Nitric oxide: role in venular permeability recovery after histamine challenge

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Al-Naemi, Hamda, and Ann L. Baldwin. Nitric oxide: role in venular permeability recovery after histamine challenge. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H2010–H2016, 1999.—Histamine is an inflammatory mediator produced by mast cells that reside close to blood vessels. It causes a transient increase in venular permeability and stimulates endothelial production of nitric oxide (NO). In this study, we investigated the role that NO plays in the permeability recovery and evaluated the response of mast cells. The mesenteric microvasculature of anesthetized rats was suffused with 10⁻⁴ M histamine for 3 min and then perfused with the NO donor sodium nitroprusside (SNP; 10⁻⁶ M), the NO inhibitor N⁵-monomethyl-L-arginine (L-NMMA; 10⁻⁵ M), its enantiomer (p-NMMA; 10⁻⁵ M), or HEPES-buffered saline containing 0.5% BSA for 15 min. This was replaced by FITC-albumin for 3 min, followed by fixative. The vasculature was visualized using epifluorescence microscopy and was stained for mast cells. Preparations treated with histamine only showed discrete FITC-albumin leaks. Subsequent inhibition of NO increased venular FITC-albumin leaks and prevented permeability recovery, whereas subsequent treatment with SNP decreased the histamine-induced venular leaks. Mast cells degranulated due to histamine and the other treatment combinations. In conclusion, inhibition of NO prevented permeability recovery and depleted mast cells of their histamine content.

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Endogenous NO has been demonstrated to mediate microvascular permeability evoked by inflammatory mediators in the hamster cheek pouch (20), rat skin (8), and guinea pig skin (24). Therefore, the role played by NO in regulating vascular permeability is not defined. In the present study we have investigated the role of NO in modulating venular permeability in a mesenteric preparation that has been exposed to histamine.

Mast cells are the major source of histamine in the microvascular environment. It has been shown that NO decreases mast cell reactivity (9, 23), but it has not been documented how mast cells react in the presence of histamine. For this reason we also studied mast cell reactivity in the presence of histamine and histologi-
Surgically evaluated the response of mast cells during the permeability recovery.

MATERIALS AND METHODS

Surgical procedure and experimental protocol. Male Sprague-Dawley rats (350–450 g) were anesthetized with an intraperitoneal injection of pentobarbital sodium (6 mg/100 g). A tracheotomy was performed, and the animals were ventilated artificially. A dose of the mast cell stabilizer sodium cromoglycate (5 mg/kg; Sigma, St. Louis, MO) was administered intravenously into the jugular vein, followed by another dose 30 min later. The mast cell stabilizer was used to inhibit mast cell degranulation in response to surgery and handling as documented by other authors (14, 26). The abdomen was slit along the linea alba, and the open blood vessels were cauterized to avoid any contact of blood with mesenteric vessels. Mesenteric windows (3–4) were selected on the basis that they had an “unbroken” adequate vascular network lying between adjacent pairs of traversing arteries and veins. The chosen windows were spread out flat over a Plexiglas platform and continuously superfused with 37°C HEPES-buffered saline (HBS; pH 7.4). The superior mesenteric artery was cannulated close to the selected series of mesenteric windows, and the appropriate bordering arteries and veins were ligated to allow perfusion only to these chosen windows. A clamp was placed around the superior mesenteric artery near the chosen windows, and then the windows were flushed clear of blood with HBS containing 1 U/ml heparin and 0.5% BSA at 37°C under an inlet pressure of 100 mmHg. Histamine (10^{-3} M) was suffused over the mesenteric preparation for 3 min, either alone or followed by 15 min of perfusion with HBS-BSA, NG-monomethyl-L-arginine (L-NMMA; 10^{-5} M), its enantiomer (D-NMMA; 10^{-5} M), or sodium nitroprusside (SNP; 10^{-6} M). The concentration of nitric oxide synthase (NOS) inhibitor (L-NMMA; 10^{-5} M) was chosen to block NO release from both constitutive (cNOS) and inducible NOS (iNOS). Laszlo and colleagues (19) reported that inhibition of both cNOS and iNOS was dose dependent of L-NMMA and that a dose of 2 \times 10^{-3} M L-NMMA was enough to inhibit >90% of both enzymes. The control group was perfused with HBS-BSA for 3 min. Seven animals were used for each treatment. Next, in all cases a solution of 0.05% FITC-BSA was perfused and incubated for 3 min. A fixative consisting of 3% formaldehyde in HBS was then perfused, the portal vein flow outlet was clamped, and the fixative was allowed to incubate for 30 min under a pressure of 40 mmHg. After the fixation, the vasculature was flushed with HBS and the mesenteric tissue was excised and mounted on glass slides with an aqueous mounting medium ( Vectashield, Dako).

Venular permeability. Changes in venular permeability were evaluated by measuring the number and area of FITC-albumin extravasalizations. Slides of mesenteric windows were visualized under epifluorescence microscopy (Zeiss Axioplan), and the mesenteric venules were scanned and videotaped using a video camera (Optronix VI 470) attached to the microscope. Images of venules were obtained by epifluorescence with the suitable FITC excitation filter (λ = 488) and emission filter (515 nm) and then analyzed by computer software (NIH Image). The length and diameter of each venule were measured as well as the number and area of leaks per venule. Data were pooled within each experimental group, and the average number and average leak area per unit length of venule were calculated.

Mast cell staining. Slides with mesenteric windows were rehydrated with distilled water and then stained with 0.1% Alcian blue (Sigma) in 0.7 N HCl for 30 min, rinsed in 0.7 N HCl, and subsequently stained with 0.5% Safranin O (Sigma) in 0.125 N HCl for 5 min. They were then rinsed in distilled water, counterstained with 0.1% eosin (Sigma) for 30 s, and gradually dehydrated in a series of 70%, 80%, 90%, 95%, and absolute ethanol. The slides were cleared in xylene and mounted with mounting medium (xylene; Fisher Scientific, Swedesboro, NJ). This staining procedure was modified from Mayrhofer (22).

Mast cell counting. A microscopic 100-square-counting grid (Carl Zeiss, Germany) was used at \times 200 magnification to count the number of mast cells within an area of 0.41 mm² around mesenteric venules of 26–50 µm in diameter. This area corresponded to the field of view of a \times20 microscope objective. For each animal (7 animals/treatment) five fields were counted. The grid was randomly placed around different venules, and the numbers of degranulated and intact mast cells were counted. All mast cells were classified morphologically as not degranulated, moderately degranulated (10–50% of the granules expelled from the cell), or extensively degranulated (50–100% of the granules expelled) as in Tromp et al. (26). All counts were pooled for each treatment, and the percentage of mast cells was calculated and expressed as the percentage of total mast cells.

Statistics. ANOVA was used for statistical comparison. Significance of difference between pairs of groups was assessed using Student’s t-test with P < 0.05 considered statistically significant and n equal to the number of venules per group. All values are expressed as means ± SE. The significance of the difference between the proportion of the counted mast cells was tested by using the z test with P < 0.05.

RESULTS

The ANOVA analysis indicated a highly significant difference (P < 0.001) among the experimental groups for venular leakage parameters.

Venular permeability. Observation of venular leaks by epifluorescence microscopy showed that the FITC-albumin leakage occurred in venules rather than in arterioles or capillaries. Very few leaky sites, or no leaks, were noticed within the control group after perfusion with HBS-BSA for 3 min (7 experiments, n = 82 venules), as shown in Fig. 1A. Venules suffused with histamine for 3 min showed many discreet FITC-albumin leaks (7 experiments, n = 82 venules), as shown in Fig. 1B (venule). Preparations suffused with histamine for 3 min and then perfused with L-NMMA for 15 min showed many small and large venular leaks (7 experiments, n = 90 venules) (Fig. 1C). Preparations treated with SNP (Fig. 1D), D-NMMA, or HBS-BSA for 15 min after the 3 min of histamine suffusion showed fewer venular leaks (7 experiments, n = 82, 129, and 82 venules, respectively). Figure 2 summarizes the effects of the different treatments on the rat mesenteric venules, expressed as the area of FITC-albumin leaks per venule length for each treatment. The average leak area per venule length increased significantly (14.19 ± 2.64 µm²/µm) after 3 min of histamine suffusion compared with the control value of 3.08 ± 0.95 µm²/µm for the same time. Inhibition of NO for 15 min after 3 min of histamine treatment caused a significant increase in the area of leaks (26.82 ± 2.16 µm²/µm compared with 14.19 ± 2.62 µm²/µm), whereas application of the NO donor SNP for 15 min after histamine treatment significantly reduced the area of leaks (5.13 ± 0.8 µm²/µm).
close to the control value. Similar decreases in the average leak area per venule length were observed after 15 min of treatment with d-NMMA (6.11 ± 0.67 \( \mu \)m²/µm) or HBS-BSA (5.13 ± 0.8 \( \mu \)m²/µm) after histamine treatment. The average number of leaky sites per unit length (µm) of venule for the control group was 6.67 ± 1.19 \( \times \) 10⁻³, which increased significantly to 26.6 ± 6.38 \( \times \) 10⁻³ after 3 min of histamine suffusion (Fig. 3). Inhibition of NO for 15 min after histamine application almost doubled the average number of the leaky sites per unit length of venule (51.93 ± 9.03 \( \times \) 10⁻³), whereas this number decreased significantly after 15 min of perfusion of SNP (12.3 ± 1.15 \( \times \) 10⁻³), d-NMMA (13.85 ± 1.08 \( \times \) 10⁻³), or HBS-BSA (13.8 ± 1.10 \( \times \) 10⁻³), as shown in Fig 3.

Mast cell degranulation. The mast cell stabilizer did not totally prevent mast cell degranulation. In the control group, 11% of the mast cells had degranulated, which is similar to previous results from our laboratory (2) and from others (26). In groups treated with histamine either alone or followed by L-NMMA, d-NMMA, SNP, or HBS-BSA, high percentages of degranulation occurred.

Histamine granules (blue stain) were released from mesenteric mast cells after histamine suffusion for 3 min (Fig. 4A), 91% of which were degranulated. Inhibition of NO with L-NMMA after histamine application caused further degranulation of mast cells (98%). Most of the cells were almost depleted of histamine, as demonstrated by the rim of blue stain around each

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**Fig. 1.** Light micrographs of FITC-albumin leakage. A: control network of venules (arrow) and arterioles (arrowhead) with no leakage. B: histamine treatment showing venule (small arrow) with leaks (large arrow) and arteriole (arrowhead) with no leak. C: histamine + N⁰-monomethyl-L-arginine (L-NMMA) treatment showing venules (arrow) with many leaks and arterioles (arrowhead). D: histamine + sodium nitroprusside (SNP) treatment showing venules (arrow) with few small leaks and arteriole (arrowhead) with no leaks. Bar = 50 µm.
mast cell (Fig. 4B, arrowhead). Few or no granules were release from mast cells in the control group (Fig. 4C). The percentages of mesenteric mast cell degranulation are shown in Fig. 5. There was a significant \( P < 0.05 \) difference in the percentage of mast cell degranulation between the control group and the other experimental groups. In the control group 9% of mast cells were moderately degranulated, whereas in the histamine group 31% of mast cells were moderately degranulated. In the control group, 2% of mast cells were extensively degranulated compared with 60% in the histamine group. There was no significant difference in the degree of mast cell degranulation between animals treated with histamine alone and those treated with histamine plus \( \alpha \)-NMMA, SNP, or HBS-BSA. However, there was a significant difference between the histamine group...
DISCUSSION

In this study, the role of NO in modulating venular permeability during the recovery period after histamine challenge was demonstrated, in situ, with evaluation of the mast cell response during this period. Our results showed that suffusion of the mesenteric microcirculation with histamine for 3 min increased venular leakiness fourfold compared with its control level, followed by a decline to normal values during the next 15 min. This finding is consistent with previous results reported from our laboratory (1, 27). The decline in venular permeability was prevented when the microvasculature was treated with NO inhibitor for 15 min. The results showed a significant increase in the leak area and in the number of these leaks, which can be reversed with the NO donor (SNP). Similar results regarding the decrease in leak area and number were obtained with D-NMMA or HBS-BSA. The similarity in the decrease that occurs in the leak area and the number of leaks with D-NMMA, SNP, and HBS-BSA is due to the effect of NO, which has been demonstrated to be present after the histamine challenge.

Previous studies performed in vivo in different animal species have indicated that histamine suffusion directly activates endothelial H1 receptors, causing the transient increase in venular permeability. It was suggested that this occurred via a phospholipase C NO/cGMP-dependent mechanism (28, 29). In our study, the histamine-induced rapid transient increase in FITC-albumin extravasation was altered by L-NMMA. Rather than peaking after 3 min and subsiding by 15 min, venular leakage was still high after 15 min. Thus the reduction in histamine-induced venular leaks is probably related to the histamine-induced release of NO.

Similar results were reported by Kubes et al. (16) from inflamed feline mesenteric vessels. They found that coapplication of NO donor (CAS 754) with platelet-activating factor (PAF) reduced the increase in venular permeability caused by PAF. Another obligatory role of NO was reported from isolated coronary venules (29). It was demonstrated that histamine induced an increase in venular permeability via NO. The contradiction between this finding and ours might be explained by the fact that in the other study the vessels were isolated from the surrounding interstitium, whereas in our experiment the vessels were kept in situ. The interstitium may contain an extravascular source of mediator(s) that would induce an increase of venular permeability with NOS inhibition, as suggested by Kubes (11). In this study we showed that mast cells release their granular contents in the absence of NO, which could account indirectly for the increase in venular permeability. Also, this contradiction could be due to

![Percentage of mesenteric mast cell degranulation after histamine (H) treatment alone or in combination with L-NMMA, D-NMMA, SNP, or HBS-BSA.](http://ajpheart.physiology.org/)

and the group treated with histamine plus L-NMMA for 15 min. There were more moderately degranulated mast cells and fewer extensively degranulated cells in the histamine group compared with the histamine plus L-NMMA group.
the fact that in the other study (29) the venules were isolated from the pig's heart, and the NO may have a different effect in different tissues. He et al. (6) showed that the increment in NO production induced by an agonist was correlated with the increase in venular permeability of frog mesenteric microvasculature. This dichotomy could be explained by the possibility that NO plays a different biological role in different animal species and in different anatomic locations in the same animal. Therefore, it is important to consider the living tissue as a whole in the different species and to include other major cellular components of the tissues.

How much NO is required to produce the recovery phenomenon? This is an important question. In this study we showed that perfusion with 10^{-6} M SNP after histamine suffusion produced venular leakage comparable to that observed after perfusion with D-NMMA or HBS-BSA. Thus the amount of NO that is produced by this concentration of SNP appears to be functionally similar to that produced endogenously.

Mast cells are strategically located adjacent to vessels within microvasculature networks. There is a wide heterogeneity in the structure and function of mast cells that has been documented both in vitro and in vivo (10). For this reason we used the Alcian blue-Safranin method for mast cell staining to differentiate between the mast cells containing proteoglycan heparin (red stain) and those with biogenic amines, such as histamine (blue stain). From our study, the histological observation of mast cell granules revealed that most of the mast cell population in the mesenteric windows with well-developed vasculature has blue granules, and few cells have both red and blue granules. However, in small mesenteric windows, with less well-developed vasculature, most mast cells contain red granules, and few have both red and blue granules. These observations indicate that the majority of mast cells in well-vascularized windows contain biogenic amines.

Mast cells that were stimulated to release their granules in the presence of histamine continued to expel more granules in the absence of NO. Kubes et al. (15) reported that 23–30% of mast cells were partly degranulated and that 17.4–31.2% of mast cells were fully degranulated after 60 min of NO inhibition, whereas we found that most of the mast cells were extensively degranulated after 15 min of NO inhibition. The difference between our results and those of Kubes et al. (15) is probably due to the combination of histamine and L-NMMA used in our study. Histamine stimulates mast cells to release their granules, and in the absence of NO this reactivity continues, causing the further loss of granules. A period of 15 min was not enough time for the mast cells to recover from the degranulation and replenish their histamine store. This was indicated by the insignificant difference between the degree of degranulation observed after 3 min of histamine treatment alone and that seen 15 min later. The type of biogenic amines that were released is uncertain. It has been reported that different stimuli result in different patterns of mast cell mediator release (25).

The increase in venular permeability after histamine application maybe due to release of mast cell mediators as well as exogenous histamine. However, there is probably not a direct link between degranulation and leak formation, because sites of leaks did not usually coincide with the exact sites of mast cell degranulation (unpublished data).

In summary, we have shown that the venular permeability recovery following the histamine challenge was, in part, due to release of NO. In addition, we have shown that mast cell reactivity is triggered by histamine and is increased in the absence of NO. These results together imply that the changes in microvascular permeability produced by exogenous histamine are not just a direct response to histamine, because the histamine also invokes the release of different biogenic amines from mast cells.

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