue. The mast cells were best observed at the ×20 optical view with a 1.13-mm² field of view. Figure 3A illustrates how the cromolyn treatment (injection and suffusion) effectively stabilized the mast cells during perfusion with DBBF-Hb. The intact mast cells were typical of the preparation as a whole. Figure 3B shows an example of the minimal mast cell degranulation that was observed when saline injection and cromolyn suffusion accompanied DBBF-Hb perfusion. One DMC can be seen (arrow). The mean number of DMC for the treatments was as follows: experiment A, 1.07 ± 0.12 (n = 458); experiment B, 1.68 ± 0.23 (n = 212); experiment C, 0.17 ± 0.08 (n = 90); and experiment D, 4.40 ± 0.40 (n = 340). There was a significant difference between the DBBF-Hb preparations treated with cromolyn (groups A and B) and the DBBF-Hb preparations with no cromolyn (group D) (Fig. 2C). In addition, group A, which was treated with cromolyn injection and suffusion before DBBF-Hb infusion, showed significantly less mast cell degranulation than group B (saline injection and cromolyn suffusion).

It was hypothesized that the fields with a high number of DMC would correlate with the fields with microvascular leaks. However, no such correlation was observed. Because the granules are expelled away from the mast cell, it was thought that venules further removed from the DMC might also be...
affected. Therefore, areas four times larger than the field of view were considered by grouping four consecutive fields of view together and overlapping two fields within each larger group (i.e., 1, 2, 3, and 4 = group 1; 3, 4, 5, and 6 = group 2). Again, no correlation was observed between the location of the DMC and the leak sites, even for the larger areas (\(R^2 = 0.0528\)). Al-Naemi and Baldwin (4) also determined that there is probably not a direct link between mast cell degranulation and leak formation induced by applying exogenous histamine. Similar to the present study, their unpublished data indicated that the leak sites did not coincide with the exact sites of mast cell degranulation.

**DISCUSSION**

This study has demonstrated that the suffusion with cromolyn sodium salt significantly reduces the numbers of DMC and microvascular leaks caused by the synthetic blood substitute DBBF-Hb. In fact, the cromolyn suffusion and preinjection, in conjunction with the administration of DBBF-Hb, proved extremely effective in significantly reducing the number of DMC below that of a previous HBS-BSA control [1.28 ± 0.20, \(n = 152\) (9)] and in minimizing the number of leaks (similar to the HBS-BSA control). If the cromolyn preinjection was substituted by a saline injection (group B), the leak number was significantly greater than that of the HBS-BSA control, although it was still only a fraction of the value obtained for DBBF-Hb alone (group D). In addition, in these animals, the number of DMC per field of view, although small, was significantly larger than for the group with cromolyn preinjection (group A). Thus it appears that preinjection with cromolyn, in addition to suffusion with cromolyn, further enhances mast cell stability. However, despite cromolyn’s mast cell-stabilizing capabilities, some mesenteric leaks still formed in response to DBBF-Hb, and their total area per unit venule length was not significantly lower than that for group D (DBBF-Hb alone). In fact, the mean area of individual leaks was increased in groups A and B compared with group D. The HBS-BSA treatments [group C and the previous HBS-BSA control (9)] both produced lower leak areas compared with any of the DBBF-Hb treatments [statistical significance for HBS-BSA control (0.02 ± 0.02 \(\mu\)m\(^2/\mu\)m venule) versus DBBF-Hb treatments in groups A, B, and D]. To the best of our knowl-
edge, this study is the first attempt to demonstrate the connection between cromolyn’s ability to stabilize mast cells and its effect on increased microvascular permeability produced by a modified Hb.

Our observation that cromolyn has the ability to reduce the number of DMC is consistent with other studies (10, 22–24, 29). By reducing mast cell degranulation, the release of mast cell mediators in the surrounding tissues will be reduced. There is ample evidence to support the theory that histamine, a mast cell mediator, causes tremendous increases in vascular permeability (28). Therefore, it was not surprising that treatment with cromolyn reduced the number of leaks produced by DDBF-Hb. However, the fact that cromolyn did not reduce the mean leak area per micrometer of venule was surprising and indicates that there is a property of DDBF-Hb that induces microvascular leaks independently of the inflammatory mediators released by mast cells. One possible suggestion for this mechanism involves the formation of ROS by Hb. Perhaps ROS are reacting directly with the venules, causing large interendothelial cell gaps and therefore increasing the permeability. It is known that a bolus injection of DDBF-Hb causes formation of ROS in intestinal tissue (8) and that reaction of ROS with microvessels increases their permeability to macromolecules (11). Leak formation by ROS would not require mast cell degranulation, but the ROS formed by oxidation of the modified Hb could diffuse out of the venules and into the tissue and then cause mast cell degranulation, which could exacerbate the leak formation.

The lack of a correlation between exact sites of mast cell degranulation and microvascular leakage suggests that the products released by mast cells in response to Hb do not necessarily exacerbate leakage in nearby venules. One possibility for this mechanism involves the formation of ROS by Hb. Perhaps ROS are reacting directly with the venules, causing large interendothelial cell gaps and therefore increasing the permeability. It is known that a bolus injection of DDBF-Hb causes formation of ROS in intestinal tissue (8) and that reaction of ROS with microvessels increases their permeability to macromolecules (11). Leak formation by ROS would not require mast cell degranulation, but the ROS formed by oxidation of the modified Hb could diffuse out of the venules and into the tissue and then cause mast cell degranulation, which could exacerbate the leak formation.

To summarize, it appears that DDBF-Hb induces venular leak formation by a process that does not require mast cell degranulation but that this process can also cause mast cells to degranulate. The lack of a direct correlation between sites of mast cell degranulation and sites of vascular leakage suggests that the endothelial cells in the same venule vary in their response to a given degree of inflammatory stimulus.

ACKNOWLEDGMENTS

The authors give special thanks to Dr. Ed Valeski for the guidance and expertise on the surgical procedures and to Ela Cudilho for aiding in data collection and analysis.

REFERENCES


